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Advanced Variety Development and Multiplication of Arabica Coffee: Challenges and Opportunities

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SUMMARY

Arabica coffees make up about 60% of current world coffee production and are generally sold at considerably better prices than Robustas on account of superior beverage quality. However, costs of production are much higher, mainly due to more stringent demands for soil and climatic conditions, crop management, primary processing and control of several pests and diseases including the potentially very destructive Arabica-specific coffee leaf rust (CLR) and berry disease (CBD).

Breeding for disease resistance in combination with vigour, productivity and quality started in the early 1920s in India, but especially in the second half of the 20th century comprehensive breeding programmes have been implemented also by research centres in several other coffee producing countries, particularly in Brazil, Colombia, Costa Rica, Kenya, Tanzania, Ethiopia and Angola/Portugal. Many of the resulting CLR- and CBD+CLR-resistant cultivars (true-breeding lines and F1 hybrids) meet the required standards of profitable and environmentally sustainable crop production. Nevertheless, in many countries the speed of replanting the traditional, disease-susceptible, varieties with these modern Arabica cultivars has been slow. Reasons for the disappointing impact of innovative variety development on arabica coffee production include: inadequate coffee extension services, limited access to credit facilities for financing costs of replanting and inputs, inefficient systems of multiplication and distribution of the new cultivars to the growers, and last but not least the persistent scepticism among coffee traders about the cup quality of disease-resistant cultivars. Challenges of more recent date are the conservation of and access to additional genetic resources of *Coffea arabica*, breakdown of host resistance to CLR in some countries and the increasingly negative impact of climate change on Arabica coffee production worldwide.

The durability of CLR resistance can be improved by strategic management of the available SH genes, through marker-assisted gene pyramiding or multilines, and by a continued search for new R genes in the *Coffea* gene pool. A novel approach to achieving durable host resistance in obligate biotrophic pathogens like CLR could be selection for "loss of susceptibility" within the host-pathogen relationship. However, the host resistance to CBD has proved to be very durable so far. Chances of detecting and successfully introgressing effective host resistances to important coffee pests, other than nematodes, from different *Coffea* species appear to be low, especially when genetic modification is not an option. Searching for heat- and drought-tolerant genotypes within the *Coffea* gene pool seems to be the preferred approach to countering the negative effects of climate change, but success under field conditions is as yet unconfirmed. On the other hand, there is ample room for improving crop water productivity in Arabica coffee, by selection and innovative crop management, without decreasing cup quality.

Functional-genomic methods of analysis open perspectives of effectively selecting genotypes combining high yields with superior beverages, e.g. identifying metabolic pathways of the aroma/flavour precursors of intrinsic cup quality.

This review discusses ways to improve the development and dissemination of new Arabica coffee cultivars, by seeds or clones, with or without growers' participation, by the public or private sector, with the objectives of contributing to sustainable, climate-change tolerant and diversified coffee production.

Advanced coffee variety development could benefit tremendously from networks of collaborating research institutes to facilitate the sharing of resources (financial, genetic and genomic), technologies and scientific information.

INTRODUCTION

World coffee production has increased from 60 (1963-67) to 148 (2012-14) million (60 kg) bags of green coffee harvested annually from some 10.5 million ha, as a result of intensified crop production and also considerable expansion on newly cleared and planted land, in Vietnam and Brazil in particular (FAO, 2012). Concurrently, the share of Arabica coffees came down from 75% to 60%. Arabica coffees (*Coffea arabica*) are generally sold at twice the price of Robustas (*Coffea canephora*) or even more, on account of superior beverage quality (ICO, 2014). However, costs of production are much higher, mainly due to more stringent demands for soil and climatic conditions, crop management, primary processing and control of several pests and diseases, including the potentially very destructive coffee leaf rust (CLR) and berry (CBD) diseases (Wintgens, 2009).

Climate change – rising temperatures, longer droughts, excessive rainfall – appears to threaten the sustainability of Arabica coffee production. Colombia's severe (30%) production decline since 2008 was the result of excessive rainfall, lasting five years, and CLR epidemics. However, its production was back to normal levels (11 million bags/y) in 2013. This rapid recovery was the result of government-backed concerted efforts of the well-organized FNC (Federation National de Cafeteros) and CENICAFE (Coffee Research Institute), including the replanting of about 45% of the total coffee area (0.9 million ha) with CLR-resistant "Castillo" cultivars (Gast, 2014). Coffee growers in many other Arabica-coffee producing regions/ countries have not been so lucky and continue to suffer considerable crop losses with insufficient access to extension services, inputs and credit facilities, e.g. in Central American coffee countries (Neill, 2013). Brazil has been facing reduced production due to severe droughts in 2014, but is likely to bounce back with its combination of strong public coffee research centres, effective extension services and a resilient private sector (Brando, 2014).

Consequences of climate change include: (a) an upward move of the lower altitude limits for growing Arabica coffee resulting in a reduction of land suitable for Arabica coffee production in equatorial regions (Läderach *et al*., 2010), (b) increasing disease (e.g. CLR) and pests (e.g. Coffee Berry and Stem Borers) problems, (c) declining productivity and cup quality and (d) threat to the livelihoods of millions of farming families.

After summarizing past achievements of selection and breeding, this review discusses the challenges and opportunities of developing and disseminating resilient (hybrid) cultivars contributing to sustainable, climate-change tolerant and diversified Arabica coffee production.

A CENTURY OF VARIETY DEVELOPMENT

The history of the geographic distribution of Arabica coffee – from its Ethiopian centre of genetic diversity to Yemen (6th century?) and from there to India, S.E.Asia, Latin America and East Africa during the 17–19th centuries – has been described by a.o. Haarer (1962), Wrigley (1988) and Pendergrast (1999). The remarkable adaptation of Arabica coffee to a wide range of environments throughout the intertropical belt, despite its genetically narrow source population, could be attributed to the allotetraploidy of its genome (Chen, 2010).

Arabica coffee production is still based to a large extent on traditional cultivars developed long ago by line selection within the Typica and Bourbon source varieties or in offspring of crosses between them (e.g. Mundo Novo, Catuai). A few, like Caturra, Sumatra and Maragogipe are single gene mutants found in Bourbon or Typica coffee fields (Carvalho *et al.*, 1969; Van der Vossen, 1985). Some of these traditional Arabica cultivars are high yielding and have a reputation for producing outstanding cup quality under optimal conditions of climate, soil and crop management. However, almost all of them are highly susceptible to the major coffee diseases and pests, which makes them increasingly difficult to maintain for economic (cost of chemical control) and ecological (pesticide pollution) reasons in many coffee regions.

Breeding for disease resistance in combination with vigour, productivity and quality started in the early 1920s in India (CLR-resistant cultivars Kents and S795), but especially during the second half of the 20th century comprehensive breeding programmes – applying Mendelian and quantitative genetics, plant pathology, crop physiology and agronomy – have been implemented by research centres in several coffee producing countries: India (CCRI), Brazil (IAC, IAPAR, UFV), Colombia (CENICAFE), Costa Rica (CATIE/PROMECAFE/CIRAD), Kenya (CRF), Tanzania (TaCRI), Ethiopia (EIAR/JARC) and Angola/Portugal (CIFC-IICT). Genetic variation was increased by introgressive breeding with other species, such as *C. liberica*, *C. canephora*, *C. eugenioides*, *C. stenophylla* and *C. racemosa* (Carvalho, 1988; Van der Vossen, 2001). A summary of progress made to-date in genetics and breeding of Arabica coffee is presented in Table 1.

Table 1. Summary of major achievements in genetics and breeding of Arabica coffee.

- Effective pre-selection for host resistance to CLR and CBD
- Genetics of resistance to CLR (9 major genes; > 50 phys. races) and to CBD (3 major genes; no physiologic races), nematodes
- The first molecular markers for CLR, CBD and nematode R-genes
- Quantitative genetic information (h^2, r_g, GCA, SCA) components of yield and quality (diallel crosses; P-F1-F2-BC1.2 sets)
- Early selection for potential yield from first 3 years production combined with few simple plant vigour parameters (index selection)
- Resilient F1 hybrids with compact growth, disease resistances, good bean size and liquor quality: high yields per ha at much lower production costs

The CIFC (Coffee Leaf Rust Centre) at Oeiras, Portugal, has played a crucial role in pre-breeding for host resistance to CLR. The main progenitors for CLR resistance were plants of "Hibrido de Timor" (HdT), an Arabica-like variety found in Timor-Leste and assumed to have arisen from natural hybridization of *C. arabica* and *C. canephora* (Rodrigues *et al*., 2000). The release in 1970 of a number of crosses, between selected HdT clones (CIFC832/1, CIFC832/2 and CIFC1343) and the compact Caturra, Catuai and Villa Sarchi varieties, enabled coffee research centres in Latin America to develop, after a decade of pedigree selection, true-breeding CLR-resistant and compact cultivars (Silva *et al*., 2006).

CIFC832-1 was the progenitor for development of cultivars like CR95 and Lempira in Central America or Cauvery in India, generally called Catimors (Bertrand *et al*., 1999; Rodrigues Jr *et al*., 2000). CIFC832/2 was the main source for development of 'Sarchimors' in Brazil, such as Obata and Tupi, and Marsellesa in Nicaragua. CIFC1343 has been used extensively in development of Catimor lines with CLR resistance in Colombia, included in the multiline Castillo. These HdT derivatives carry different resistance genes not only for CLR but sometimes also for other important diseases like CBD (Rodrigues Jr *et al*., 2000). By massive RNAseq data analysis, Herrera *et al.* (2014) demonstrated that CIFC1343 carried more *C. canephora* genomic DNA than CIFC832-1 or CIFC832-2. There are other CIFC-HdT derivatives (clones 19759, 19758, CIFC 420, 7963/3, 419/20, 2252/28), as well as germplasm similar to HdT found in a neo-natural coffee tree population in New Caledonia (Mahé *et al*., 2007) that may be used as sources for CLR-resistance and other useful genes.

Introgressing new progenitors in the breeding programmes should increase genetic diversity among cultivars. Nevertheless, Setotaw *et al*. (2013) showed that the genetic base of 121 cultivars released in Brazil between 1939 and 2009 was defined by only 13 ancestors. The situation is more or less the same for Latin America as a whole (85% of the world production of Arabica coffee). The limited number of parental lines included in the early stages of *C. arabica* breeding, the frequent use of the variety Bourbon Vermelho as recurrent parent and th
C

cv. KP423
susceptible to CBD + CLR

F1 hybrid cv. Ruiru 11
resistant to CBD + CLR

Figure 1. Results of breeding for host resistance to CBD and CLR in Kenya.
Fig. 1: Results of breeding for host resistance to CBD and CLR in Kenya

The advantages of hybrid cultivars, even for the autogamous *C. arabica*, were demonstrated for the first time in the Kenyan breeding programme. Simultaneous combination of compact plant type, hybrid vigour for growth and yield, high beverage quality and host resistances to CLR and CBD was realized in the F1 hybrid cultivar "Ruiru 11" (Walyaro, 1983; Van der Vossen, 1985; Fig. 1). Breeding strategies of developing hybrid cultivars of Arabica coffee have subsequently been adopted in Ethiopia (Bellachew, 1997), Central America (Bertrand *et al.*, 1997, 2011) and Tanzania (Teri *et al.*, 2004)

Tall-stature Arabica cultivars with CLR-resistance developed from interspecific hybridization between *C. arabica* and *C. canephora*, followed by recurrent backcrossing to the Arabica parent, include Icatu in Brazil and S2828 in India. For a detailed description of traditional and modern cultivars of Arabica coffee, reference is made to Tables 2.2 & 2.3 in the handbook "Espresso Coffee" (Illy & Viani, 2005).

CHALLENGES AND OPPORTUNITIES

Genetic Resources

Past efforts of coffee genetic resources conservation have been impressive, considering all the hard work of individual coffee scientists, starting with Thomas (1942), Sylvain (1953) and Lejeune (1958), then the FAO Coffee Mission to Ethiopia in 1964 (Meyer *et al.*, 1968) and another by ORSTOM in 1966 (Guillaumet and Hallé, 1967; Charrier, 1978). Since 1972 large collections of *C. arabica* germplasm have been planted also in field gene banks at Choche (BCRI), Gera & Jimma (IAR/JARC) in Ethiopia. Most of these materials have been thoroughly studied in many coffee research centres in Africa, Latin America and Asia and described in numerous reports and journal papers, such as the report "Conserving coffee genetic resources" by Bioversity International, with emphasis on the CATIE (Costa Rica) coffee germplasm collections (Engelmann *et al.*, 2007). This last report also indicates the real potential of long-term *ex-situ* conservation by cryopreservation of coffee seeds.

Of course, all *C. arabica* germplasm available in *ex-situ* collections may represent only a fraction of the total genetic diversity of the remaining wild and semi-wild forest coffees in S.W. Ethiopia (Labouisse et al., 2008). Nevertheless, breeders of Arabica coffee do have already a pretty good idea of the potential and limitations of Ethiopian (ET) germplasm, in particular in regard to host resistances to diseases and pests. For instance, none of the modern Arabica cultivars with host resistances to CLR derive these from ET germplasm. Also cultivars resistant to CBD outside Ethiopia do not have Ethiopian germplasm as progenitors, while nematode resistance found in ET accessions provide only limited protection to the severe nematode problems in Central America (Anzueto *et al.*, 2001). On the other hand, ET germplasm may be a good source of intrinsic cup quality. The excellent cup quality of the new F1 hybrid cultivars developed for Central America is said to derive largely from one of the two parents, being a selected ET accession of the FAO-1964 collection (Bertrand *et al.*, 2006). Silvarolla *et al.* (2004) found three coffee plants in offspring of Ethiopian Germplasm, which were nearly caffeine-free. Male sterility has also been detected in a few ET accessions, a character very useful for F1-hybrid seed production (Georget *et al.*, 2014a).

Other *Coffea* species which have already contributed useful genes to the *C. arabica* gene pool are *C. liberica* (SH3 gene) and *C. canephora* (several SH genes conditioning CLR resistance; the T gene for CBD resistance; resistance to parasitic nematodes), *C. racemosa* and *C. stenophylla* (leaf-miner resistance), *C. salvatrix* (drought tolerance), *C. eugenioides* (beverage quality). The IAC at Campinas, SP Brazil, has had extensive experience with introgressive breeding programmes including the six mentioned species (Carvalho, 1988). Nagai *et al.*

(2008) reported low-caffeine, tetraploid (Arabica-like) coffee plants selected from the GCA-programme of interspecific hybridization including *C. eugenioides*, *C. canephora* and *C. arabica*, carried out in Madagascar over the past 30 years.

Host resistance to major diseases and pests

CLR (Hemileia vastatrix)

The host resistance to CLR acquired from introgressive breeding with HdT clones as donor parent (SH6-9+? genes) has shown to be non-durable in some countries, especially in ecosystems very favourable to the pathogen (e.g. India). However, the current CLR crisis in C. American coffee regions is more likely caused by poor crop management than by the occurrence of new physiologic races, since most coffees grown are still traditional cultivars without SH genes. The durability of CLR resistance can be improved by strategic management of the available SH genes, through marker-assisted gene stacking or multilines, and by a continued search for new R genes in the *Coffea* gene pool (Zambolin *et al*., 2005). Recent insights into the genomic basis for complex plant-CLR interactions (Vieira *et al*., 2012; Fernandez *et al*., 2012) should contribute to attaining these objectives. On the other hand, a novel approach to achieving durable host resistance in obligate biotrophic pathogens like CLR could be the selection for "loss of susceptibility" within the host-pathogen relationship (Pavan *et al*., 2010; Visser, 2014).

CBD (Colletotrichum kahawae)

CBD is a hemibiotrophic pathogen. Host resistance to CBD – from the Arabica accession Rume Sudan in particular, from certain HdT genotypes and also present in Ethiopian (ET) germplasm – has proved to be remarkably durable (Van der Vossen and Walyaro, 2009). No breakdown of resistance has been reported in Africa, since the first release of CBD-resistant cultivars in Kenya in 1985 and subsequently in Ethiopia and Tanzania. Molecular markers for the R gene in HdT have already been detected (Gichuru *et al*., 2008; Gichimu *et al*., 2014) and its genomic basis of resistance has been studied (Figueiredo *et al*., 2013). Similar molecular research work remains necessary for the R genes present in Rume Sudan and ET accessions. This would considerably increase selection efficiency, in particular also for the breeding programmes in Latin America, where CBD is still absent (quarantine disease). On the other hand, further searches for alternative sources of CBD resistance may be given low priority for the time being.

Other diseases

There are a number of locally important diseases, for which host resistance is not (yet) available, for instance: Ojo de Gallo (*Mycena citricolor*) in C. America and Colombia; Black Rot (*Corticium koleroga*) in India on Arabica and Robusta coffees; Bacterial Leaf Scorch (*Xylella fastidiosa*) in Brazil; Bacterial Blight or Elgon Dieback (*Pseudomonas syringae*) in Kenya and Brazil (Wrigley, 1988). Would there be new opportunities from genomic approaches and or selection for "loss of susceptibility"?

Nematodes and Insect Pests

Host resistances to root-knot (*Meloidogyne* spp.) and root-lesion (*Pratylenchus* spp.) nematodes within *C. arabica* and *C. canephora* germplasm (Anzueto *et al*., 2001; Bertrand *et al*., 1998) and a leaf miner species (*Leucoptera coffeella*) introgressed form *C. racemosa* (Guerreiro Filho, 2006) are now available, but not for the globally important coffee berry borer (CBB, *Hypothenemus hampei*) and stem/twig borers (e.g. *Xylotrechus quadripes*,

Xylosandrus compactus). Fairly effective methods of IPM (integrated pest management) have been developed, but these are often too sophisticated for application in smallholder coffee plots. Genetic modification appears to offer good opportunities, e.g. GM Robusta plants with resistance to leaf miners based on Bt genes (Leroy *et al*, 2000), but continues to be rejected by the coffee industry and consumers.

Drought tolerance

Several centuries of (un)intentional selection in Yemen and subsequently in East Africa has yielded Arabica coffee cultivars (e.g. SL28 in Kenya) with relatively higher drought tolerance (DT) than most of the original Ethiopian germplasm (Van der Vossen and Browning, 1978). However, *C. arabica* lacks the basic physiological characteristics of veritable DT plant species and more severe conditions of water (and high temperature) stress will unavoidably affect productivity and quality (DaMatta, 2004, 2006).

Marraccini *et al*. (2011, 2012) have provided interesting scientific evidence of the molecular mechanisms of plant responses to drought stress in coffee, but do not relate this to mature coffee trees in regard to yield and beverage quality. Instead, adapting the ecosystem to the physiological requirements of the coffee plant, f.i. by planting shade trees or in Agroforestry systems, will probably be much more effective. Hybrid cultivars were shown to maintain productivity under shaded conditions much better than pure-line cultivars (Fig. 2), probably due to homeostatic effects of heterosis (Bertrand *et al*., 2011; Maro *et al*., 2014).

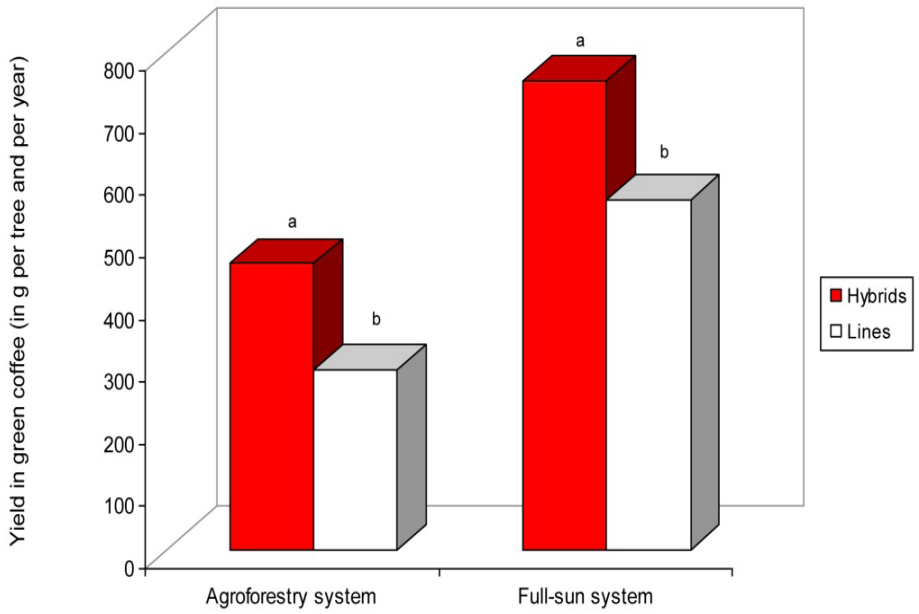

Figure 2. Considerably higher yields for hybrids compared to pure-lines under shade and in full sunshine (Bertrand *et al*., 2011)

In warm lowland areas with marked dry seasons, the use of grafting Arabica on Robusta rootstock (hypocotyledonary) may result in productivity gains of around 30% (B. Bertrand, pers. comm.). Besides, there is also ample opportunity for improving crop water productivity (kg green coffee per m^3 water used) in Arabica coffee, by selection and innovative crop management, without decreasing cup quality (Van der Vossen, 2013).

Beverage quality

In addition to the genotype of a coffee variety, various environmental factors determine the final beverage quality. Arabica coffee originated from the highlands of southwestern Ethiopia and its mild and pleasant beverage is best preserved under similar growing conditions.

High altitudes are critical for the successful production of quality Arabica coffees in equatorial regions. Lower temperatures, and their larger daily amplitudes, induce slower growth and more uniform ripening of the berries, and result in larger and denser beans, an increase of important precursors of aroma and flavour in the bean and superior beverage quality (Avelino *et al*., 2005; Bertrand *et al*., 2006, 2012). In Central America, the beverage quality of samples from a large number of cultivars and accessions taken over a period of 10 years at different altitudes, indicated that there are significant varietal differences. However all cultivars/accessions perform much better at altitudes above 1200 m a.s.l. At the highest altitudes, the main characteristics of Caturra, Mundo Novo or Catuai were much similar. They gave a beverage with medium body and chocolate attributes. Geisha and some Ethiopians accessions or F1 hybrids gave an exceptionally fruity taste and a very good balance between acidity and body. Catimor CR95 gave a cereal note to 'standard' cup, the Catimor T5175 gave a beverage with 'medicinal notes' and the Sarchimor 'Marsellesa' gave a very high acidity. At altitudes below 800 masl, the cvs. Caturra, Mundo Novo or Catuai delivered a flat to standard beverage as Geisha and the majority of the Sudano-Ethiopian accessions. The Catimor CR95 gave a strong cereal note, the catimor T5175 gave a very medicinal cup, but the Sarchimor Marsellesa continued to be more acid than Caturra. Shade has a similar positive effect on coffee quality, particularly at medium altitudes, but also reduces yields (Decazy *et al*., 2003; Guyot *et al*., 1996; Muschler, 2001; Vaast *et al*., 2006).

Rainfall requirements for Arabica coffee production are at least 1200 mm per year with a maximum of 2500 mm. Coffee plants grow and yield better if exposed to alternate cycles of wet and dry seasons, and moreover, a period of water deficit is important to synchronize flower bud differentiation. Areas with excessive precipitation, especially during crop maturation, have a tendency to produce lower quality coffee due to irregular cherry ripening and poor conditions for drying the crop after harvesting. In years of severe dry seasons, shoot dieback and premature ripening of the berries will result in light beans producing a liquor with immature and astringent notes.

Coffee can be cultivated on a wide range of soil types. High-quality, acidic Arabica coffees are mostly produced on soils of volcanic origin. A balanced nutrient status of the coffee tree is essential for sustained production of high quality coffee (Mitchell, 1988; Van der Vossen, 2005). For example, excess N-fertilization may have a negative effect on cup quality. The effective control of pests and diseases is also essential for the production of quality coffee.

Inherently high-quality coffee can easily be degraded by suboptimal harvesting, processing and storage practices. Washed Arabicas from East Africa (Kenya, Tanzania, Ethiopia) and Latin America (Colombia, Guatemala, Costa Rica) are characterized by mild to pointed acidity, light body and intense aroma. Natural dry-processed Arabicas from Brazil and Ethiopia have low acidity, less marked aroma, but much stronger body, which is sought after in espresso coffees (Wintgens, 2009).

The beverage quality of disease-resistant (hybrid) cultivars issued from Arabica breeding programmes will have to be comparable to that of the best traditional varieties to ensure market acceptance. The screening of breeding material for cup quality has always depended entirely on the visual and sensorial evaluation of thousands of carefully prepared green coffee

samples by expert coffee tasters, a tedious exercise with sometimes variable results (Van der Vossen, 2009). Fragrance, aroma, acidity, body and flavour are the major beverage characteristics being scored numerically, while the coffee tasters use additionally a vocabulary for describing specific tastes and off-flavours (Wintgens, 2009).

Accurate characterization of the cup quality of Arabica coffees has so far defied all serious efforts to develop quantitative chemical profiles (Bertrand *et al*., 2006). None of these correlate well enough with sensorial tests. Functional genomic approaches in identifying molecular determinants of coffee quality characteristics appear to open new perspectives for selecting for superior coffee beverages without having to rely exclusively on cup testing experts (Joët *et al*., 2012; Joët, 2014). Caffeine content can be determined accurately by a standard chemical test, which is useful when selecting for low caffeine content.

Multiplication and distribution

Most cultivars of the self-pollinating *Coffea arabica* species are true-to-type (e.g. Brazil, Colombia, India, Ethiopia) and can be fairly easily multiplied by collecting seed form multiplication blocks. These seed blocks are usually managed by the national coffee research institutes and seeds or seedlings are sold at fairly low (subsidized) prices to the growers. However, dissemination of (traditional and modern) cultivars is often unsatisfactory in many countries, with the result that especially the smallholder coffee growers have access to a limited number of cultivars and receive seed or plants of low genetic purity.

For the disease-resistant hybrid cultivars, which have been developed in East Africa and Central America, there are the options of producing F1 seed by (1) hand-pollination (Kenya, Ethiopia), (2) seed blocks with a male sterile female line (in development in C. America: Georget *et al*., 2014a), or (3) plants by vegetative propagation. In Tanzania, a decentralized system of vegetative propagation, by rooted cuttings and grafting, with full participation of some 600 farmers' groups has been successful (Magesa *et al*., 2013). On the other hand, commercialization of large-scale somatic embryogenesis (s.e.) in central laboratories followed by hardening off in plastic houses annex nurseries, is in progress in Central America (Etienne *et al.*, 2012). Technically, this scheme works very well, but apparently there are some financial problems as the costs of s.e. plants still exceed the sales price. Georget *et al*. (2014b) have altered the process by adding a stage, which may significantly reduce costs. Generally, however, low profitability remains a major deterrent from privatizing the multiplication and distribution of coffee cultivars.

PROSPECTS

Genetic resources

The autogamous *Coffea arabica* is an allotetraploid (2n=4x=44) resulting from natural hybridization of ecotypes of the allogamous diploid species *C. eugenioides* (E genome) and *C. canephora* (C genome), which took place in relatively recent times in eastern Africa (Lashermes *et al*., 1999), and probably from an unique event of hybridization (Lashermes *et al.*, 2014). Consequently, the genetic diversity of *C. arabica* is low and unrestricted access to its genetic resources (and also other *Coffea* spp.) is crucial to advanced variety development.

The challenges of *C.arabica* germplasm conservation in Ethiopia are well reviewed by Labouisse *et al.* (2008, 2012). Much more than climate change (Davis *et al.*, 2012), regional overpopulation appears to be the main cause of accelerated destruction of the montane forests of S.W. Ethiopia and with that the disappearance of all remaining wild germplasm of *C.*

arabica. Rescuing this material before it is too late remains an elusive issue, as it seems very difficult to agree on an internationally supported (and funded) programme with full consent and collaboration by the Ethiopian authorities. According to the UN Convention on Biological Diversity, Ethiopia has sovereign rights over its natural resources. Any agreement on participatory conservation and sharing of this germplasm with other coffee producing countries should, therefore, be based on adequate financial compensation. Determining the exact monetary value of this germplasm will remain difficult (Hein and Gatzweiler, 2006), as its value largely depends on potential benefits to future variety development, but at least it should be more than the costs of collection and conservation in Ethiopia. In this respect, the proposal of a "global coffee genetic resource conservation initiative" by the Inter-African Coffee Organization (Bellachew and Sacko, 2008) deserves serious consideration by all main stakeholders in the supply chain of Arabica coffees.

Creating genetic variability by germplasm collection, gene-introgression and pre-breeding are essential for long-term substantial progress in variety development. However, the final product of any breeding programme will have to be uniform cultivars (homozygous lines, or heterozygous F1 hybrids and clones) for the purpose of certification (c.q. plant breeder's rights) and acceptance by the growers.

Development and release of improved varieties

Disease-resistant cultivars have contributed considerably to ecologically sustainable coffee production and to socio-economic benefits for the growers of Arabica coffee. Nevertheless, in many countries the speed of replanting the traditional, disease-susceptible, varieties with modern Arabica cultivars has been slow. Colombia (Gast, 2014) and Tanzania (Magesa, 2014) are clearly exceptions. Major reasons for the disappointing impact of innovative variety development on Arabica coffee production include: (1) the (mostly smallholder) coffee growers are unaware of the advantages of the new cultivars due to poorly operating coffee extension services, (2) limited access to credit facilities for financing costs of replanting and inputs, (3) inefficient systems of multiplication and distribution of the new (hybrid) cultivars and (4) last but not least the persistent scepticism among coffee traders of the cup quality of disease-resistant cultivars (Van der Vossen, 2009).

Coffee breeding has always been the exclusive domain of public research institutes in coffee producing countries. The main reasons are the very long-term nature of variety development, the strategic importance of coffee to national economies, and also because the financial resources of coffee growers, smallholders in great majority, are limited. Coffee breeding programmes are usually financed by a combination of government subventions, contributions by the growers (coffee cess) and often also by international donor assistance. Consequently, the new cultivars issued from such programmes are considered a national property and export of seeds or clones is usually prohibited, except for small quantities as germplasm exchanges between coffee research institutes. Examples are the government ban on distribution of cv. "Ruiru 11" seed from Kenya to neighbouring countries since 1986, and very recently the Brazilian refusal to supply CLR-resistant cultivars to Central American countries (Padua-Martin, 2014).

Genomic breeding

A complete genome sequence and high-density genetic map of *Coffea canephora* was published recently (Denoeud et al., 2014) and similar results are expected to become available soon for *C. arabica* (ICGN, 2014a). This will offer opportunities for enhancing breeding progress to increase crop quality and yield, as well as to protect the coffee crop from major

losses caused by diseases, insect pests and abiotic stresses related to climatic changes. Genes and their arrangement are currently being deciphered and their role is explored by studying the genome's expression at the transcriptomic, proteome and metabolome levels (Andrade, 2014; Gongora *et al.*, 2014; Lashermes *et al.*, 2014; Moncada *et al.*, 2014; Mueller *et al.*, 2014, Fig 3).

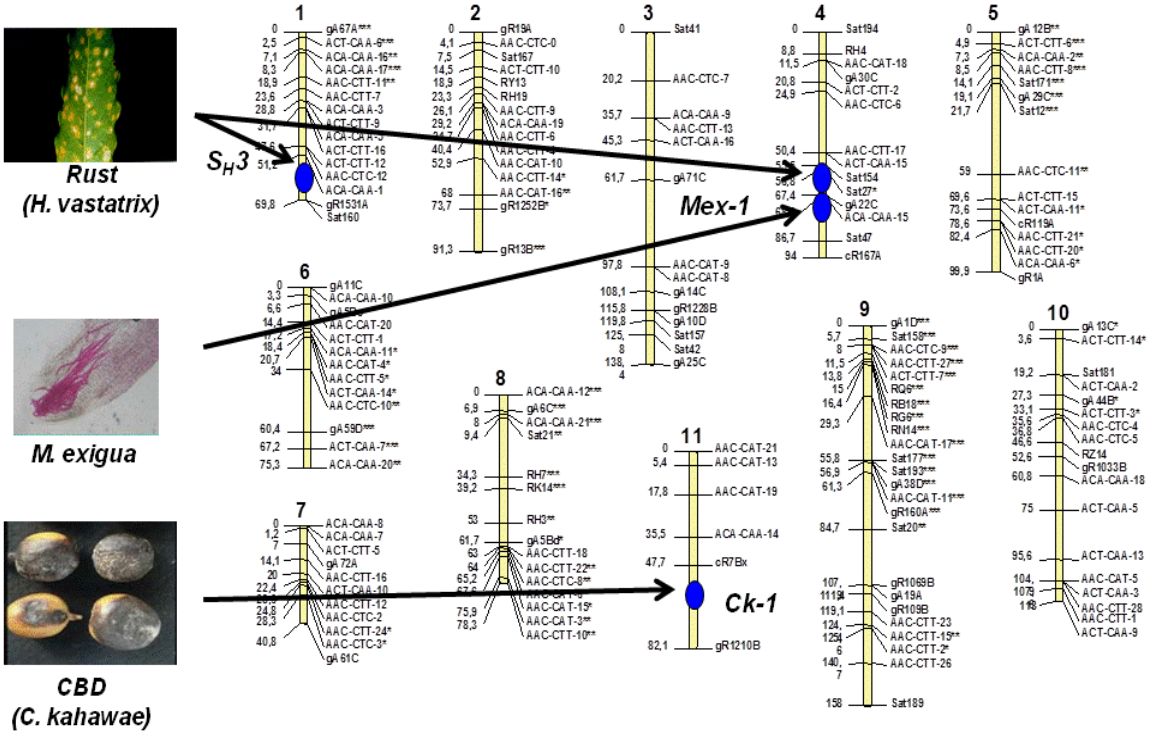

Figure 3. Partial genetic map of *C. arabica* with molecular markers for CLR (*S_H3*), CBD (*Ck-1*) and nematode (*Mex-1*) resistance genes (P. Lashermes, pers. comm.)

The application of molecular marker technologies has certainly accelerated selection progress of mono-/oligogenically-inherited plant characteristics, but has so far failed to significantly improve polygenically-inherited quantitative traits, such as yield, quality and tolerance to abiotic stresses. Tremendous progress in high-throughput genotyping at low costs and powerful statistical methods, that enable the simultaneous estimation of genome-wide marker effects, stand at the basis of a novel approach called "genomic selection", which has the potential of greatly advancing breeding efficiency for these quantitative crop traits (Jannink *et al.*, 2010). The value of candidates available for selection is no longer determined on the basis of the genotype with a small number of QTL (quantitative trait loci), but by estimating the effect of thousands, or even hundreds of thousands, of markers in a phenotype. The absence of *a priori* assumptions about causal relationships between markers and target traits allows the breeding of complex traits whose genetic basis is not completely known (Heffner *et al.* 2009). At the same time, marker-assisted selection allows massive and rapid transfer of targeted alleles or chromosomal segments from one gene pool to another and to test the transfer's phenotypic impact. It thus provides new insights in the effects of pleiotropy and epistasis and consequently leads to a better understanding of agronomic behaviour and biological processes that underlie them, while producing material of great agronomic value.

Research networking

Advanced coffee variety development will benefit tremendously from networks of collaborating coffee research centres, to facilitate the sharing of resources (financial, genetic and genomic), technologies and scientific information at the pre-breeding stages. There are

currently two active global networks on specific coffee research projects, viz. the CIFC (Coffee Leaf Rust Research Centre) and the ICGN (International Coffee Genome Network), but both are currently hampered by inadequate funding. CIFC has been supporting the coffee countries since 1955 in solving the CLR problem, by characterizing the regional variability of the pathogen and by pre-breeding for host resistance (Zambolim *et al*., 2005). The ICGN was established in 2005 with the objective of sequencing the coffee genomes and to develop molecular tools and resources to advance the development of improved coffee cultivars and so contribute to sustainable coffee production (ICGN, 2014b). Examples of regional and bilateral cooperative coffee research programmes are PROMECAFE (C. America) – CIRAD, ICCRI (Indonesia) – CIRAD & Nestlé-Research and CENICAFE – American Universities. World Coffee Research was founded in 2012, with public-private funding, aimed at variety development and multiplication serving all Arabica coffee regions in the world (WCR, 2014).

CONCLUDING REMARKS

Durable resistance to CLR, by pyramiding dominant SH genes and/or exploiting pathogen-specific susceptibility-genes, remains top priority in breeding programmes of Arabica coffee.

It is unrealistic to propose centralized variety development and multiplication programmes serving all Arabica coffee regions in the world. Development of new cultivars well-adapted to the local environment should be left to national or regional coffee research centres.

International cooperation should be restricted to the pre-competitive stages of Arabica coffee breeding: genetic resources, genomic technologies, sharing of scientific information and pre-breeding for specific characters (disease and pest resistances, tolerance to drought and other abiotic stresses, beverage quality, etc.).

Adequate coordination and funding of such cooperative projects by public and private entities are prerequisites for successfully contributing to the development of resilient hybrid cultivars needed for sustainable coffee production under changing conditions of diseases, pests and climate.

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Combining CBD Resistance with High Yields and Good Cup Quality: Success Case in Ruiru 11 Cultivar

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SUMMARY

Coffea arabica cv. Ruiru 11 is a composite of sixty six (66) F1 hybrid sibs each derived from a cross between a specific female and male population. The pedigree of Ruiru 11 consist of CBD resistance donors, Rume Sudan (*R* gene), Hibrido De Timor (*T* or *Ck-1* gene), Catimor (*T or Ck-1 gene*), K7 (*k* gene), SL4 and the high yielding, good quality but susceptible cultivars such as N39, SL28, SL34 and Bourbon. Ruiru 11 sibs reportedly present significant variability in terms of resistance to CBD, yields and quality. The objective of this study was to select for CBD resistance, high cherry yields and good quality within *Coffea arabica* L. cultivar, Ruiru 11. Thirty four hybrid sibs of Ruiru 11 cultivar grown in three different locations in Kenya were used for the study. Two entries of SL28, one of which was sprayed with fungicides against CBD, were used as checks. The experiment was conducted between 2009 and 2011. Ripe cherries were harvested, bulked per replication, weighed and yield data recorded before subjecting them to wet processing, drying, hulling and grading. Beverage quality was determined following the sensory evaluation procedure of Specialty Coffee Association of America (SCAA). Evaluation of CBD resistance was conducted in the laboratory using hypocotyl inoculation method. The study confirmed earlier reports that Ruiru 11 sibs differ in quality aspects, yields and resistance to CBD but some sibs that combine all these desirable traits were identified.

Strengthening Arabica Coffee (*Coffea arabica* L.,) Quality Breeding Research Strategy in Ethiopia through Optimum Use of Coffee *Terroir*

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SUMMARY

Arabica coffee (*Coffea arabica* L.,) breeding research strategy has been under continuous modification since the inception of the program in the late 1960 at the then Jimma Agricultural Research Station of Ethiopia. Recurrent prevalence of problems associated with coffee production and productivity, the presence of genetic and environmental diversity, the growth of speciality coffee industry and the presence of considerable genotype x environment interactions for economically important traits are some of the major driving forces among many others causing the progressive adjustment of coffee research strategy in the country. In this regard, the local landrace development program which has been in place since 2002 is the latest and an advanced coffee research strategy in the country. This program has demonstrated remarkable advances pertaining to effective use of local genetic diversity, maintaining typical quality profile of regions, alleviating local adaptation problems and maintaining farmers' preference for improved local varieties. The current intention is to strengthen the application of this strategy with great emphasis on quality improvement and profile mapping through optimal use of coffee *terroir*. The implication of *terroir* concept on coffee quality improvement, profile mapping and development of origin based speciality coffee varieties are discussed in this paper.

INTRODUCTION

Coffee is the economic power for Ethiopia that has been contributing the highest of all exports and shares the largest national revenues (MOFED, 2011). In terms of production, Ethiopia is the fifth largest coffee producer in the world after Brazil, Vietnam, Indonesia and Colombia with a production volume of 396,000 tonnes during the 2012/13 crop year (ICO, 2014). The country supplies mainly *arabica* coffee type to the national and international coffee markets. Arabica coffee has its origin in Ethiopia with an immense genetic diversity that is available distributed in different regions of the country (Anthony et al., 2001; Lashermes et al., 1996a; BRYNGELSSON et al., 2003a; Tesfaye et al., 2007). Particularly, South-Western part of the country is the richest coffee growing corridor for coffee genetic diversity. *Keta Muduga*, which is a well known coffee growing locality under Limu coffee type since ancient time in the south western part of Ethiopia, is known as the birthplace of arabica coffee (*Coffea arabica* L.,). It is located in Oromia regional state in Jimma Zone, Goma Woreda near Agaro town. For that matter, the Oromia regional state laid a foundation for coffee museum in 2007 announcing officially *Keta Muduga* as a birth place for arabica coffee and the coffee discoverer and goat herder, *Khalid*. Taking into account the existing genetic diversity in the area, the national coffee research center was established near to Jimma town at a village

called *Fisho* (7°40'03.5"N 36°47'12.6"E). Since the establishment of Jimma Agricultural Research Center (JARC) in 1958, different coffee research strategies have been followed to address coffee production and productivity related problems in the country. Coffee Berry Disease (CBD) resistant variety development program in early in 1970's (Girma et al., 2008) and local landrace development strategies since 2002 (Bellachew and Labouisse, 2007) have played great roles to safeguard the Ethiopian coffee industry. In this regard, coffee berry disease, adaptation and quality adulteration problems have been addressed.

As far as coffee is the economic power for millions of small scale farmers, great emphasis should be given to sustain the coffee industry of the country. The available genetic diversity is a stock for coffee researchers, growers, producers and the coffee community as a whole to conserve and utilize it systematically in promoting the Ethiopian coffee industry a step forward on the stages of international coffee markets. Since quality is among the other economically important traits which are available diversified in different agroecological coffee growing regions of the country (Bellachew and Labouisse, 2007; Dessalegn et al., 2008; Bekele et al., 2009; Getu et al., 2009; Bellachew, 1997), further investigation, discovery and promotion of the intact potential of Ethiopian coffee quality profile should be at the top of the agenda of coffee research institutes for the improvement of coffee research and development strategy of the country. Past experiences indicated that improvements from such diversity have brought great opportunities to develop very high quality cultivars for sale at exceptionally higher price (Bellachew and Labouisse, 2007). However, these great potentials have not yet been utilized effectively from the perspective of specializing products and breeding for quality.

In spite of the advancement of the program, existing opportunities with regard to genetic diversity, environment and market are still indicating the importance of further fine-tuning of the local landrace strategy to the context of *terroir* for better improvement of coffee quality breeding strategy in the country. Here, complementing coffee local landrace development program through coffee quality profile mapping and geographical indication system will be the last resort to advance coffee research and development approach of Ethiopia. Therefore, the objective of this paper is to indicate strategies for coffee quality breeding research program within local landrace variety development program to strengthen sustainable and traceable speciality coffee production systems in the country.

COFFEE QUALITY PROFILE MAPPING AND USE OF GEOGRAPHICAL INFORMATION: *TERROIR*

The presence of rich genetic diversity (BRYNGELSSON et al., 2003b; Lashermes et al., 1996b; Aga et al., 2005; Hein and Gatzweiler, 2006; Labouisse et al., 2008) with its significant economic value (Hein and Gatzweiler, 2006) and possessing diversified and economically important traits is one of the main opportunity for further improvement of the crop. Source of coffee improvement for any traits of interest such as yield, disease and pest resistance, drought/stress tolerance, typical quality, caffeine content, and others are available distributed in different physical environment and production systems of the country. Despite the advanced knowledge on coffee diversity for economically important traits, little is known about their growing environment, exact whereabouts (geographic locations), social and cultural setups affecting the expression of these traits; particularly quality profile of a given production system. For example, it is hardly possible to trace back the story of any coffee quality profile for its origin, variety type, production environment and any other related information. Since there has never been a well demarcated region of production, improved coffee technologies from research centers have been disseminated with no boundaries. This has been already observed and clearly indicated the nature and efforts made with regard to

coffee quality improvement program in the country. Thus, this dimension of coffee research is at its infant stage crippling still on the blanket recommendation of varieties rather than channelling varieties in to a well demarcated production system.

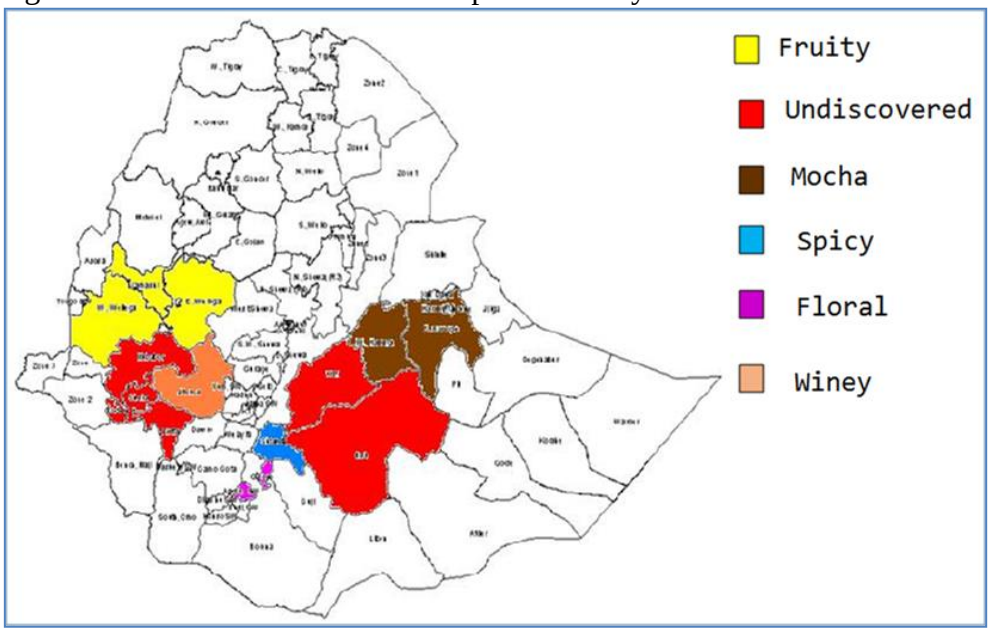

Figure 1. Coffee quality notes and their localities in Ethiopia.

So far, wide ranges of quality characteristics with respect to region have been pledged to the international market from Ethiopia. Spicy, floral, fruity, winey and Mocha flavour notes have already been known to the national and international coffee markets (Fig 1). Moreover, there are still untapped potential of coffee quality profiles that needs to be discovered and promoted. In spite of variable flavour notes, information pertaining to physical environment, detail quality profile, genetic diversity, geographical information, social setup and cultural practices associated with each region of distinct flavour note is scanty. As depicted on the map, there is no clear geographical boundaries for these flavour notes. Therefore, Ethiopian coffee quality profile mapping and demarcation needs to be the first step to lay basis and subsequently implement coffee quality improvement research and development activities within each region.

By considering the geographical locations of major coffee growing regions, areas of coffee collection history (JARC coffee genetic collection database), agroecological nature of the regions (rainfall distribution, soil type, and others) (MoA, 2000), information on grading and classification of coffee arrivals from different region by the national coffee quality liquoring unit of Ethiopia (Desse , 2008), and coffee local landraces development strategy (Bellachew and Labouisse, 2007), currently the national coffee research center is gearing its coffee research and development strategy towards specific localities by quality profile mapping and coordinated variety and verification trials. With a bigger theme of "Demand Driven and Export Oriented Coffee Technology Generation and Promotion" for south-western, western, eastern, southern , south-eastern and new potentials coffee growing areas of Ethiopia, the national coffee research center is heading towards developing the following coffee *terroirs (Fig 2-4).*

Coffee quality profile mapping is the process of characterizing coffee quality characteristics (physical, sensorial, biochemical, molecular) of an area in relation to its physical environment (soil, weather and others), coffee genetic diversity, ecological composition, social setup and cultural practices of coffee production, and finally the application of geographical indicator

system (GIS) to delineate a region to be used as a *terrior*. Various studies indicated the potential use of *terroir* in coffee production system (Mawardi et al., 2005; Avelino et al., 2002; Silva et al., 2014). Application of *terroir* in coffee is among the major opportunities available for coffee improvement programs of different countries around the world (Folmer, 2014); particularly Ethiopia is exceptionally endowed with rich natural coffee genetic diversity by growing under different production systems.

In the south-western and western coffee growing regions *terroir* classification, various factors were considered to start with classification of coffee producing localities to different *terroirs*. Continuum of geographical set up between regions, ecological compositions, production systems, altitudinal ranges, flavour notes already known to markets (fruity and winey flavour notes of Wallagaa and Limu respectively), registration as biosphere international heritage sites (Yayu and Bonga), uniqueness in terms of special varieties (like Geisha originated from Tepi and its surroundings *terroir*), disease hot spot area (CBD and CLR in Gera and Tepi respectively), soil type (dominant red soil type in Wallagaa) and other personal observations during research activities by the coffee research team and particularly by the authors, a total of seven coffee *terroirs* were nominated for further research, development and promotion activities.

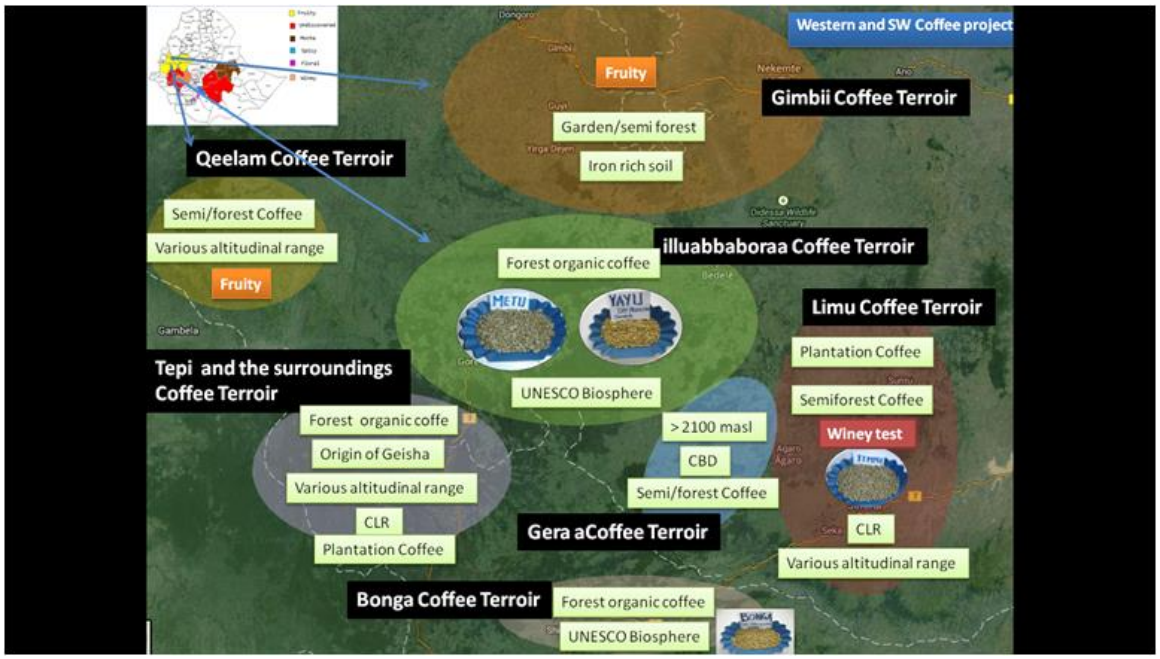

Figure 2. South-western and western coffee growing regions *terroir* classification in Ethiopia

The seven *terroirs* in this region hereafter referred and promoted as Qeelam Coffee Terroir (QCT), Gimbii Coffee Terroir (GiCT), Illuabbaboraa Coffee Terroir (ICT), Limu Coffee Terroir (LCT), Gera Coffee Terroir (GeCT), Bonga Coffee Terroir (BoCT), and Tepi and Its Surroundings Coffee Terroir (TSCT) (Fig 2).

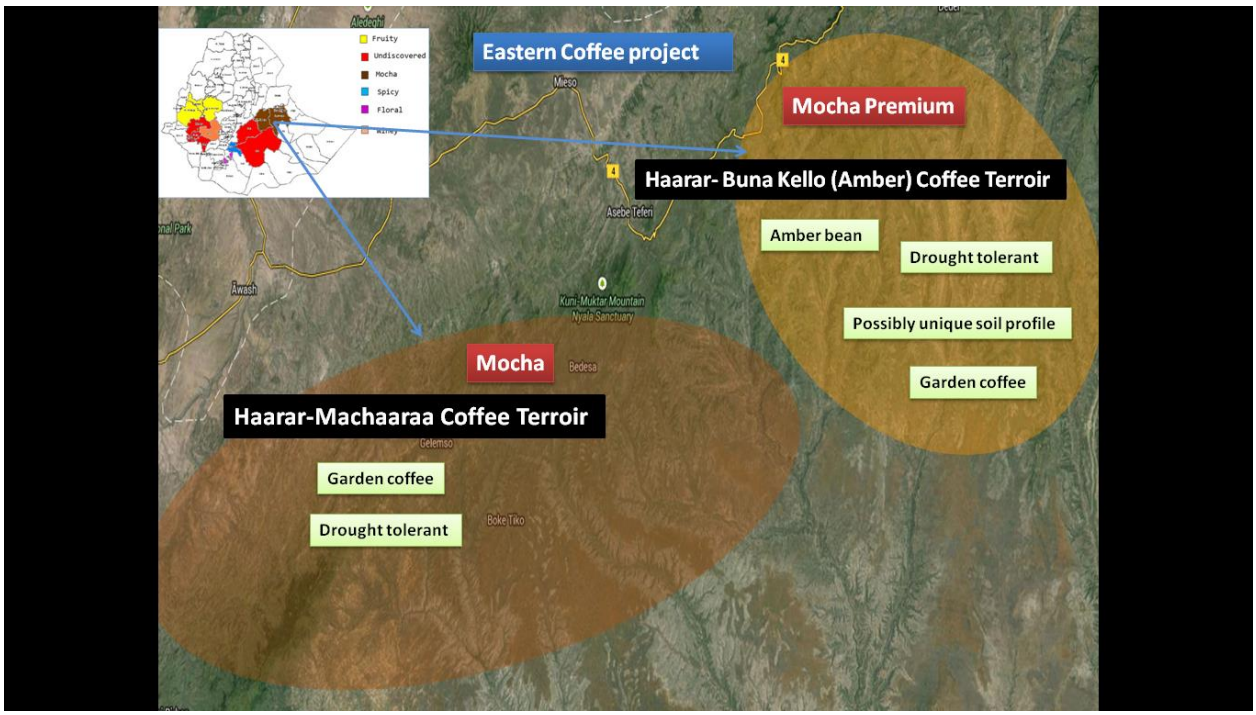

Figure 3. Eastern coffee growing regions *terroir* classification in Ethiopia.

Coffee lots originating from the eastern part of the country, mainly from Haararghe is well known to the international consumers for its dominant Mocha flavour. Sundried natural coffee from this region has been channelled on segmented markets for Haaraar coffee as amber-haaraar coffee beans that fetches extremely premium prices while other supplies from the region have standard haaraar coffee market prices. Considering the presence of market price differentiation, coffee research should jump in to understand profile map of those regions within Haarar to further upgrade and promote the product on the basis of *terroir* concept. So far on the basis of market segmentation and research experience in the area, two Haaraar coffee *terroirs* namely Haarar-Mechara (HM) and Haarar-Buna Kello(Amber) (HBK) coffee *terroirs* were targeted by the national coffee research to further investigate and promote the region as a *terroir* (Fig 3).

In the southern and south-eastern coffee research project, the well known Sidama, Yirgacheffe and Bale coffee types have been addressed. Despite of the popularity of Sidama and Yirgachefe coffee types in the international coffee markets, their exact whereabouts is known poorly to researchers, producers and consumers. Particularly, the Bale coffee type was not so far promoted beyond the national market. With the introduction of coffee *terroir* concept to these regions, the whereabouts of floral and spicy flavour notes from Sidama and Yirgachefe respectively can be demarcated. Moreover, new coffee quality notes from Bale can be supplied as a speciality coffee segment to the national and international coffee markets. Accordingly, the national coffee research center is striving forward to further promote coffee types from these regions as Sidama (SCT), Yirgacheffe (YCT), and Bale (BaCT) coffee *terroirs* (Fig 4).

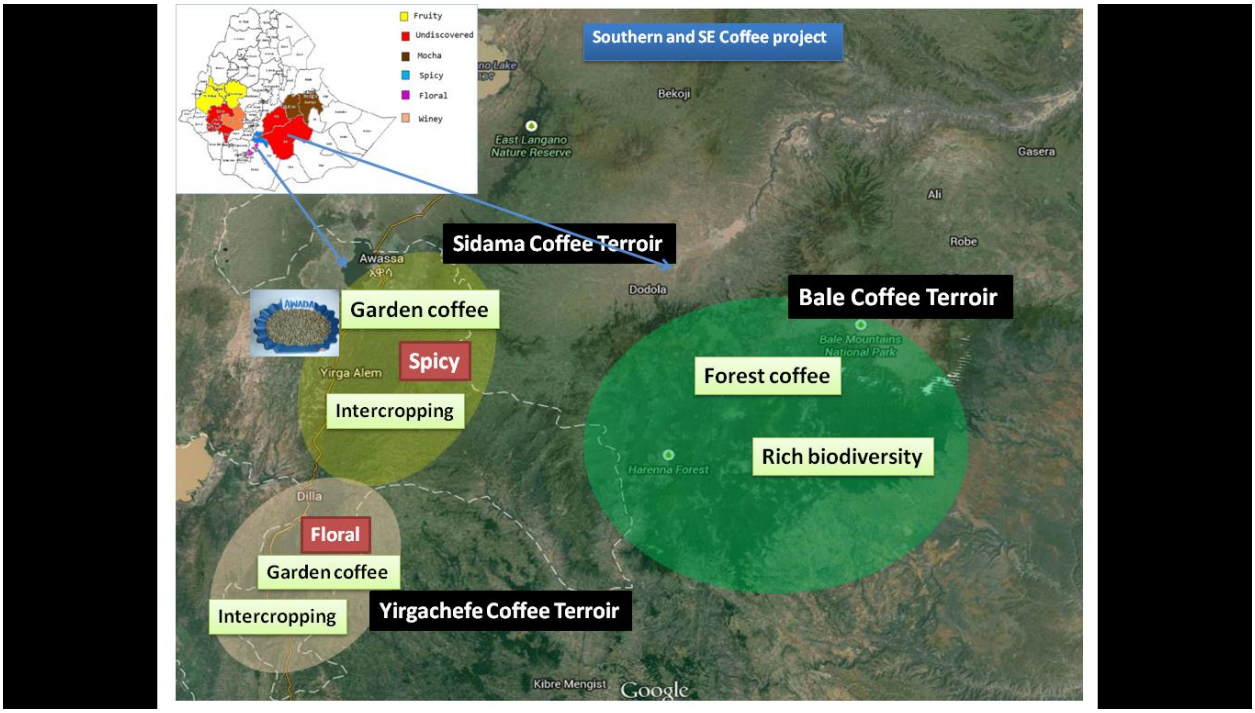

Figure 4. Southern and South-eastern coffee growing regions *terroir* classification in Ethiopia.

Moreover, there is a great potential of establishing coffee *terroir* for different traits like caffeine. Ethiopian coffee varieties with low bean caffeine content were identified so far that indicated the presence of untapped potential in the gene pool for further improvements (Silvarolla et al., 2000). Besides, environment plays a great role for differential profile of caffeine level in coffee varieties (Getu *et al*., 2009). As a result, caffeine can be profiled and produced under special *terroir* meant for zero or low caffeine production. However, further research should be carried out in depth prior to establishing and promoting such *terroirs*.

Generally, coffee quality profile characterization (mapping) and demarcation (*terroir* formation) is a mechanism to effectively utilize a naturally gifted coffee quality of an area that would help to produce wide ranges of product to be supplied to the international markets. With the establishment of *terroir*, respective coffee research and development activities can be carried out within a well delineated area to generate and disseminate coffee technologies. In this regard, breeding for coffee quality is the integral part of *terroir* concept.

COFFEE QUALITY BREEDING RESEARCH STRATEGY

Improved coffee variety development and channelling varieties to its appropriate area of production has been made through local landrace development program in Ethiopia. The destiny of the improved coffee varieties have been mainly decided on the basis of administrative boundaries rather than on a well characterized and delineated *terroirs*. For this matter, breeding for coffee quality needs a prior knowledge on the geographical reference points that indicate physical position of a given coffee quality profile of a region. Then, the presence of such prior foundation would help to direct collection, testing and distribution of improved coffee varieties to be carried out within these well delineated geographical regions. As a result, distinct, traceable and unadulterated coffee quality products will be proliferated and delivered to the international coffee market. This necessitates the application of coffee landrace development strategy within a *terroir.*

Pure line speciality coffee variety development within a *terroir*

Currently the national coffee research centre at Jimma is coordinating and conducting coffee research and development activities at the national level on five different projects, which are classified on the basis of coffee growing administrative regions. As indicated in Table 1, coffee variety development research activities have been carried out for respective administrative regions to develop varieties that represent the classical quality profile of an area. In this approach, each project has a wide range of agroecological zone representation within less characterized region.

Table 1. Project components of national coffee research program in Ethiopia.

Project	Region	Research centers	Coffee type	Quality profile[+]
Western	Oromia	Haru and Anfilo	Wallagaa coffee	Fruity[+]
South Western	Oromia and SNNP	Jimma, Agaro, Gera, Metu, Tepi,	Jimma, Limu,Illuababora, Gera, Bonga(Kaffa), Tepi	Winey and various undiscovered flavour notes
Southern	SNNP	Awada, Wenago	Sidama, Yirgacheffe, and others	Spicy and Floral[+]
Eastern and South-Eastern	Oromia	Mechara	Eastern and Western Hararghe, Arsi, Bale	Mocha [+]
New potentials	Different regions	In collaboration	To be studied	To be discovered

[+] *More flavour notes can be discovered*

So far, pure line (selection) and hybrid coffee variety development programs have been the two most commonly used approaches in the national coffee research coordination center of Ethiopia (Bellachew and Labouisse, 2007). In any crop improvement program, securing diverse starting material is at the first priority to improve targeted trait(s) of interest. For coffee, securing diverse local landrace breeding materials that best fits the production system of a region; henceforth called *terroir*, is important to develop pure line and subsequently hybrid speciality coffee varieties. Starting from 1966 to 2009, a total of 6385 coffee accessions were collected by Jimma Agricultural Research Center and other institutions (Table 2). This coffee genetic resource has been conserved *ex situ* at JARC and its sub centers. A wide range of economically important traits viz., coffee berry disease resistance, drought tolerance, stress tolerance, coffee leaf rust resistance, yield, caffeine, physical and sensorial quality, insect-pest resistance, early ripening, late ripening, coffee wilt disease resistance and others were noted in this gene pool. In Labouisse et al (2008), it is reported that a total of 5196 mass collected coffee accessions by Institute of Biodiversity Conservation (IBC) were also conserved *ex situ* at *Choche* around *Agaro* in 2006. The full description of the genetic resources is given in Table 2. Despite the fact that these collections are sources of such valuable traits for further coffee improvement, significant percent of tree loss (*ex situ* genetic extinction) is recorded mainly because of biotic and abiotic stresses and poor field conservation practices. In order to safeguard the resource, it is important to consider analysing the diversity at a molecular level to establish core collections and thereby design appropriate conservation strategies like the application of cryopreservation techniques.

Table 2. Original number of coffee accessions collected or selected for coffee improvement program in Ethiopia.

Collection program	Collected by	Main target	Regions addressed	Years of collection/selection	Original Number of accessions	Conservation sites by JARC
National collection to catch existing coffee genetic variability	JARC	Diversity, some CBD resistance, and some adaptation to low altitude	Oromia, SNNPR, Gambella, Tigray, Amahara	1970-1974, 1977-1979, 1981-1985, 1987, 1989, 1990, 1994-1997	1149	Melko, Gera, Awada
French & Semu Negus	FAO	Diversity	Oromia & SNNPR	1966	73	Melko and Gera
Ministry of Coffee and Tea Development (MCTD)	MCTD	Diversity	Oromia	1985	95	Melko
International	JARC	Diversity & Rust resistance	India, Tanzania, Portugal, Brasil, Costa rica, Cuba	1967, 1968,1979, 1984	100	Melko and Gera, Tepi
CoCE collections	CoCE project	Different study	Oromia, SNNPR	2004	60	Melko
CBD resistant selections and Wide adaptation	JARC	CBD resistance and yield	Oromia, SNNPR	1973-1975,1980-1982,1985,1987	1041	Melko and Gera
Stress tolerant	JARC	Stress tolerance	SNNPR	1988	45	Melko
Landraces selections based on mother tree CBD reaction	JARC	CBD resistance and diversity	Oromia	2000-2001, 2003-2005	407	Agaro, Haru, Mechara
Landraces variability	JARC	Landrace diversity	Oromia, SNNPR	1998-1999, 2002, 2004, 2007-2009	3415	Haru, Gera, Melko, Tepi, Jinka, Mechara
Total number of accessions (JARC)					6385	
Total number of accessions (IBC)					5196	
Grand Total					11,581	

So far, with the implementation of various coffee improvement strategies by JARC, a total of 37 coffee varieties have been developed from such diverse coffee gene pool. Basically, less number of traits was targeted despite of the wide range of economically important traits. For example, zero caffeine, drought tolerant, stress tolerant and coffee wilt disease resistant varieties should have been among the list of released varieties in the catalogue (Table 3). In terms of percentage representation by improved varieties, south-western coffee growing region takes the largest share (67.6%) while western, eastern and southern coffee growing regions each has 10.8%.

Table 3. Lists of released Arabica coffee (*Coffea arabica* L.,) varieties in Ethiopia.

Strategy	Variety Group	No.	Varieties	Year of release	Yield (Qt/ha)	Recommended altitude	Recommended area of production
Landrace development	Wallagaa Speciality	1	Haru-1	2010	15.66	1750-2100	Wallagaa province(4 zones)
		2	Challa	2010	15.55	1750-2100	
		3	Sende	2010	16.08	1200-1750	
		4	Manasibu	2010	16.43	1200-1750	
	Haarar Speciality	5	Harusa	2010	16.02	1200-1750	Hararghe (West)
		6	Mocha	2010	13.46	1200-1750	
		7	Mechara-1	2010	11.89	1200-1750	

		8	Bultum	2010	17.06	1200-1750	
	Sidama/Yirgacheffe Speciality	9	Fayate	2010	20.21	1740-1850	Sidama and Gedeo
		10	Odicha	2010	19.47	1550-1750	
		11	Koti	2010	11.6-21.15	1740-1850	
		12	Angafa	2006	20.4	1550-2100	
Highland coffee development	Gera Highlands Speciality	13	Merdacheriko	2006	15.4	1750-2100	Gera Highlands
		14	BunoWashi	2006	23.5	1750-2100	
		15	Yachi	2006	19	1750-2100	
		16	Wushush	2006	16.4	1750-2100	
Coffee Leaf Rust Resistance	CLR Resistance	17	Catimor J-19	1998	16.6	1000-1400	Bebeka, Tepi
		18	Catimor J-21	1998	19.4	1000-1400	
		19	Gesiha	2002	18-25.4	1000-1400	
Hybrid	Hybrid	20	Ababuna	1998	23.8	1000-1750	Metu, Jimma, Goma
		21	Melko-CH2	1998	24	1000-1750	
		22	Gawe	2002	26	1550-1750	
CBD resistance	CBD Resistance	23	741	1978	12.2	1550-2100	Jimma, Gera, Agaro
		24	744	1979	16.6	1550-2100	Jimma, Gera, Metu
		25	7440	1979	16.2	1000-2100	Gera, Jimma, Tepi
		26	7454	1980	18.3	1000-2100	Gera, Tepi
		27	7487	1980	23.8	1550-2100	Gera
		28	74110	1979	19.1	1550-2100	Gera, Jimma, Metu
		29	74112	1979	18.1	1550-2100	
		30	74140	1979	19.7	1550-2100	
		31	74148	1980	18	1550-2100	
		32	74158	1979	19.1	1550-2100	
		33	74165	1979	17.3	1550-2100	
		34	754	1981	14.8	1550-2100	
		35	75227	1981	17.9	1550-2100	
		36	Dessu	1998	18.2	1000-1750	Jimma, Metu, goma, Tepi
		37	Mi'oftu	2002	21.2	1550-1750	Jimma, Metu

Coffee science and knowledge advance and the prevalence of various forms of problems in coffee production system are the main driving forces behind recurrent coffee variety development strategies over time. Since 1970's, breeding for coffee berry disease resistance, hybrid development, coffee leaf rust resistance (CBD) and local landrace variety development approaches have been the main breeding strategies that resulted in the development of 37 improved coffee varieties. CBD and yield were the major traits addressed in those strategies. However, quality was basically a secondary trait of interest which had been evaluated at the final phase of verification plots. In the concept of *terroir*, quality is an integral part of variety development program that should be considered starting from the early phase of an experiment.

Improved coffee variety was released in the country at a rate of one variety per year. On average, only one coffee growing area could be addressed by receiving a single variety each year. Despite the fact that Ethiopia is ranking at the top by releasing large number of improved *arabica* coffee varieties, as compared to the availability of diverse agro ecology and genetic material, the number of released coffee varieties so far are extremely less. In this regard, from a total of 6305 *ex situ* conserved coffee genetic resource, 0.6% of the gene pool has been released. Moreover, few traits were only addressed in spite of several economically important traits available in the gene pool. Breeding for quality on the basis of *terroir*, would create various well demarcated coffee growing profiles that alone need large number of improved varieties per *terroir*. Therefore, this strategy triggers the country's coffee research

program to utilize its coffee genetic resource effectively and make available large number of varieties at the disposal of coffee growing farmers.

Table 4. Assessment of released coffee varieties from the perspective of terroir and local landrace concept.

Variety Group	Project region	Quality profile of the region	Variety vs quality profile of an area match	Terroir Concept
Wallagaa Speciality	Western	Fruity	Compliant	Establish Geographical Information for the area
Haarar Speciality	Eastern and South Eastern	Mocha	Compliant	
Sidama/Yirgachefe Speciality	Southern	Floral and Spicy	Compliant	
Gera Highlands Speciality	South-western (Specific for Gera)	Several flavour notes (winey)	Compliant	
CLR Resistance*	South-western	Several flavour notes	Non-compliant	Quality profile of an area to be characterized and re-assessment of these varieties for quality
Hybrid*				
CBD Resistance*				

**Re-assessment and re- recommendation of this group of varieties*

Since the noncompliant matches between improved coffee varieties and their region of production on the basis of local landrace and *terroir* concept (Table 4), reassessing the quality of those varieties on their area of current production or further re-recommendation back to their region of origin (collection site) needs to be accomplished. This pattern of mismatch with the concept has been observed particularly for south western coffee varieties which were released from the period 1977 to 2002. Adjustment of these varieties back to their region of origin is highly recommended or development of new varieties and replace those farms in the region will be the other option. For this reason, JARC has already started variety development activities specifically within south western coffee growing regions by classifying localities as Limu Coffee, Gera Coffee, Illuababora Coffee, Keffa Coffee and Tepi and its surroundings Coffee.

To advance the local landrace variety development strategy, we introduced the approach of coordinated variety and verification trial for the development of pure line and hybrid speciality coffee varieties within a *terroir*. Since there is always large number of collections available for the preliminary screening, the experiment can be established from the very beginning in two sets of experiments at two testing sites within a well-delineated coffee production *terroir*. From previous coffee improvement experiments, we could learn that most of those released varieties were among top performing genotypes consistently in a series of experiments viz., preliminary screening, variety, and verification trials. Therefore, this trend is an indicator for coffee researchers to refocus on coordinating preliminary, variety and verification trials on one platform called foundation experimental plot. The concept of coordinated variety and verification trial within a *terroir* is still in line with the local landrace coffee improvement research strategy. In a coordinated experiment, coffee accessions are evaluated and tested for traits of interest on a single plot that can serve simultaneously as a

foundation experimental platform to assess accessions and release best performing ones as a speciality coffee variety within a *terroir*. The foundation experimental plot will serve as a conservation, characterization, variety trial, verification trial, crossing block and seed orchard. The following coordinated variety and verification trial of local landrace development strategy describes the procedure for developing *arabica* coffee pure line speciality coffee varieties within a *terroir.*

Step 1. Preparation and molecular study phase (Preparation year)

- Access starting breeding materials (landrace collections/others)
- Raise seedlings (nursery)
- Collection of leaf samples for molecular study (Next generation Sequencing is currently best) (nursery)
- Selection of two representative testing sites within a *terroir*

Step 2. Field establishment phase (Preparation year)

- Field experiment establishment at two sites (simple lattice or RCBD with three replications)

Step 3. Field experiment management and observations (Year 2)

- Observation on seedlings and leaf disease (CLR, insect pests)
- Molecular data should be ready within one year (publish molecular genetic diversity and identification of molecular markers)

Step 4. Fly crop data (Year 3)

- Yield, quality, disease, soil, weather data

Step 5. 1st -6th year crop (Year 4 to 9)

- Yield, quality, disease, soil, weather data
- At 6th year, select promising accessions and establish seed orchard to be used immediately after variety release **(optional)**
- Select best parental lines to develop **hybrid speciality coffee varieties** (Coordinated variety and verification trial: evaluate hybrids at two testing sites following the procedure of pure line variety development program and variety to be released at 9th year of the experiment. Mapping population study can be designed for molecular study in this hybrid experiments)
- Association studies (phenotype and genotype data will be available at this time)
- Marker assisted breeding program designing

Step 6. Variety release approval (Year 9-10)

Step 7. Multiplication and distribution of varieties within a terroir

Hybrid Speciality Coffee Variety Development

Intra specific F1 hybridization of arabica coffee is one of the breeding approaches followed in Ethiopia to develop hybrid coffee varieties (Bellachew and Labouisse, 2007; Ameha and Belachew, 1984). The approach has been practiced shortly after the outbreak of CBD in Ethiopia in 1970's. Despite of all the progress made so far to secure high yielding and disease resistant coffee hybrids, quality profile representation by hybrid varieties still need further investigation. Thus, study on mode of inheritance of coffee quality traits is one of the basic approaches to develop speciality coffee hybrids that can represent typical coffee quality

profile of a *terroir*. The pure line speciality coffee variety development strategy described above will be used similarly for hybrid coffee variety development. Based on the performance of accessions at 6th year of pure line speciality coffee development program, promising parental lines will be selected and crossing is carried out at the seventh year. Then, F1 seeds are collected to establish experimental plots for hybrid speciality coffee variety development. The same number of experimental sites, years of evaluation and types of data with pure line development program will be considered.

POSSIBLE CHALLENGES

Since there is no a single institute being responsible to coordinate coffee research, development and marketing together in Ethiopia, the hope of specializing and advancing the coffee industry and improving the life of poor coffee growing farmers is diminishing over time. Most of coffee producing countries around the world have a focal entity as a board or institute to organize coffee industry as a whole. However, despite of such a very good experiences around the world, the coffee sector of Ethiopian has remained scattered around lacking focal point, common interest among coffee actors along the value chain and integration to overcome problems in coffee. As a result, bringing the coffee industry to a better place is hardly possible if the trend continues. Therefore, the *terroir* concept can only be made real if and only if there is a well organized coffee institute to utilize naturally available coffee resource and make a great opportunity out of it for poor coffee growing farmers and the country as a whole.

CONCLUSION

The presence of great opportunities in the coffee industry of Ethiopia; particularly pertaining to genetic and agro ecological diversity, and high market demand should encourage the country to utilize its untapped potential by producing different coffee types for commodity, speciality and organic coffee market. For this reason, a well organized coffee research institute needs to be functioning to accomplish coffee quality profile mapping and application of geographical information to develop coffee *terroir*. Afterwards, this is the basic transition point to target coffee breeding research strategy gearing towards complementing local landrace development program by *terroir* concept and racing for developing distinct and traceable high quality coffee varieties. Henceforth, the idea of speciality coffee variety development is capitalized within *terroir* concept. Accordingly, **speciality coffee variety** is defined as a *pure line or hybrid coffee variety that grows under a well delineated and traceable coffee terroir by possessing a distinct quality (physical, sensorial, biochemical) profile.*

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A bitter cup: Climate Change profile of global Arabica and Robusta production

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SUMMARY

Coffee has proven to be highly sensitive to climate change. We employ a spatially explicit machine learning based ensemble modeling approach to generate global climate change impact scenarios for the two commercially dominant coffee species *Coffea arabica* and *Coffea canephora*. Functions of current climatic suitability are derived from a comprehensive database of geo-referenced production locations. Using a multi model mean of models with several parameter combinations enhances the robustness of our analysis. The models suggest that higher temperatures may affect *C. arabica* while *C. canephora* could suffer from increasing intra-seasonal temperature variability. The analyses of the distribution of climate change impacts on coffee suitability supports the argument that on a global scale climate change has negative effects at low latitudes and low altitudes. Impacts at higher altitudes and higher latitudes are less pronounced, though not positive. The world's dominant production regions in Brazil and Vietnam may experience substantial reduction of suitable area due to climate change. Opportunities for coffee production from positive suitability changes occur in East Africa and Asia. In the latter region these gains are located in mostly forested areas, which could pose a challenge to mitigation efforts.

INTRODUCTION

The majority of coffee production is based on two distinct species. Robusta coffee (*Coffea canephora* var. Robusta) is generally seen to be more tolerant towards heat, but more susceptible to low temperature than Arabica coffee (*Coffea arabica).* The predominant Arabica coffee production has previously been shown to be negatively affected by climatic changes (e.g. Gay Garcia et al. 2006, Zullo Jr.et al. 2011, Schroth et al. 2009). The suggested latitudinal (Zullo Jr.et al. 2011) or altitudinal (Schroth et al. 2009) migration of major coffee production regions could be a serious threat to the livelihoods of coffee producers (Baca et al. 2014) and ecosystems (Läderach et al., 2009). Furthermore, among stakeholders uncertainty prevails to what extent the *C. canephora* species may be able to replace the higher quality *C. arabica* based production on commodity markets.

Thus, previous studies on climate change impacts demonstrate possible drastic impacts on coffee cultivation: Latitudinal migration, altitudinal migration or complete abandonment of coffee production. However, results are limited to local levels and global trends remain unclear.

Two studies by Davis et al. (2012) and Schroth et al. (2009) investigate climate change impacts on *C. arabica* using the MaxEnt species distribution modeling software (Phillips et al. 2006) that is based on machine learning concepts. It has been suggested that such models are prone to fitting to a biased representation of suitable climate when not used with

appropriate parameter choices. To overcome this limitation the use of ensemble outputs of various models has been shown to be more robust and allow for explicit uncertainty analysis (Araujo and New 2007).

MATERIALS AND METHODS

To enable the classification of current and future climate data as suitable or unsuitable for coffee production we first assemble a comprehensive global dataset of known presence locations of either coffee species. Then we use three popular machine-learning algorithms (Support Vector Machines (Karatzoglou et al. 2005), Random Forest (Breiman 2001) and MaxEnt (Phillips et al. 2006) and train 45 distinct parameter combinations each, resulting in a total of 135 models. Finally, the models are extrapolated on interpolated climate data of current and future climatic conditions and the mean suitability score for each global pixel cell derived. We analyze impacts for latitude, altitude, regions and land-use classes to hypothesize future impact scenarios in global coffee production.

Climate variables

For the current climate (1950–2000) we used the WorldClim global climate data set on 2.5 ArcMin resolution (Hijmans et al. 2005). The dataset provides interpolated climate variables representing patterns found in monthly weather station data, e.g. annual temperature and precipitation extremes, seasonality and means. Future climate data layers are generated by downscaling the native outputs of 5 GCM's (GFDL-ESM2M, HadGEM2-ES, IPSL-CM5A-LR, MIROC-ESM-CHEM, and NorESM1-M) from the 5th assessment report for the representative concentration pathways (RCP) 2.6, RCP 6.0 and RCP 8.5 using the delta method as in Ramirez and Jarvis (2010).

Presence data

Presence location data locate climates that are currently suitable for coffee production. The presence points in our database are derived from three principal sources: (i) Geo-referenced coffee farms, (ii) geo-referenced coffee producing municipalities for large coffee growing areas without coffee farm data and geo-referenced coffee growing areas identified from satellite data (google earth) where data source (i) and (ii) was not available.

After stratification the resulting presence dataset included 2861 unique pixel cells for *C. arabica* distributed over 26 countries that together account for 92% of global Arabica output in the period '98-'02 (USDA 2012). For *C. canephora* the presence dataset included 364 unique pixel cells distributed over 11 countries that together account for 92% of global Robusta output in the period '98-'02 (USDA 2012). Figure 1 shows the distribution of coffee locations and major production regions.

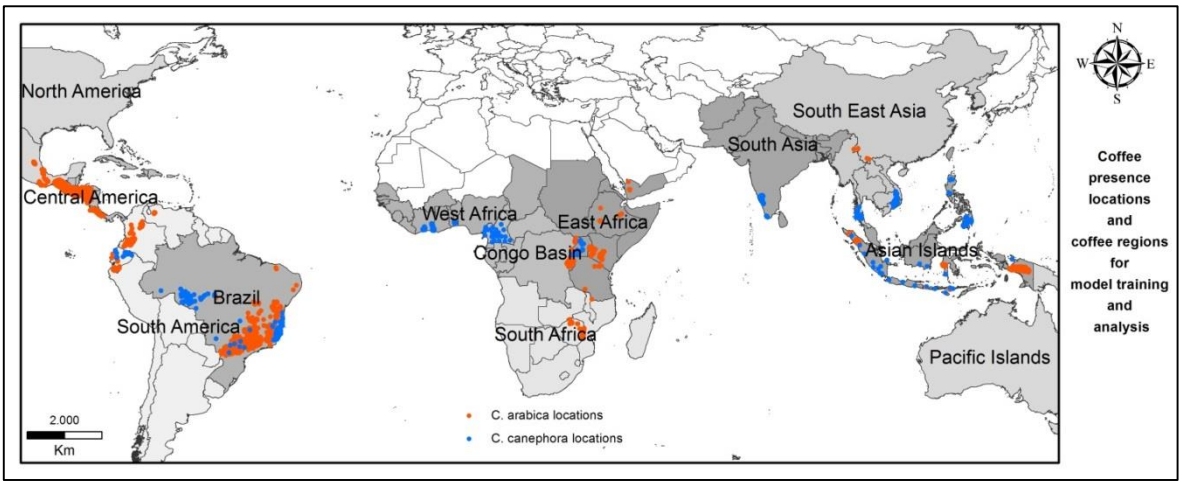

Figure 1. Global coffee location database and major coffee growing regions. Blue points represent *C. canephora* presence locations; orange points locations of *C. arabica* based production. Grey shading and bold names represent regions of coffee production.

Background sampling

In order to fit a function that describes suitable climates the classification algorithms compare the variable pattern found at presence locations with the pattern found in potentially suitable environments. To characterize these environments random samples are taken from locations that are not known presence locations.
We employed three different background concepts, a political one, a biophysical one and a geographic one. The first background is defined over all Robusta or Arabica producing countries (USDA 2012) respectively, the second by limiting the environment to the observed spread of annual mean temperature for each species location sample (*C. arabica*: 14°C – 26.4°C; *C. canephora* 19.2°C – 27.8°C annual mean temp.) and the third by using a 4.5° buffer around presence locations.

The literature agrees that the ratio of background samples to presence locations should be at least 1:1. Too few background samples do not allow for a clear distinction between presence and background, commonly leading to an over-prediction of distribution, while too many background samples result in under-prediction (Barbet-Massin et al. 2011). We use presence location to background sample ratios 1:1, 2:1, 4:1, 6:1, 8:1.

Model training

For the climate suitability mapping we relied on the classification probabilities as provided by three popular machine-learning algorithms: MaxEnt, Support Vector Machines (SVM) and Random Forest. MaxEnt (Phillips et al. 2006) is one of the most popular species distribution modeling software in ecology (Merow et al. 2013). SVM is among the most widely used classification algorithms. We use the implementation in the R package "kernlab" (Karatzoglou et al. 2005). Furthermore, Random Forests (Breiman 2001) are increasingly popular and have been shown to be useful in ecology (Prasad et al. 2006).

Machine learning algorithms include a regularization parameter that allows the user to adjust a trade-off between optimal model fit and generalization. For the MaxEnt regularization parameter β we choose 0.01, 5 and 20: for SVM's c-cost parameter 1, 0.5 and 0.05; and for the number of variables picked at nodes by Random Forest 8, 4, and 2.

The three algorithms are trained using the above described parameter spaces. For each of the three extents we employ five different background to presence ratios and three regularization choices. Thus, we train 3*3*5*3= 135 distinct models per species.

Model Evaluation

To assess the performance of the individual models we use two measures: The threshold independent area under the receiver characteristic curve (AUC), and a calibrated AUC measure (cAUC) derived from a trivial null model based Hijmans (2012). Variable importance is estimated by computing AUC on each predictor variable individually using the Caret package in R (Kuhn 2008).

Impacts

The model outputs are continuous probability scores whether a pixel cell belongs to the absence or presence class from 0 to 1. We compare impacts across latitude and altitude classes. The suitability score is summed up across 1° latitude classes and 100m altitude classes. Regional analysis of impacts is done for 12 regions of coffee production (Figure 1). We use the GLC2000 global land cover database (European Commission 2003) to partition suitability changes to land with forest cover (GLC2000 global categories 1-9) and land without forest cover and agricultural land (GLC200 global categories 10-18). A conversion from natural forest to coffee plantations would result in a negative environmental impact. On the other hand, this would not necessarily be the case in a conversion from open land to coffee plantations.

RESULTS

Model validation

AUC values are consistently high across all model set ups. The lowest AUC value for Arabica coffee is .92 and .73 for Robusta, indicating that they perform much better than chance at discerning presence from background locations. Considering the values that are compared to the performance of a simple null model, cAUC, the majority of the models perform better than the distance based model. All models for Arabica coffee are better than the null model according to cAUC. The Robusta models perform better than a null model in 74% of the cases according to cAUC.

Variable contribution

Across all models for Arabica the mean temperature of the warmest quarter is ranked the most influential variable that contributes most to the suitability distribution. This is followed by the maximum temperature of the warmest month. Among the temperature variables the two that indicate temperature variability (Bio 2 and Bio 7) are least influential. The latter contrast with the Robusta models where the mean diurnal range of temperature (Bio 2) and the annual temperature range (Bio 7) are consistently ranked high. Least important are the temperature in the coldest quarter (Bio 11) and the precipitation during the coldest quarter (Bio 19) respectively.

Current coffee suitability

The trained and tested models are extrapolated on raster data for the 19 bioclimatic variables from WorldClim resulting in a global map of current suitability for coffee production (

Figure 2). The current distribution of *C. arabica* based production centers on the Brazilian Minas Gerais province, Central America and shows high suitability values in the Ethiopian highland region. Madagascar is also seen to be highly suitable despite not being a major producer today. Asian origins, and many African origins, are rated as predominantly of intermediate climatic suitability for Arabica production. Larger patches of highly suitable area for *C. canephora* based production can be found in the Brazilian Espirito Santo region, West Africa, the lower regions of Central America and in mountainous locations in Asia, especially the Philippines, Indonesia and Vietnam.

Future coffee suitability

Application of the models to climate information for the 2050s period (2040-2069) yields maps for the future distribution of coffee production. These maps are averaged and the difference to current suitability conditions is calculated.
Figure 3 (A-D) shows the changes by 2050 in the RCP 6.0 climate change scenario for the dominant production regions of *C. arabica* in Latin America, Brazil, Asia and the species origin in East Africa. **Errore. L'origine riferimento non è stata trovata.**(E-G) shows the changes in suitability for *C. canephora* based production by 2050 in the RCP 6.0 scenario GCM outputs for Brazil, its region of origin West Africa, and the most important region of Robusta production in South East Asia and the Asian island states.

Distribution of climate change impacts

The suitability scores indicate how likely it is that a location is climatically suitable for coffee production. A higher sum of scores therefore means more suitable area. The sum of suitability scores across latitudinal meridians for current climate conditions and GCM outputs for scenario RCP 2.6, RCP 6.0 and RCP 8.5 is shown in **Errore. L'origine riferimento non è stata trovata.**. *C. arabica* loses suitability across all latitudes. Only in very high latitudes the losses are not as pronounced. For *C. canephora* losses seem to occur mostly at low latitudes.

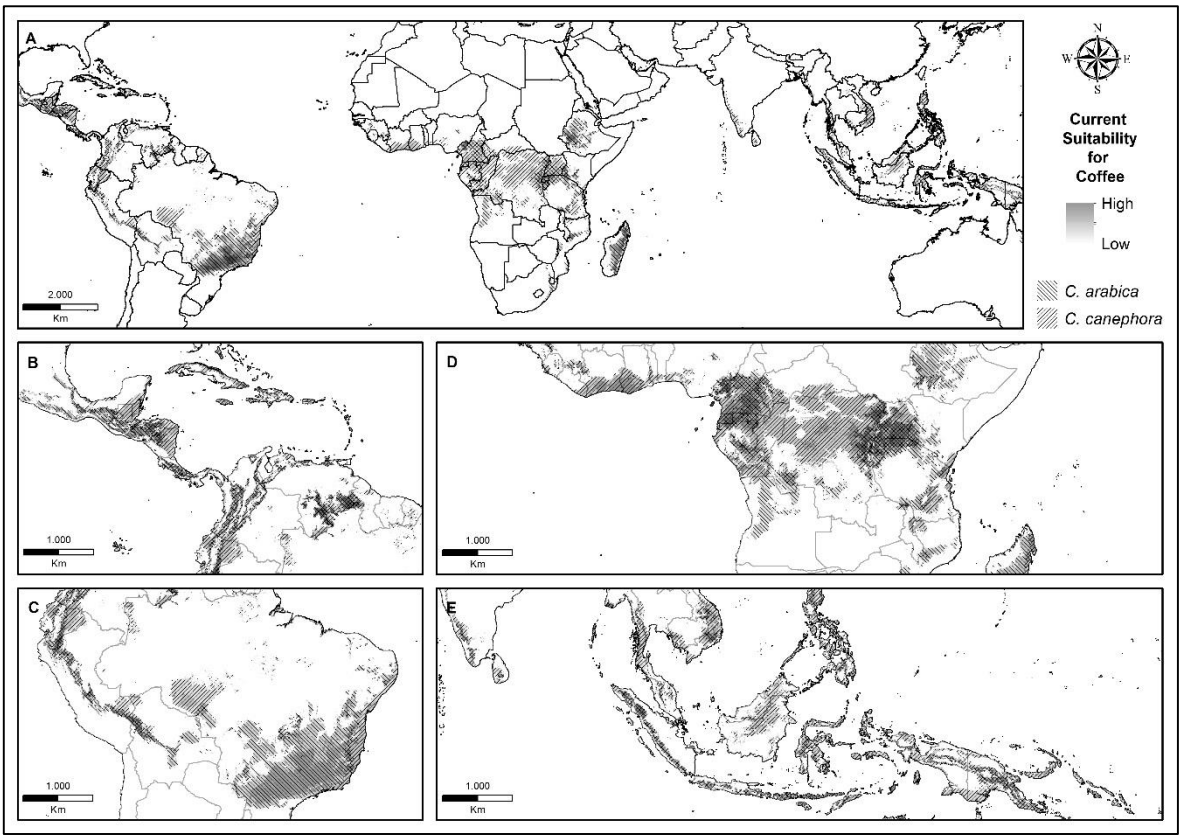

Figure 2. Current suitability distribution for coffee. Dark grey indicates high suitability, light grey intermediate suitability. Hatching indicates the species.

The sum of suitability scores in discrete altitude classes for both *C. arabica* and *C. canephora* by 2050 in the mean of RCP 2.6, RCP 6.0 and RCP 8.5 scenario GCM outputs and under current conditions is shown in
Figure 4b. Both species lose large shares of total suitability mostly in low altitudes below 1000 m.a.s.l. while relative losses in higher altitudes are not as drastic.

The sum of suitability scores for major coffee production regions for current conditions and of the mean of GCM outputs for the scenarios RCP 2.6, RCP 6.0 and RCP 8.5 by 2050 is shown in **Errore. L'origine riferimento non è stata trovata.**c. The largest loss of suitability can be observed in Brazil and South East Asia for Arabica coffee. In these regions the accumulated suitability score lost ranges between 85% in the RCP 8.5 scenario at least 30% in the RCP 2.6 scenario.

The least impact on Arabica is projected for East Africa and the Pacific Island region with 10% of suitability lost in the RCP 2.6 scenario and up to 30% in the RCP 8.5 scenario. *C. canephora* suitability is lost in the Congo basin. There 60% (RCP 2.6) to 95% (RCP 8.5) of total suitability may be lost in the species origin. Again, East Africa is projected with the least impact. In the RCP 2.6 scenario 16% of suitability may be lost here, and 30% in the RCP 8.5 scenario.

In
Figure 5 the changes in suitability are distributed according to land use classes in the coffee producing regions by 2050 in the RCP 6.0 scenario GCM outputs. Most losses and gains in suitability are equally distributed across area with forest cover and without forest cover, while gains are modest. In Brazil suitability losses are entirely observed for areas without forest

cover, while in East Africa substantial areas without forest cover show suitability gains. In contrast in Asia most suitability gains are in areas with forest cover.

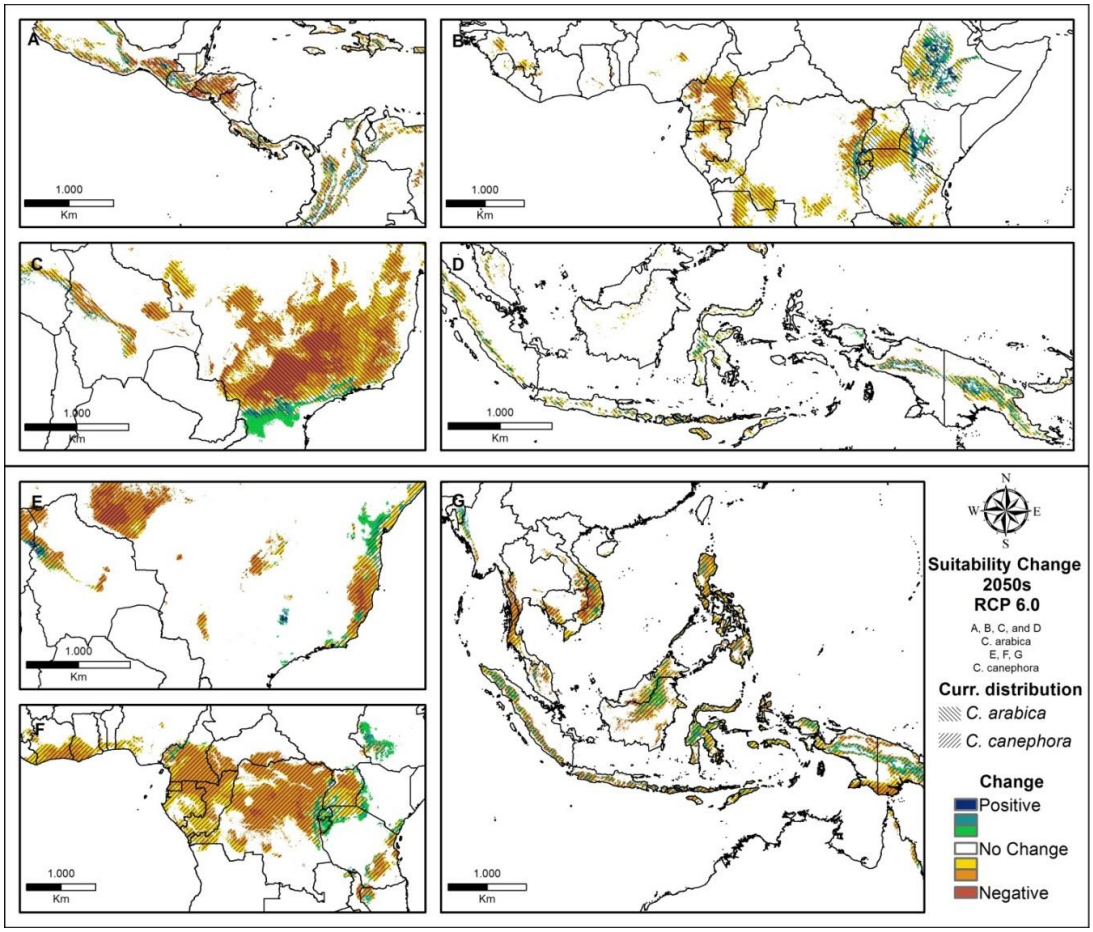

Figure 3. Suitability changes by the 2050s in the RCP 6.0 scenario; A-D: Arabica, E-G: Robusta. Hatching indicates the current suitability distribution; Warm colors represent areas with negative climate change impacts and cold colors positive changes.

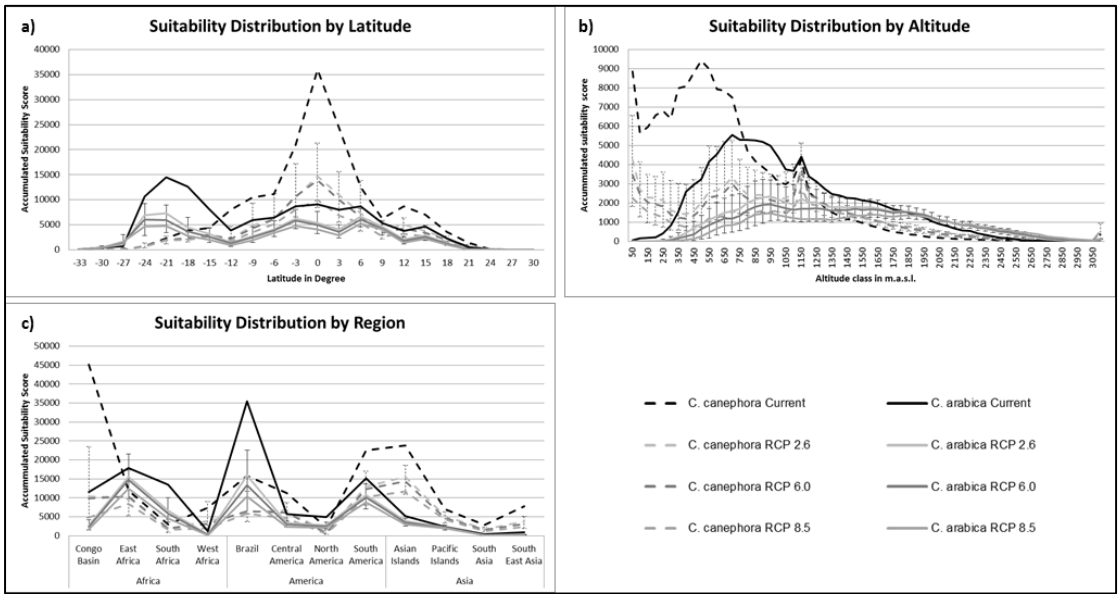

Figure 4. Distribution of suitability changes by a) latitude, b) altitude, c) coffee regions; Continuous lines represent *C. arabica*, dashed lines *C. canephora*, black lines the current

distribution, grey lines future distribution; the error bars indicate the minimum and maximum across RCP 6.0 model means

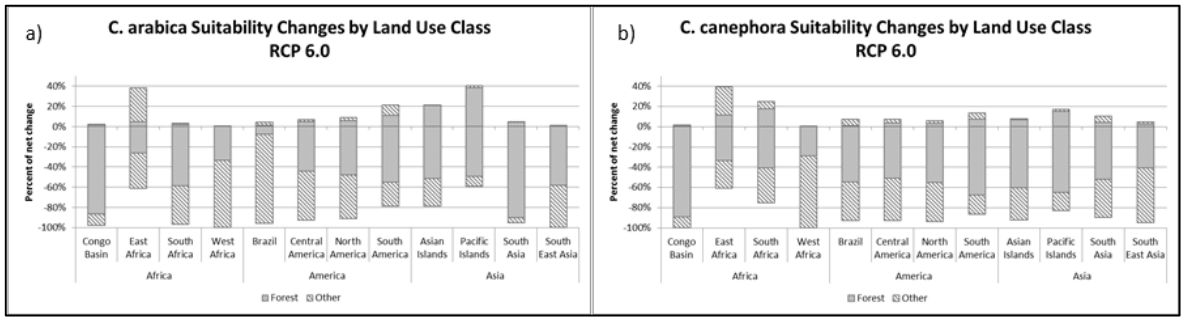

Figure 5. Distribution of suitability changes by region and the land use classes with forest cover and without forest cover by 2050 under RCP 6.0; a) *C. arabica* b) *C. canephora.*

DISCUSSION

The goal of this study was to examine the implications of climate change for global coffee production. We developed a methodology that is based on the notion that an ensemble of models captures more relevant information than a single model could. By using a mean of models based on several feasible parameter combinations rather than a single model our analysis is more robust than previous regionally limited studies.

It has been hypothesized that Robusta production may be able to replace in part the losses in Arabica production. While such a measure may be viable in some regions, our models draw attention to *C. canephora*'s need for climates with little intra-seasonal variability. As climate may not only become hotter, but also more variable, this may negatively affect Robusta coffee production.

Both species show significant changes in accumulated suitability scores along lower latitudes and less negative, albeit not positive, impacts at latitudes further away from the equator. We find a similar latitudinal migration trend in Brazil as was proposed in a regional study (Zullo Jr.et al. 2011). However, we did not find similar climate change impacts in other regions. The suitability gains in Southern Brazil may not be substantial enough to make up for suitability losses in the large production regions. In a similar pattern, losses in suitability occur mostly at low altitudes while at higher altitudes also positive changes are possible. Such altitudinal migration was also found in other studies, e.g. for Arabica in Central America (Schroth et al. 2009) or Robusta in Uganda (Simonett 1988). Here, we confirm these findings and show that altitudinal migration of coffee production is a global trend.

Analysis of suitability changes in several important coffee production regions for the RCP 6.0 emission scenario shows that for Arabica coffee production especially Brazil may see harmful climatic changes. Also Robusta may strive less well in important regions in Brazil and Vietnam. These losses are little counterbalanced with gains in other regions. East Africa and the Asian island states appear to be the only regions with substantial gains in suitability for the two species. This finding partially contrasts with a proposed substantial reduction in climatically suitable area for indigenous Arabica varieties in Eastern Africa by Davis et al. (2013). This difference suggests that commercial production has been adapted to a broader range of climatic conditions than can be found in Arabica's native range.

Interestingly, the East African areas with positive suitability changes are currently not covered with forest. In contrast the Asian areas that gain suitability are currently under forest. Our models show that the currently highly productive regions of coffee production in Brazil and Vietnam may in the future become unsuitable. This would create economic opportunities in East Africa, but may induce additional deforestation in Asia, where Coffee is already a frontier crop.

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A Review of The Status and Progress in Management Research of the Black Coffee Twig Borer, *Xylosandrus compactus* (Eichhoff) in Uganda

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SUMMARY

Coffee is the backbone of Uganda's economy, contributing about 18% of foreign exchange earnings valued at US$ 446 annually. Additionally, nearly 15 million people, draw their livelihood from from coffee-related activities along the value chain. Currently, production of the crop is threatened by a relatively new pest, the black coffee twig borer, *Xylosandrus compactus*, (Eichhoff) (Coleoptera: Scolytidae). (Here-after referred to as BCTB). A country-wide survey conducted during 2012/13 showed that the pest is rapidly spreading from its secondary epicenter in Kayunga and Mukono districts (Central Uganda) to most of the Robusta coffee growing regions of the country. The survey results further revealed 8.6% death of primary branches that translates into 8.6% loss of coffee export volume and foreign exchange valued at US$40 million annually at the current market prices and production volumes. In related studies, higher BCTB incidences and damage was recorded on shaded than on un-shaded coffee trees. In addition, suveys for its alternate hosts revealed that more than 30 plant species which are commonly intercropped in coffee are alternative hosts of BCTB. These include shade trees like *Albizia* spp. that have been actively promoted for their excellent shade structure and nitrogen-fixing capacity. Being a relatively new coffee pest in Uganda, there is generally limited information on BCTB and its management. Research efforts to-date have focused on surveillance of its spread and impact with a view of preventing further spread to new areas and minimizing its impact in already infested ones, identifying bio-ecological drivers of its population dynamics, and developing integrated pest management (IPM) packages for its management. A preliminary IPM package has been assembled for use by coffee farmers. This combines community-based phyto-sanitary actions and chemical sprays using imidocloprid (IMAXI or KOHINOR or CONFIDOR) mixed with tebucozanole (ORIUS), eliminating known alternate hosts (including *Albizia* spp.), proper management of shade trees and coffee canopies, and proper soil fertility and soil moisture management. Future research strategies for better management of BCTB are briefly discussed here.

GENERAL INTRODUCTION

Coffee is the most important cash crop in Uganda contributing 3% of the Gross Domestic Product (GDP) and 18% of annual foreign exchange earnings valued at US$ 466 million in 2010/11 (UCDA, 2012). Coffee is grown by over 1.3 million households and provides

employment to over 5 million Ugandans through coffee-related activities along the value chain (Hill, 2005). However, productivity of coffee is still far below the attainable yield potential due to a number of constraints (Musoli *et al*., 2001). Coffee Wilt Disease (CWD) which broke out in early 1990's and wiped out more than 50% of coffee stand (Adipala-Ekwamu *et al*., 2001) has been the major constraint to Robusta coffee production. CWD has been partly contained through the recent development and release of 7 new resistant varieties. However, this achievement is likely to be rolled-back by the emergence of a relatively new pest, the Black Coffee Twig Borer, *Xylosandrus compactus* (Eichhoff) (Coleoptera: Curculionidae). This paper therefore presents an overview of the pest status, progress and future research strategies towards management of the BCTB pandemic in Uganda.

OVERVIEW OF THE BIOLOGY OF *X. COMPACTUS*

The twig borer attacking coffee in Uganda is *Xylosandrus compactus* (Eichhoff; Egonyu *et al*., 2009). It is a coleopteran belonging to the family scolytidae and typical "ambrosia" beetle because it lives in association with an ambrosia fungus (Ngoan *et al*., 1979). Males are flightless and remain in brood galleries for their entire lifespan whereas; females leave the brood gallery after mating to initiate infestation of other branches or hosts. They excavate a tunnel into host plant leaving pin-sized entry holes; inoculate host galleries with an ambrosia fungus for feeding their larvae (Hara and Beardsley, 1976; Ngoan *et al*., 1976). Females can also reproduce parthenogenetically (without mating), in which case the offspring are all males (Hara and Beardsley, 1979). The lifecycle of *X. compactus* is completed in about one month (Ngoan *et al*., 1976). Adult females emerge through entrance holes of parent beetles and can be dispersed at least 200 meters in a single flight. However, it is likely that dispersal over several kilometers is possible, especially if wind-aided (Entwistle, 1972). Based on its prolific reproductive potential and dispersal capacity, *X. compactus* is therefore a high risk invasive pest species for areas not yet infested (Egonyu *et al*., 2009; Pennacchio *et al*., 2012).

PROGRESS IN RESEARCH ON *X. COMPACTUS* IN UGANDA

Status of *X. compactus*

X. compactus is believed to have originated from Southeast Asia (Brader, 1964) but currently distributed worldwide, particularly in the tropics and sub tropics (Rabaglia *et al*., 2006). It is a highly invasive and damaging pest that spreads far and wide over a short period of time (Pennacchio *et al*., 2012; Kagezi *et al*., 2013a,c). In Uganda, it was first reported in 1993 in Bundibugyo district located in the west of the country neighbouring the Democratic Republic of Congo (DRC), coinciding in place and time with the advent of CWD (Adipala-Ekwamu *et al*., 2001). Second report was in 2002 in southwestern districts of Rukungiri, Kanungu and Bushenyi, and the third and fourth reports were in the central districts of Mukono in 2007, and Mukono and Kayunga in November 2008 (Egonyu *et al*., 2009). A survey was conducted by the National Coffee Research Institute (NaCORI) and International Institute of Tropical Agriculture (IITA) in 2011/12 in 25 districts covering the 5 major coffee growing regions of Uganda. It shows that the pest was present in all the five districts sampled in the central region and 50% of the districts in the southwest. However, the pest was not observed in West Nile, northern and Mt. Elgon regions of the country (Kagezi *et al*., 2013c). Another survey conducted in 2012/13 in 26 districts covering the major Robusta coffee growing regions indicate that BCTB was present in all regions and in all districts apart from Namayingo in Busoga sub-region (Kagezi *et al*., 2013a). This shows that the pest is rapidly spreading away from where it was first reported and its epicenter (Egonyu *et al*., 2009), to new infestation areas (UCDA, 2012; Kagezi *et al*., 2013a,c). Incidentally, the affected regions produce the

bulk of Robusta coffee, *Coffee canephora* in Uganda, which contributes >80% of the coffee export volume in the country (Musoli *et al*., 2001).

Impact of *X. compactus* on coffee production

Severe losses in coffee caused by BCTB have been reported elsewhere. For example, Ramesh (1987) observed losses of 21% on 45-year-old coffee plants and 23.5% on young plants in India. Similarly, Lavabre (1959) reported losses of about 20% of coffee crop in Cameroon. In Uganda, the 2012/13 survey shows national averages of 68.8% of coffee farms, 40.3% of coffee trees and 8.6% of primary branches infested by BCTB (Kagezi *et al*., 2013a). Out of the 26 districts surveyed, 42.3% of them had all the coffee farms (100%) infested. The survey further indicates that in the southern and central regions which produce more than half of Uganda's Robusta coffee (Musoli *et al*., 2001), 88.3% of the coffee farms, 58.9% of coffee trees and 15.3% of primary branches were infested. These results show that BCTB poses a big threat to coffee production in Uganda if no comprehensive mitigation measures are put in place (Egonyu *et al*., 2009; UCDA, 2012; Kagezi *et al*., 2013a,c;). Although an objective assessment of national losses caused by BCTB is yet to be conducted in Uganda, the current 8.6% death of the primary branches (Kagezi *et al*., 2013a) which produce berries translates to 8.6% reduction in coffee production, and export volumes and value. This implies that at this rate, Uganda will lose about US$40.1 million of her coffee foreign exchange earnings per year if the pest is not contained. This will definitely have adverse effects not only on the national coffers but also to the more than 5 million Ugandans who derive their livelihoods from various coffee activities along the value chain (Musoli *et al*., 2001; Hill, 2005; Kagezi *et al*., 2013a,c). This again emphasizes the economic importance BCTB posses on the Uganda's coffee industry (Egonyu *et al*., 2009; UCDA, 2012; Kagezi *et al*., 2013a,c).

Ecology of *X. compactus*

Population dynamics of BCTB have been reported to fluctuate throughout the year with peaks occurring during dry season and at harvesting period (Ngoan *et al*., 1976; Burbano, 2010; Egonyu *et al*., Unpublished data). This could probably be due to physiological stress on trees since they receive little water and nutrients (Smith, 2003). However, farmers usually give contradicting views concerning BCTB infestation peaks; some claim to be higher in wet season and others dry season. Probably this could be due to the fact that although more damage might be manifested in the dry season, symptoms are more evident in the wet season when there is active vegetative growth (Personal observ.). Understanding the spatial distribution of *X. compactus* population and infestation levels is vital in designing appropriate monitoring and IPM strategies. This information is particularly important if spot application of chemical and/or release of natural enemies are to be done (Chong *et al*., 2009; Kagezi *et al*., 2013b). Preliminary results in Uganda show that BCTB infestation is higher down-slope than up-slope (Kagezi *et al*., 2013b; Smith, 2003), on primary branches located in lower third than upper portions of coffee canopy (Kagezi *et al*., 2013; see also; Chong *et al*., 2009) and on basal third than tip parts of infested primary branches (Kagezi *et al*., 2013b).

To diminish the BCTB problem, farmers must always trim off and burn or burry all infested plant materials in their coffee fields (Hara and Tenbrink 1994; Smith, 2003; Egonyu *et al*., 2009; Kagezi *et al*., 2013a,c). This method has been reported to control *X. compactus* to some level in Uganda and elsewhere. For example, Jones and Johnson (1996) reported almost 90% mortality of the beetles where phyto-sanitary methods had been employed in Hawaii. In Uganda, farmers in Masaka and Rakai districts have reported significant reduction (though not quantified) in BCTB infestation where they use phyto-sanitary methods. Also, a farmer in Buyende sub-county, Jinja district, reported a 90% reduction in BCTB-infestation where

phyto-sanitation combined with application of chlorpyrifos had been used. Similarly, BCTB infestation was reduced by at least 29% in phyto-sanitary coffee fields compared to non-phyto-sanitary ones by Twekembe Coffee Farmers Field School in Mukono district (Kyamanywa *et al*., 2012). However, this method is labor intensive and may be uneconomical (Smith, 2003). Secondly, many farmers do not use appropriate methods to destroy the infested plant materials, thus leaving an important re-infestation source in the fields. In addition, for this tactic to be effective needs collective efforts by all farmers at community level in order to avoid re-infestation from neighboring farmers who are not controlling the pest (Kagezi et al., 2013a). This might call for by-laws at local levels (see; Kubiriba et al., 2012).

Differences in cropping and farming practices may create variations in field conditions that might favor or disfavor multiplication and development of insect pests (Kucel et al., 2009). Our preliminary results show that BCTB infestation is higher on coffee plants grown under shade or coffee which is closely planted, un-pruned or inadequately de-suckered (Kucel et al., 2011; Kagezi et al., 2013b).These conditions probably provide micro-environments that may favor development and completion of its life cycle (Kucel et al., 2011; Kagezi et al., 2013b). Humid microclimates may also facilitate development of associated ambrosia fungus (Wintgens, 2009). But also, a number of shade tree and plant species commonly intercropped in coffee have been reported to be alternate host species for BCTB in Uganda (Kucel et al., 2011; Kagezi et al., 2013b). Good tree care to promote vigor and health may help in resisting BCTB infestation or recovering from infestation (Smith, 2003). Farmers in Kyingo Farmer Field School (FFS) in Rakai district have reported reduced BCTB infestation (though not quantified) in coffee fields where they use trenches, mineral fertilizers (Urea) and mulch (see also; Pennacchio et al., 2012). This agrees with the findings of researchers from the University of Hawaii College of Tropical Agriculture and Human Resources who removed all infested branches from coffee trees and then applied fertilizers once a week through irrigation system. After 4 months, no more borer damage was observed on coffee but when they stopped applying fertilizers for just one month, the borer quickly returned (Smith, 2003).

Host plant utilization by *X. compactus*

X. compactus is a polyphagous insect infesting more than 224 plant species in about 62 families worldwide including those listed as threatened and endangered (Rabaglia et al., 2006). In Uganda, although BCTB was initially mainly reported on Robusta coffee (Egonyu et al., 2009), recent data show that it attacks Arabica coffee as well (Kagezi et al., 2013c). In addition, more than 30 plant species which are commonly intercropped in coffee have been confirmed to be alternate hosts of BCTB (Kagezi et al., 2013c). These include commercial and food crops, ornamentals and tree species (forest, shade, fodder and shrubs). For example, a survey conducted in cocoa growing districts located in the Lake Albert Crescent Zone (LACZ) in mid-western Uganda during January 2014 shows that more than 50% of the cocoa plantations, 13% of cocoa trees and 3.8% of their branches were infested by BCTB (Kagezi et al., Under review). Another concern is that the common shade trees (particularly Albizia chinensis) that have been for long actively promoted by research and extension for their excellent shade structure and nitrogen-fixing capacity (Mukiibi, 2011) are some of the best hosts for BCTB (Kucel et al., 2011; Kagezi et al., 2013c). This presents a lot of management implications as farmers have to either protect their coffee from BCTB infestation by eliminating alternate hosts or maintain them for various purposes (Kagezi et al., 2013c). Secondly, most farmers in Uganda (Egonyu et al., 2009; Kagezi et al., 2013a) and elsewhere (Smith, 2003) are currently relying on cutting off and burning and/or burying of infested plant materials and/or removing them from the vicinity of coffee trees in order to control the beetle. However, these cultural methods require a thorough understanding and knowledge of host

plant range and utilization by the pest in question. Therefore, occurrence of alternative hosts may influence the ecological dynamics and biology of pest (Tanwar et al., 2010) and thus complicate cultural control strategies (Kagezi et al., 2013c).

DISSEMINATION OF INFORMATION ON *X. COMPACTUS*

In response to the recent countrywide outbreak of BCTB in Uganda, National Agricultural Research Organisation (NARO)/Uganda Coffee Development Authority (UCDA)/Ministry of Agriculture, Animal Industry and Fisheries (MAAIF)/National Agricultural Advisory Services (NAADS) formulated several action plans for its management. Due to the gravity of the problem, a short-term strategy of embarking on an intensive program of sensitizing stakeholders along the value chain was implemented in a number of districts (see; Tushemereirwe et al., 2006). Awareness campaigns began with mobilization and sensitization of local leaders because they are the opinion leaders in their localities and as such, local masses easily believe in the message they convey. Local leaders also possess a more reach-out effect on farmers compared to researchers and extension workers, thus can easily out-scale the information. It is also vital to involved local leaders if community-based approaches are to be implemented because it might necessitate formulation and enforcement of local by-laws (Kubiriba et al., 2012). This was done in Jinja, Iganga, Luuka, Kamuli, Mayuge, Rakai and Masaka districts. The National Agricultural show held in Jinja district and annual coffee days held in Lwengo, Rakai, Ntungamo, Luwero, Butambala and Mpigi districts were also utilized as a platform for sensitizing various stakeholders. In addition, Livelihoods and Enterprises for Agricultural Development-United States Agency for International Development (LEAD-USAID) funded workshops for farmers and extension in its operational areas in the districts of Iganga, Kamuli, Mukono, Masaka, Luwero, Kiboga, Mubende, Ibanda and Bushenyi also sensitized farmers and extension workers.

CONTEMPORARY INTEGRATED PEST MANAGEMENT (IMP) PACKAGE FOR *X. COMPUCTUS* IN UGANDA

The overall goal of BCTB research efforts is to reduce current and future coffee yield losses caused by the pest in Uganda through development and implementation of a cost-effective and environmentally-sound Integrated Pest Management (IPM) package. IPM measures are vital for preventing BCTB from spreading to and also protect coffee fields in free (threatened) zones, halt further advances in 'frontline' zones and suppress pest population and infestation in already infested ('endemic') zones. A preliminary IPM package for BCTB management based on cultural and chemical options has been assembled and developed into extension materials in form of brochures, posters and leaflets by NaCORI-NARO/UCDA and currently being implemented in Uganda. These materials have been mass produced with assistance from Uganda Government and LEAD-USAID project. This package includes:

- Step number one in controlling BCTB is for farmers to inspect their coffee plantations regularly and frequently to check for any outbreaks and signs of infestation
- All BCTB-infested plant materials should be trimmed off (not breaking!) with a pair of secateurs and burned or buried and/or removed from the vicinity
- Avoid 'bushiness' in coffee fields by planting the coffee at correct spacing, maintaining 3-4 stems and removing all unwanted suckers (proper pruning and de-suckering) and the lower un-productive primary branches to eliminate conditions which promote infestations
- Clean weeding coffee plantations to bury fallen BCTB-infested plant parts
- Improving soil fertility and moisture through application of animal manure or mineral fertilizers, mulch and/or cover crops

- Eliminating all known alternate hosts within and near coffee plantations
- Planting only non-alternate host shade trees at recommended spacing. In the meantime, some shade trees notably; *Albizia chinensis*, *Maesopsis eminii* and *Markhamia lutea* should not be planted in coffee fields until research advises.
- Shade trees should also be pruned regularly to avoid too much shade (but not totally eliminate)
- Spraying coffee trees with imidacloprid (IMAXI or KOHINOR or CONFIDOR) at a rate of 4 mls per liter mixed with tebuconazole (ORIUS) at 6 mls per liter. The imidacloprid kills the beetle whereas tebuconazole suppresses the associated fungi.

However, the decision by farmers to use chemicals for controlling pests is highly influenced by environmental and human health concerns (Pimentel, 2005). It should also be emphasized that spraying with the chemicals should be combined with phyto-sanitary measures because these chemicals are systemic and thus will not be translocated through the BCTB-dried plant materials. Additionally, these chemicals may not be readily available and/or unaffordable by a majority of smallholder coffee farmers yet they are the producers of over 90% of Uganda's coffee (Musoli et al., 2001). For example, at present market price, Imaxi costs US$40-52 whereas Orius is US$10-12 which might be far beyond what farmers can afford. All in all, it is recommended that community-based approaches be the main-stay in managing BCTB. This is necessary so as to avoid re-infestation from neighboring farmers who are not practicing control measures. In this case it might even necessitate the local governments to embark on the establishment of community task forces at local levels. This should be coupled with formulation and enforcement of by-laws in order to combat the rampant spread of BCTB (see also; Kubiriba et al., 2012).

ON-GOING AND FUTURE RESEARCH ON BCTB IN UGANDA

Basing upon the information presented herein, it is recommended that the following aspects considered for further research in order to develop and disseminate an economically-viable, environmentally- and farmer-friendly package for managing BCTB.

Biology of *X. compactus*

Preliminary studies show that the black coffee twig borer associated with coffee in Uganda and elsewhere is *Xylosandrus compactus* Eichhoff (Entwistle, 1972; Hara and Beardsley, 1976; Ngoan et al., 1979; Egonyu et al., 2009). However, a number of *Xylosandrus* species have been recorded elsewhere (CABI, 2005). Likewise, the fungus isolated from the female BCTB mycangium and the associated coffee galleries is Fusarium solani (Mart.) Snyd. and Hans. (Hara and Beardsley, 1976; Ngoan et al., 1976; Egonyu et al., 2009). Research is now focused on establishing inter and intra species diversity of the BCTB and its associated ambrosia fungus collected from the various coffee agro-ecological zones (AEZ's) of Uganda. In addition, other pathogenic fungi which might be associated with the twig borer are also to be isolated and characterized. The actual cause of wilting and eventually death of plant hosts attacked by BCTB is still unknown. It could be due to either disruption of water and nutrient movement across galleries made by the beetle (direct physical damage; Masuya, 2007) or inoculation of coffee trees with a fungal pathogen (pathogenicity; Daehler and Dudley, 2002). Indeed, F. solani is a well-known plant pathogen that causes cankers, root rot, or rapid wilt syndrome in coffee, depending on the strain (Venkatasubbaiah et al., 1984). This would mean that death of coffee twigs could be avoided by early treatment using systemic fungicides to suppress growth of ambrosia in BCTB tunnels in coffee tissues. Research activities are therefore focusing on determining pathogenicity of fungi isolated from insect and infested plant materials collected from different coffee AEZ's of Uganda. The advent of CWD in

Uganda in 1993 coincided with the first episode of BCTB outbreak and in the same district of Bundibugyo (Adipala-Ekwamu et al., 2001). Another outbreak of BCTB in Mukono and Kayunga districts of central Uganda in 2007/8 (Egonyu et al., 2009) also was concurrent with CWD resurgence on replanted coffee in the area. In addition, the F. solani associated with BCTB (Hara and Beardsley, 1976; Ngoan et al., 1976; Egonyu et al., 2009) belongs to the same genus as Fusarium xylarioides Steyaert (Gibberella xylarioides Sacc and Heim), causal organism of CWD (Adipala-Ekwamu et al., 2001). This suggests a close relationship between BCTB and CWD. BCTB might be playing a vital role in the transmission of CWD or otherwise. Current research is embarking on further studies of X. compactus samples collected from CWD-infected and non-infected coffee trees from different coffee growing AEZ's to establish the role of BCTB in CWD transmission.

Chemical ecology of *X. compactus* and its Plant Hosts

Plants produce volatiles that variously attract or repel insect pests (Farré-Armengol *et al*., 2013). It is through these volatiles that insects recognize their hosts. Preliminary studies at NaCORI on extracts from coffee, ambrosia fungi and other BCTB alternate hosts, using an olfactometer, have isolated six (6) sets of volatiles (alkaloids) in these materials that attract BCTB (Egonyu *et al*., Unpublished data).. Further studies are therefore envisaged in order to identify the isolated volatiles, and to evaluate them for efficiency of BCTB trappings, in order to consider their use in BCTB traps. It is also planned to continue efforts to isolate volatiles with BCTB repellent properties, in order to complete a BCTB repellant management protocol. The olfactometer procedure shall also be used to determine the chemical basis of host preference by BCTB, and to explicate the observed relative susceptibility of coffee varieties to BCTB.

Management of *X. compactus*

Cultural control

Farmers in Uganda and elsewhere are currently relying mainly on cultural options to manage BCTB (Smith, 2003; Egonyu et al., 2009; Kagezi et al., 2013a,c). However, this method depends a lot on the ecological knowledge of the pest. Therefore, further search for more alternate host plant species, and studies of the bio-ecological drivers of the population dynamics of BCTB and associated fungi are being conducted in the diverse AEZ's of Uganda. This information is vital in refining the available cultural control options.

Trapping and repellent technology

Lures such as ethanol, manuka oil and (-)-α-pinene have been used in traps to attract ambrosia beetles including X. compactus for purposes of monitoring, studying population dynamics, predicting outbreaks, and mass trapping to reduce damage. Likewise, repellents such as verbenone and limonene could be used in combination with the attractants (Burbano, 2010; Burbano et al., 2012). This could act in a push-pull manner to deter settling of dispersing beetles while the attractant might lure repelled beetles to a trap placed some distance from protected plants. Brocarp traps used in management of the Coffee Berry Borer (CBB; Kucel et al., 2009) have been modified and adopted for managing X. compactus and various sources of alcohol (including local gins), trap color, trap designs and placement methods are being tested on-station and on-farm.

Varietal resistance

Varietal resistance could provide desired cheap and environmentally friendly solution to X. compactus problem in Uganda. However, limited attempts have been made so far by research to develop varieties that are resistant to coffee pests including BCTB despite the vast diversity of coffee materials in on-station germplasm collections, on-farm and in wild forests (Kucel et al., 2009). Nevertheless, varietal influence on BCTB infestation has been observed in Uganda. Robusta coffee is more attacked than Arabica coffee within the same location (Kagezi et al., 2012). Research shows that BCTB infestation was significantly less on 236/26 Robusta line than 258/24, 1s/3 and 1s/6 lines (Kucel et al., 2011). Similarly, variations in BCTB infestation within Arabica coffee germplasm collection at NaCORI were observed (Kagezi et al., Unpublished data). Thus, a more compressive search for varietal resistance needs to be conducted within the centers of origin of both Robusta and Arabica coffee.

Biological control

Available literature mentions very few natural enemies for controlling X. compactus population (Greco and Wright, 2012; Pennacchio et al., 2012). In Uganda, three formicid ants namely Pheidole megacephala, Plagiolepsis sp. and Cataulacus sp. have been found to be associated with BCTB (Egonyu et al., Under review). Furthermore, farmers in Rakai and Masaka districts have reported that they often release colonies of some ant species (not yet identified) into their coffee fields to prey on BCTB immature stages. Entomo-pathogenic fungi, Beauveria bassiana and Metarhizium anisopliae have also been reported to cause some mortality of BCTB (Balakrishnan et al., 1994). Research is geared towards exploring into ways of exploiting these predators and entomopathogens as bio-control agents of X. compactus.

Chemical control

It is extremely difficult to control X. compactus using contact pesticides because males spend their entire lives in concealed habitats of hosts and females will only emerge to locate a new gallery or host (Ngoan et al., 1976). This creates a barrier between the beetle and the chemicals. Therefore, chemicals need to be applied prior to borer's arrival, should be systemic and have long residual activity in order to be effective (Pennacchio et al., 2012). In addition, chemical control of scolytid pests of coffee is discouraged in Uganda by policy that promotes production of specialty coffee, and protects the environment and human health (Kucel et al., 2009). Thus, research on chemical usage needs to strike a balance between efficiency, environmental protection and human health concerns (Kucel et al., 2009).

CONCLUSION

The East and Central African coffee producing countries are currently faced with the threat of X. compactus pandemic, a serious risk to the very basis of these economies. In Uganda, X. compactus is due to overtake coffee wilt disease (CWD) as the single most important constraint to coffee production, already occasioning 10% loss in total national coffee out-put and value. Various surveys show the pest has rapidly spread from locations of its first sighting and subsequent epicenters, to new infestation areas. Incidentally, the affected regions to-date contributes >80% of the Robusta coffee export volume in the country. In a related development, the cocoa, tea, horticultural and forestry industries are also under threat as many species in these categories have also been found to carry infestations. It is therefore imperative that quick decisive actions are taken to contain the X. compactus pandemic in order to minimize damage to the coffee industry and negative impacts on Uganda's economy.

This is however complicated by limited information and knowledge on the bio-ecology and control of X. compactus since it is relatively new in the country. While considerable progress has been made in understanding its biology and ecology in the context of Uganda's agro-ecologies, and to develop sustainable IPM packages, much remains to be done. Currently, a provisional IPM package, hurriedly assembled by NaCORI entomologists, is in use but this needs to be elaborately refined to fully embrace the key considerations essential for any such broad-scale IPM applications. In this regard therefore, key research agencies and universities engaged in IPM research, and development partners are urged to interest and rally themselves in the collective effort to combat the X. compactus menace.

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Superimposed Impacts of Enhanced [CO_2] and High Temperature on the Photosynthetic Metabolism of *C. arabica* and *C. canephora* Genotypes

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SUMMARY

Coffee crop has been predicted to become threatened by future climate changes and global warming conditions. Yet, the long-term effects of elevated [CO_2] on this plant remain to be fully elucidated. In this context, this work aims at linking coffee biochemical responses to environmental changes of [CO_2] and temperature on genotypes from the two major producing species, using the photosynthetic metabolism as probe to evaluate the plant acclimation ability. Potted plants from *C. arabica* cv. IPR 108 and of *C. canephora* cv. Conilon Clone 153 were grown under environmental controlled conditions, either at 380 or 700 μL CO_2 L^{-1} air, for 1 year, without water, nutrient or root development restrictions. After that the temperature was gradually increased from 25/20 ºC (day/night) up to 42/34 ºC. The effects of elevated [CO_2] and enhanced temperature on the photosynthetic structures were assessed through the characterization of the lipid components of chloroplast membranes, whereas the leaf metabolic performance was evaluated through the thylakoid electron transport rates (involving both photosystem (PS) I and II), and the activities of enzymes (ribulose 1,5-biphosphate carboxylase/oxygenase and ribulose 5-phosphate kinase), as well as through stable isotopes of C and N. The activities of respiratory enzymes (NADH-dependent malate dehydrogenase and pyruvate kinase) were also analyzed. The results pointed for a higher functional status along the experiment in the plants grown under elevated [CO_2], with special relevance at 37 and 42ºC in IPR108. These results could be related to the qualitative changes of the membrane lipid matrix that might have helped to preserve suitable membrane fluidity

for the membrane bound events (*e.g.*, thylakoid electron transport). The PSs and enzyme data reflect an enhancement of the energetic metabolism (both photosynthesis and respiration), mostly, until 31 ºC for IPR108 and 37 ºC for CL153 at normal [CO_2]. Yet, under enhanced [CO_2] it was found an increase in the temperature (to 37 ºC) at which maximal values of some parameters in IPR108 (MDH, PSs activities, RuBisCO) were observed, concomitantly with the maintenance of high performance in other parameters when compared to the 380 plants. Under the highest temperature (42 ºC) the enzymes were the most sensitive point, displaying the strongest reductions, irrespective of genotype and [CO_2] treatments. The temperature promoted changes in leaf $\delta^{13}C$, irrespective of genotype and [CO_2], reflecting a decrease in WUE with heat. The changes in $\delta^{15}N$ values may indicate different limitation steps of N assimilation, requiring further investigation. It was concluded that the coffee plants grown under elevated [CO_2] apparently showed a better endurance to high temperatures, what is quite relevant in a context of predicted climate changes and global warming scenarios.

INTRODUCTION

Coffee is one of the world's most traded agricultural products, currently growing in ca. 80 countries, and modeling studies have foreseen that climate change and global warming will strongly impact the suitability of current cultivation areas and coffee biodiversity, especially of C. arabica. Yet, these studies have not considered possible mitigating effects of the increasing atmospheric [CO2], as no biological information is available regarding the long-term effects of [CO2] and temperature enhancements on the coffee plant.

The photosynthetic pathway is a key metabolism as regards plant survival and acclimation to environmental variations. Under enhanced growth [CO2], C3 plants often present net photosynthesis (Pn) increases above 50% and changes in stomatal conductance (gs), as also recently reported for Coffea spp.. This Pn stimulation results from a simultaneous higher RuBisCO carboxylation rate, due to increased substrate availability, and a competitive inhibition to O2, reducing the oxygenation rate and, subsequently, decreasing the CO2 loss and energy costs associated with the photorespiratory pathway. In C3 plants the impact on photorespiration under CO2 enrichment is expected to enhance Pn to a greater degree at high than at low temperatures, thereby, at least partially, offsetting the negative effects of supra-optimal temperatures on yield. Even so, a partial down-regulation (negative acclimation) of the photosynthetic apparatus might occur, often related to limitations on sink strength that prevents the plant from fully utilizing the higher photosynthate production. That may lead to non-structural carbohydrates (NSC) accumulation, which could in turn depress the gene expression and the amount/activity of photosynthetic enzymes, including that of RuBisCO, and may decrease the components of the photosynthetic apparatus. None of them were found in coffee plants. Such high [CO2] impact would also depend on the interactions with other environmental limitations, namely water availability and enhanced temperature, changing as well with the plant developmental stage.

Currently, stable isotope analysis is a powerful tool in physiological and ecological studies to trace, record, source, and integrate environmental parameters. With regard to carbon isotopes, the basis for much of the observed variation in δ13C of organic samples derives from two metabolic processes, photosynthesis and respiration. In the case of nitrogen, variation in the δ15N in its cycle processes has been increasingly studied. Knowledge of how the isotopes of N fractionate during catabolic reactions in soils and in plants in relation to N utilization, transformation, and fixation elucidate the pathways and interactions that many times result from not only particular symbiotic associations at root level (i.e., mycorrhizae and bacteria), but also N-fluxes and sources as well land-use and agricultural practices.

As modifications in atmospheric [CO2] affect fundamental plant processes and may alter plant growth, agronomic yields and quality, it is of crucial importance to gather information concerning the biological extent of the impact on the coffee plant and their capability to respond and cope with new environmental conditions. Therefore, to our knowledge, we report the first results concerning the underlying biochemical responses to enhanced [CO2] and high temperature in two genotypes of the major coffee producing species.

MATERIALS AND METHODS

Plant material and experimental design

Plants with ca. 1.5 years from *C. arabica* L. cv. IPR 108 (IPR108) and *C. canephora* Pierre ex A. Froehner cv. Conilon Clone 153 (CL153), were transferred into walk-in growth chambers (EHHF 10000, ARALAB, Portugal) and grown in 28 L pots under controlled conditions of temperature (25/20 ºC, day/night), irradiance (ca. 650-800 μmol m-2 s-1), RH (75%), photoperiod (12 h), and either 380 μL CO2 L-1 (380) or 700 μL CO2 L-1 (700) air for 1 year, without water, nutrient or root development restrictions. Thereafter the temperature was increased from 25/20 ºC up to 42/34 ºC, at a rate of 0.5 ºC day-1, with a 7 days temperature stabilization at 31/25, 37/30 and 42/34 ºC to allow analysis. The temperature was then set to 37/30 ºC for 2 months (37/30 Long-Term, LT) and the plants further analyzed. Analyses were performed on newly matured leaves.

Thylakoid electron transport rates

To obtain sub-chloroplast fractions and determine the in vivo electron transport rates associated with both PSI (DCPIPH2→MV) and PSII, including (H2O→DCPIP) or excluding (DPC→DCPIP) the oxygen evolving complex (OEC), measured polarographically using an LW2 O2 electrode (Hansatech, UK) at 25 °C, optimized methods for coffee leaves were followed.

Carbon and nitrogen isotopes

Carbon and nitrogen stable isotope ratios were determined on an Isoprime (Micromass, UK) isotope ratio mass spectrometer coupled to an elemental analyzer (EuroVector, Italy), accordingly to standard methods. Isotope ratios were calibrated against international standards (IAEA CH6 and IAEA CH7 for carbon isotope ratio, IAEA N1 for nitrogen isotope ratios. Precision (standard deviation of the set of standards analyzed in each batch) was 0.06‰ for carbon and 0.08‰ for nitrogen isotope ratio determinations.

Photosynthetic and respiratory enzymes

The activities of enzymes were determined in four freshly cut leaf discs (0.5 cm2 each). The homogenization procedure and the evaluation of the total activities of ribulose-1,5-biphosphate carboxylase oxygenase (RuBisCO; EC 4.1.1.39), ribulose 5-phosphate kinase (Ru5PK; EC 2.7.1.19), both related to the photosynthetic pathway, and of NADH-dependent malate dehydrogenase (MDH: EC 1.1.1.37) and pyruvate kinase (PK: EC 2.7.1.40), both related to the respiratory pathway, were performed as described in Ramalho, J.C. et al. (2013).

Chloroplast membranes lipids

The homogenization (ca. 4 g of freshly cut leaf tissue), extraction, separation, identification and quantification of fatty acids (FAs) from chloroplast membranes were performed as

previously optimized for coffee leaves [18]. The double bond index (DBI) was calculated as DBI = [(% monoenes + 2 x % dienes + 3 x % trienes / (% saturated FAs)].

RESULTS AND DISCUSSION

Thylakoid electron transport rates

Concerning the functioning of the photosynthetic apparatus, the rate of thylakoid electron transport at PSI and PSII level was promoted under high growth [CO2] at 25 ºC in both genotypes, suggesting a higher investment in the photochemistry structures (Fig. 1). With the increase in temperature up to 37 ºC, a stimulation of thylakoid electron transport occurred in both photosystems and genotypes, but the [CO2] effect was noted only in CL153. Upon 42 ºC exposure the potential activity of PSI and PSII decreased roughly to control levels. Yet, in IPR108 a lower impact was observed in the high [CO2] plants. Notably, the CL153 plants were the only ones to show significant negative after-effects (at 37 ºC LT).

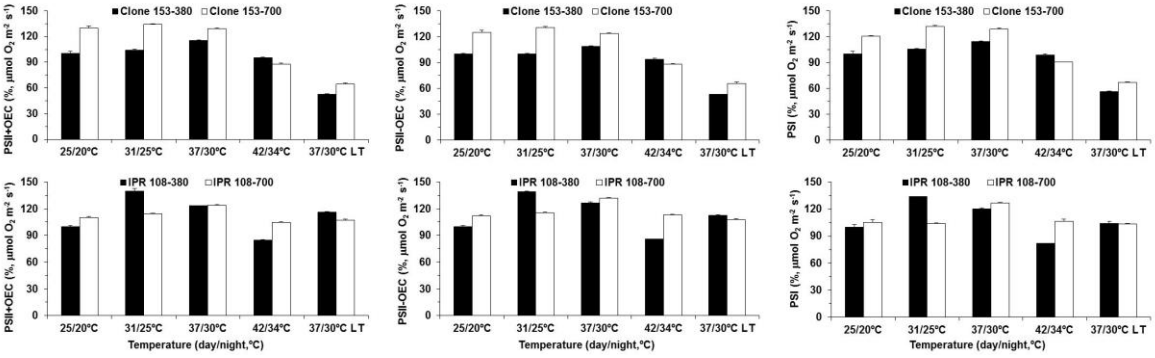

Figure 1. - Changes (in %, from results expressed in µmolO_2 m^{-2} s^{-1}, within each genotype, relative to 380 µL CO_2 L^{-1} at 25/20 ºC) for the *in vivo* electron transport rates associated with PSII, including (PSII+OEC) or excluding (PSII-OEC) the oxygen evolving complex (OEC), and PSI. Each value represents the mean ± SE (n=4-5).

C-isotope discrimination and N isotope values

The carbon and nitrogen isotope ratios in plants were linked to the prevalent climatic conditions during growth, mainly water and nutrient availability along with light intensity and temperature, which is in agreement with earlier studies that showed the potential use of the stable isotopes as tracers of coffee geographic origin. This study also points to the value of the application of stable isotopes for medium to long-term adaptation to both temperature and elevated CO2 conditions. During photosynthesis there are two major processes that lead to changes in δ13C in plant material: RuBisCO carbon fixation discriminates against 13CO2 of up to 29‰, and a lower diffusion of 13CO2 through stomata. Thus, it is expected that the δ13C in plant material reflects differences in WUE; more enriched 13C (less negative δ13C) leaf tissue is associated with a higher WUE, linked, e.g., to prolonged stomatal closure periods with CO2 restriction to the carboxylation sites, but also with lower H2O consumption rates.

The slight but consistent decrease in leaf δ13C, irrespective of genotype and [CO2], accompanied the temperature rise (including the 37 ºC LT) (Fig. 2) do reflects a reduction in WUE, agreeing with a larger increase in gs than in Pn (data not shown). Yet, the plants under high [CO2] displayed a much more negative δ13C than those at 380 µL CO2 L-1 (close to -37 and -27‰, respectively), at all temperatures, what could point to a lower WUE linked to an

enhanced growth [CO2]. However, this was not confirmed through gas exchanges assessments, both at 25ºC or at higher temperatures, where the 700 plants denoted higher iWUE than the 380 ones. In fact, the direct comparison [CO2] treatments can be misleading and should be avoided in the present case, since the 700 plants received more CO2 from a bottled gas mixture less enriched in 13CO2 (δ13C = -37.6‰) than the 380 ones (that received a mix of the same bottled air with common air, the latter with an approximate composition of δ13C = -8‰).

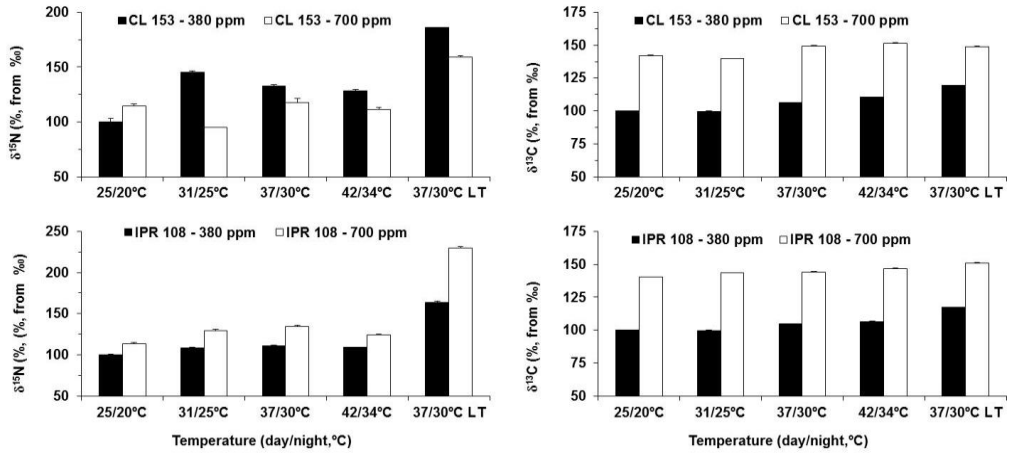

Figure 2. Changes (in %, from results expressed in ‰, within each genotype, relative to 380 µL CO_2 L^{-1} at 25/20 ºC) for the leaf isotope composition of C ($\delta^{13}C$ in negative values) and N ($\delta^{15}N$) Each value represents the mean ± SE (n=4).

On the other hand, more enriched δ15N values were observed at 25 ºC for both genotypes in the plants grown at higher [CO2] (Fig. 2). With the temperature rise, that difference was maintained in IPR108, but was inverted in CL153, which maybe related with different limiting metabolic assimilatory steps. Furthermore, both genotypes irrespective of [CO2], tended to slight higher values of δ15N with higher temperature, which paralleled an N increase in both [CO2] and genotypes with the temperature rise (data not shown). The stronger δ15N increase was observed in the 37 ºC LT. All that δ15N changes upon heat exposure, further suggests different impacts on the N-assimilation of both genotypes, as the δ15N composition reflects nitrogen sources, as well as plant 15N fractionation, and depends on assimilatory steps, such as enzymatic ammonium or nitrate assimilation. It has been shown that such processes discriminate against the isotopically heavier N substrates, resulting in 15N -depleted products and 15N-enriched residual substrates. Efflux of these 15N-enriched residual substrates renders the plant 15N depleted, contrary to what was found here with temperature increase. Plant 15N fractionation has been shown to depend strongly on the ratio between plant N demand and N supply, or on external N concentration relative to assimilatory capacity. Further studies are needed to clarify the results.

Analysis of some photosynthetic and respiratory enzyme activities

The activities of the enzymes related to the photosynthetic pathway, RuBisCO and Ru5PK, increased until 37 ºC (Fig. 3). At this point, the RuBisCO activity increased 55% (380) and 97% (700) in CL153 and 21% (380) and 85% (700) in IPR108, as compared to 25ºC-380 □L CO2 L-1 of each genotype. Furthermore, Ru5PK activity increased 42% (380) and 55% (700) for CL153 and 13% (380) and 53% (700) in IPR108. Upon 42 ºC a strong impact was observed in the activity of both enzymes, although higher values were usually maintained in the high [CO2] plants. In fact, for both enzymes and genotypes the high [CO2] plants denoted

higher activities along the entire experiment (except in CL153 at 42 ºC). By decreasing the temperature from 42 ºC to a long period at 37 ºC, a recovery in relation to the respective control was usually found. Yet, such recovery was only partial when compared to the previous exposure to 37 ºC.

Concerning the MDH and PK activities, a pattern similar to that of the photosynthetic enzymes was observed (Fig. 4). The higher activity in 700 plants was particularly clear in MDH (IPR108) and PK (CL153), with maximal increases of 2 fold (at 37 ºC) and 1.5 fold (at 31 ºC), respectively, in relation to the 380 plants at those temperatures.

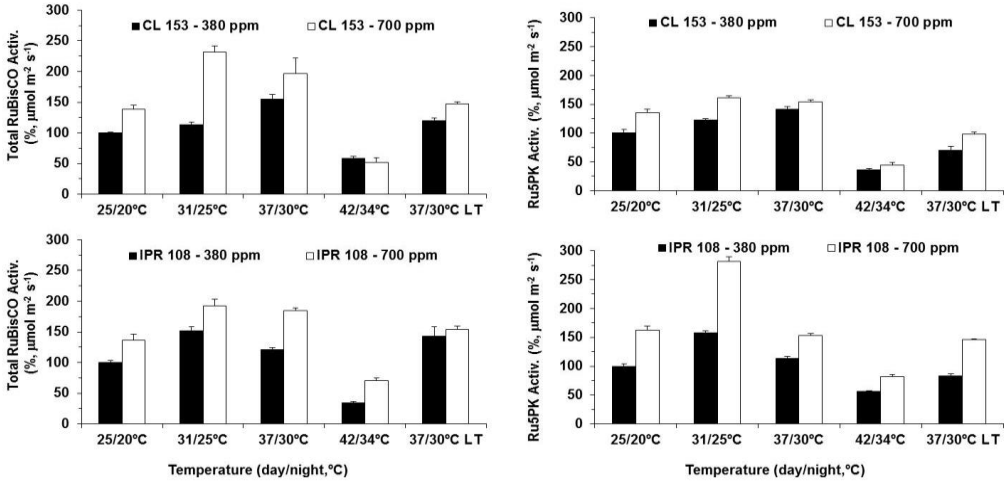

Figure 3. Changes (in %, from results expressed in µmol m^{-2} s^{-1}, within each genotype, relative to 380 µL CO_2 L^{-1} at 25/20 ºC) for the potential activities of RuBisCO and Ru5PK enzymes. Each value represents the mean ± SE (n=4).

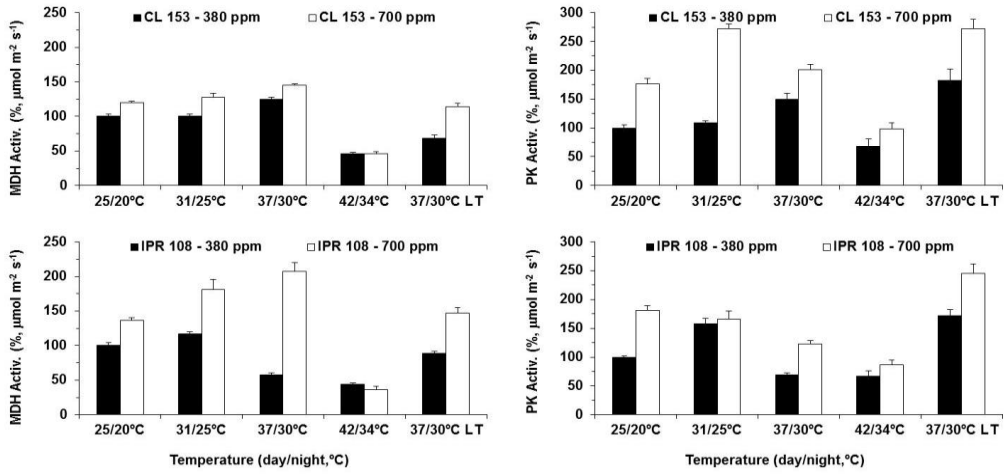

Figure 4. Changes (in %, from results expressed in µmol m^{-2} s^{-1}, within each genotype, relative to 380 µL CO_2 L^{-1} at 25/20 ºC) for the potential activities of MDH and PK enzymes. Each value represents the mean ± SE (n=4).

As pointed for coffee under enhanced growth [CO2] and 25 ºC [3], the overall reinforced biochemical potential (including enzymes and the electron transport), related to photosynthesis (and with respiration enzymes), might have contributed to prevent photosynthesis down-regulation. In fact, this down-regulation has been linked to reductions in N allocation to RuBisCO, RuBP regeneration and proteins associated with electron transport,

which were absent in the coffee plants. Still concerning these four enzymes, at 380 μL CO2 L-1, the CL153 plants showed their maximal activities at a higher temperature (37 ºC) than the IPR108 plants (31 ºC). Furthermore, IPR108 activities decreased at 37 ºC when compared to 31 ºC. That agrees with the higher temperature optimum of *C. canephora* when compared to *C. arabica*, and the higher heat sensitivity of the latter [23]. Still, for the IPR108-700 the maximal activity values for MDH was at 37 ºC, whereas the total RuBisCO activity at this temperature was similar to that at 31 ºC. Therefore, the higher [CO2] allowed the IPR108 plants to sustain a higher level of activity of these two enzymes under higher temperatures. Yet, at 42 ºC the enzymes were clearly the more sensitive targets with falls higher than 50%, when compared to the respective value at 25 ºC.

Chloroplast membranes lipids

Some alterations in total fatty acid (TFA) content and in the unsaturation level of the chloroplast membranes were noted (Fig. 5). With high temperature, IPR108 showed a TFA increase under both [CO2] until 37 ºC and a decrease at 42 ºC, reflecting a higher lipid dynamics in *C. arabica* than in *C. canephora*, as also found under cold stress for these two species [18,24]. Upon the recovery period at 37 ºC, the TFA content increased again in both [CO2]. Also, the high [CO2] plants tended to lower TFA contents after control, except upon the severe 42 ºC exposure. In CL153-380 plants some TFA decrease was observed up to 37 ºC (and at 37 ºC LT) but not at 42 ºC where a value similar to control was observed. Also, at increased temperatures the 700 plants showed somewhat lower TFA values (ca. 24%) relative to CL153-380 at 25 ºC, except at 37 ºC, when an increase was found. Therefore, CL153 presented lower TFA values in the plants grown in enhanced [CO2] along the experiment, except at 37 ºC and 37 ºC LT.

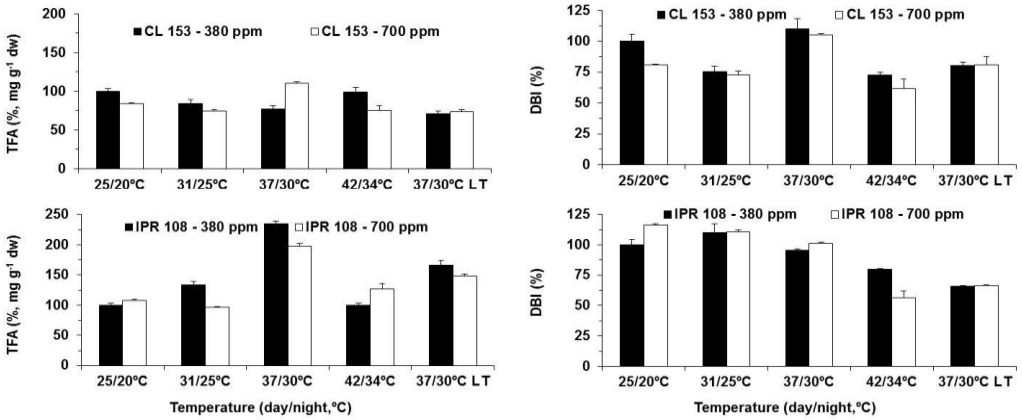

Figure 5. Changes (in %, from results expressed in mg g-1 dry weight for TFA and % for DBI, within each genotype, relative to 380 μL CO2 L-1 at 25/20 ºC) for the potential activities of MDH and PK enzymes. Each value represents the mean ± SE (n=3-4).

Concomitantly to these quantitative variations, some qualitative adjustments were found. Both genotypes displayed a gradual decrease of the unsaturation (DBI) level from 25 to 42 ºC (except at 37 ºC in CL153). This would help to rigidify the chloroplast membranes, counteracting the higher physical fluidity promoted by the increasing temperatures, presumably contributing to preserve appropriate membrane fluidity level and, thus, the membrane-associated functions. Upon 37 ºC LT a reverse trend was observed (except in IPR108-380). Comparing the [CO2] treatments, both CL153-700 and IPR108-700 plants tended to lower the DBI values at 42 ºC, when compared to the respective 380 plants. Therefore, the 700 plants could have benefit from a lower fluidity, what could have

contributed to maintain a higher performance of the photosynthetic apparatus, as reflected, namely, in the higher photosynthetic capacity values (data not shown). Also, the lower absolute DBI value in IPR108-700 (2.3) than in CL153-700 (3.7), could have contributed to maintain higher PSI and PSII activities at 42 ºC (Fig. 1). Such lowered DBI values at 42 ºC were linked to changes in individual FAs, mainly with increases in the more saturated palmitic acid, C16:0 (and a linolenic acid, C18:3, reduction) (data not shown).

In conclusion, the higher photosynthetic metabolic/functional status under elevated [CO2] was related to the up-regulation of several components of the photosynthetic machinery, both under control and under high temperatures (37-42ºC). Such higher performance could also be related to qualitative changes (e.g., higher lipid saturation) in the membrane lipid matrix, which might have preserved an appropriate membrane fluidity level for the membrane-bound events (as electron transport). The PSs and enzymes activity data clearly point to an enhancement of the energetic metabolism (considering both photosynthesis and respiration), mostly, until 31 ºC for IPR108 and 37 ºC for CL153 at normal [CO2]. However, the enhanced growth [CO2] seemed to be implicated in an increase in the temperature (37 ºC) at which maximal values of some parameters were found in IPR108 (MDH, PSs activities, RuBisCO), concomitantly with the maintenance of higher performance in other parameters when compared to the 380 plants. Notably, at 42 ºC the enzymes were the most sensitive point for both genotypes and [CO2] treatments. The changes in leaf δ13C, observed in both genotypes irrespective of growth [CO2], accompanying the temperature rise (including the 37 ºC LT), reflected a decrease in WUE. Although less clear, the δ15N values may indicate different N assimilation processes and limitation steps that requires further investigation. Although a clear distinction was not found between these, IPR108 seemed to have an increased heat tolerance promoted by enhanced [CO2], what is quite relevant in the context of predicted scenarios of increased [CO2] and global warming.

ACKNOWLEDGEMENTS

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Improving Pathogen Resistance by Exploiting Plant Susceptibility Genes in Coffee

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SUMMARY

Coffee is an important commodity. *Coffea arabica* and *Coffea canephora* are the two main coffee species cultivated in the intertropics. Though *C. arabica* is better tasting and more profitable, it is also more susceptible to pests and diseases. Molecular work has shown little genetic diversity and little resistance against pests and diseases in *C. arabica*. Research performed in other plant species concerning disease resistance by deploying susceptibility (S) genes could be transferred to *C. canephora* (of which the genome was recently published) and to *C. arabica* (of which the genome sequence is being worked on). Potential candidate S-genes could be tested by RNA-interference or mutation induced knock outs in obtaining a broad and durable resistance against pests and diseases in coffee.

INTRODUCTION

In nature many plant diseases are known (> 5000), but most plants are resistant to most diseases. Resistance is often shown by an entire plant species to all genetic variants of a pathogen (Heath, 1997). In principle each plant species suffers only from a limited number of diseases (~10 - 30), so one can conclude that susceptibility as such is rare. Furthermore, many pathogens are specialised on certain groups (clades, species, and families) of plants. Over the past 50 years lots of research has been done to introgress resistance from wild relatives into cultivated crop species and this was mostly focused on dominant (major) *R*-genes (Jones & Dangl 2006, see Figure 1). However, these resistances are easily broken; e.g. downy mildew resistance in lettuce (every year new *R*-genes are required) and *R*-genes in potato against *Phytophthora infestans* (broken after only few years). Hence there is a real need for new resistances which are more sustainable and thus less easily broken.

Many insects, nematodes, bacteria and fungi have been identified to consider the coffee plant as a host. Coffee is dependent on Integrated Pest Management- techniques involving firstly resistant or tolerant plants, secondly common farming practices that have major pest and disease control benefits (including pruning, biological control etc.) and thirdly pesticides (Segura *et al*. 2004; Waller *et al*. 2007).

Resistance and susceptibility are opposite sides of the same coin. It is assumed that, in the absence of functional *R*-genes, pathogen effectors interact with host-factors to suppress the plant innate defense, and hence induce susceptibility. Therefore, resistance in principle can be achieved in two ways: one is by the presence of *R*-genes to recognize the pathogen effectors and the other is by the absence of plant susceptibility (*S*) genes, plant genes which are required by pathogens to promote disease. We proposed in 2010 an alternative approach for obtaining durable and broad-spectrum resistance in crops by disabling plant *S*-genes (Pavan *et al*. 2010, Figure 1). Susceptibility genes (or *S*-genes) are plant genes which are required by pathogens (i.e. viruses, fungi and oomycetes, nematodes and bacteria) to promote disease. *S*-

genes can be classified roughly in so called negative defence regulator genes (e.g. the *MLO* gene; Büschges *et al*. 1997) and in susceptibility factors (e.g. the upa20 gene; Kay *et al*. 2007). To date more than 40 *S*-genes are cloned (mostly from *Arabidopsis thaliana*) and they are further grouped by van Schie & Takken (2014) based on their involvement in the process of pathogen infection: 1) *S*-genes providing early pathogen establishment; 2) *S*-genes interfering with host defense responses; 3) *S*-genes involved in feeding of the pathogen.

Recessive resistances resulting from mutations in plant *S*-genes can be immunity-unrelated when they serve demands of the pathogens in the process of pathogen development, accommodation and propagation (Lapin and Van den Ackerveken 2013; Hückelhoven *et al*. 2013). A number of the *S* genes have been used already for over thirty years in common agriculture, such as the *mlo* gene in modern European barley cultivars (Acevedo-Garcia *et al*. 2014).

Certain *S*-genes (e.g. the *MLO* gene) have been shown to be functionally conserved in a broad range of crop species like barley, apple, tomato, potato, cucumber, pepper and pea (Pessina *et al*. 2014), which offers possibilities to look for orthologs among different crop species. The expectation is that they can be edited relatively easy to accommodate the user's wishes, even in polyploid genomes like wheat (Wang *et al*. 2014) .There are however also drawbacks including the fact that not all homologous genes give similar effects in different crop species and -most importantly- the pleiotropic negative effects which seem to accompany them.

In *Arabidopsis*, many loss-of-function mutations are known to cause pathogen resistance e.g., *pmr* (powdery mildew resistance; Vogel and Somerville, 2000; Vogel *et al*. 2002) and *dmr* (downy mildew resistance (*Hyaloperonospora parasitica*, McDowell *et a*l. 2005). To provide proof of concept that disabling *S*-genes in crops could help to achieve a durable and broad-spectrum resistance, we have tested in tomato and pepper the susceptibility function of several *S*-genes identified in *Arabidopsis*. Silencing orthologs of *Arabidopsis MLO* in tomato (*PMR4* and *DMR1*) and pepper (*CaMLO2*) gives rise to resistance against the mildew fungi *Oidium neolycopersici* and *Leveillula taurica*, respectively (Huibers *et al*. 2013; Zheng *et al*. 2013). In addition to susceptibility to powdery mildews pepper, the pepper *CaMLO2* is involved in susceptibility also to other pathogens including *Xanthomonas campestris* pv. *vesicatoria*, *Pseudomonas syringae* pv. tomato and *Hyaloperonospera arabidopsidis* (Kim & Hwang 2012). Thus, silencing the right *MLO* orthologs *S*-genes in other plant species can potentially give resistance to different pathogens.

In addition to powdery mildews, we aimed also to identify *S*-genes for susceptibility to *Phytophthora infestans* in tomato and potato. Our results so far demonstrated that silencing certain orthologs of Arabidopsis *S*-genes in tomato and potato leads to resistance to the oomycete *P. infestans*. Thus, our data show that the *S*-genes identified in Arabidopsis are conserved for their function as susceptibility factors in other plant species and for a broad range of pathogens. Therefore, it is very promising to deploy altered host *S*-genes for durable resistance in controlling plant diseases.

Although fitness costs may be a disadvantage of using altered host *S*-genes, it is specific to plant species and can be overcome by both classic (e.g. searching for allelic variants) and advanced approaches (e.g. editing *S*-gene). Also in coffee this approach may be promising for e.g. bacterial blight, coffee leaf rust, coffee berry disease and major pests, such as the coffee berry and stem borers (Van der Vossen 2001, Damon 2000; Van der Vossen and Walyaro 2009). Since the genome sequence of one of the coffee species has been elucidated and published (Denoeud *et al*. 2014) and that of *C. arabica* is underway, there will be sufficient leads to look for interesting candidate genes. One way to obtain loss-of-function mutants in

the candidate *S*-genes is depicted in Figure 2. One could look for natural mutations (e.g. in the genes encoding *MLO*, *XA5* and *XA13*, *eIF4E* and *eIF4G*) or apply a mutagenesis approach to suitable coffee clones. For more directed approaches one could use RNAi techniques for candidate genes or more advanced gene editing techniques like ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering (Li *et al*. 2012; Gaj *et al*. 2013).

CONCLUSION

With the issues discussed above it seems possible to achieve sustainable/durable resistance in coffee against major diseases including the most notorious one; coffee leaf rust disease. This will not only be achieved by deploying *S*-genes (without negative pleiotropic effects) but rather by combinations of *S*-genes and (stacked) *R*-genes and as yet other unknown approaches. For *S*-genes it is clear that a wealth of potential candidate genes is awaiting further research and time will tell how many of them truly will make a difference in conveying sustainable resistance.

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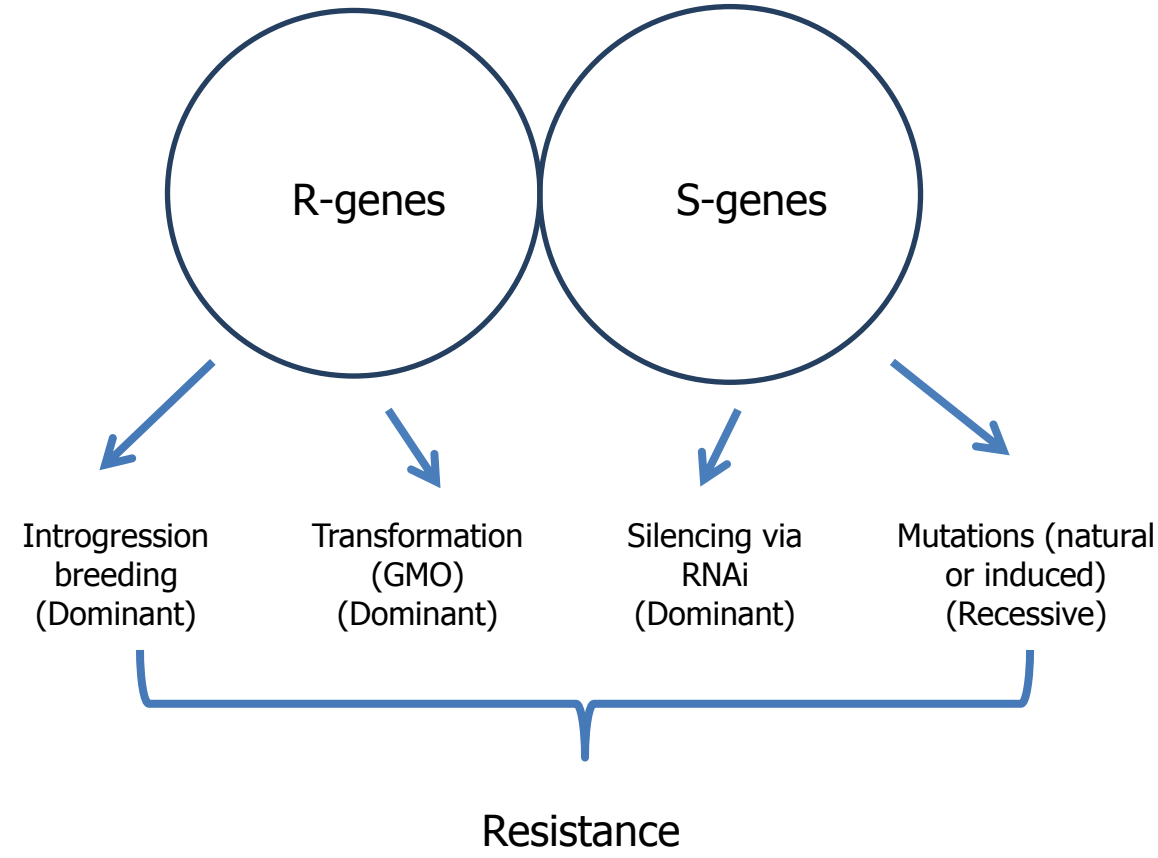

Figure 1. Different strategies used in breeding to obtain resistance either by using major *R*-genes or by using *S*-genes. Examples of different crops in which S genes are or could be deployed (barley, tomato, pepper, apple, grape, cucumber, pea and coffee).

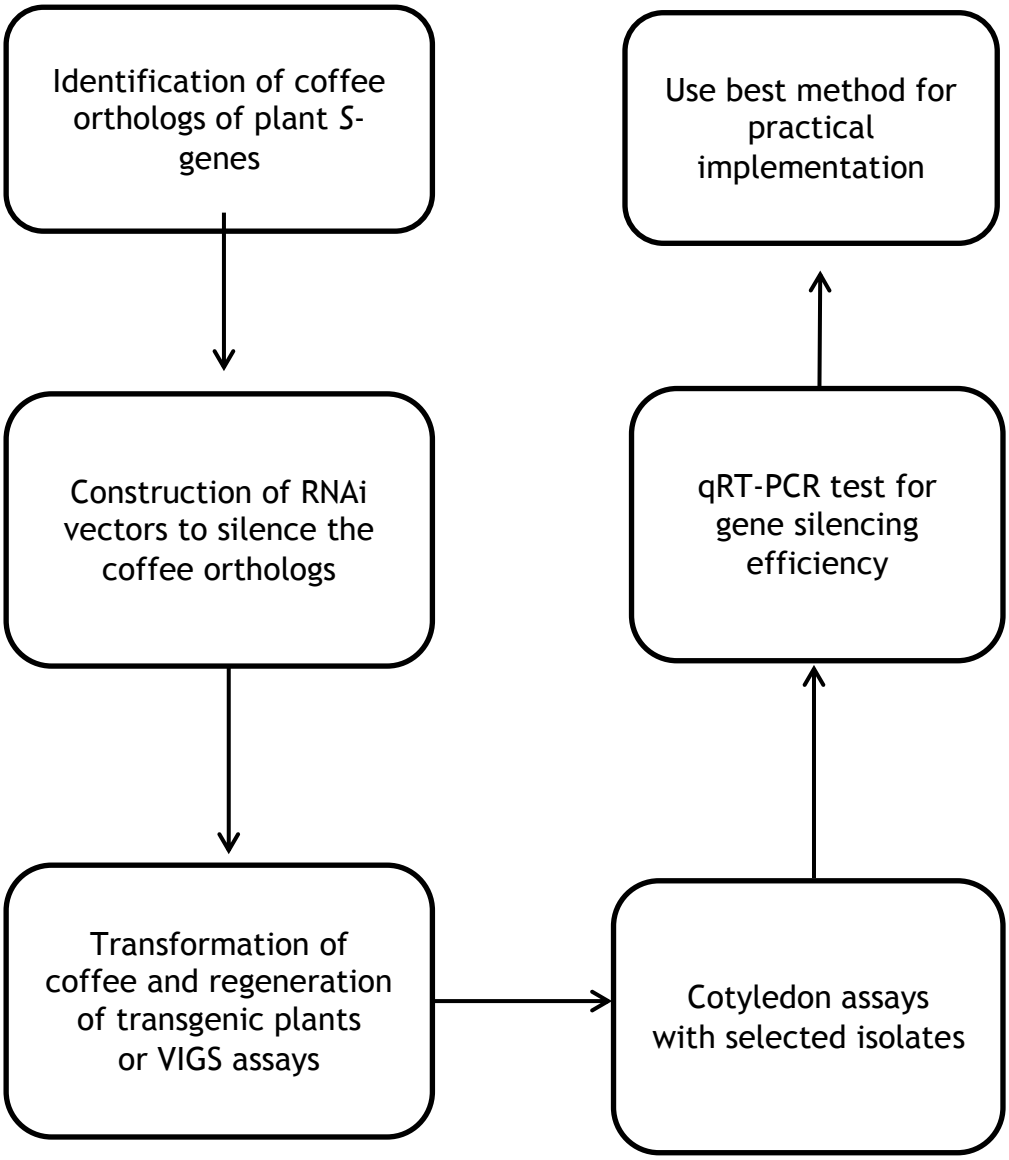

Figure 2. Proposed *S*-gene approach in coffee. RNAi: RNA based interference, VIGS: Virus Induced Gene Silencing.

Greenhouse Gases Assessment of Brazilian Green Coffee Production: a Case Study of the State of Minas Gerais

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SUMMARY

Consumers are more and more concerned about the quality of food products and now looking for those with a low environmental impact, with a particular attention to greenhouse gases emissions (GHG), a sustainability indicator related to agricultural products. Although coffee production in Brazil is responsible for about a third of all coffee consumed worldwide, there are few studies regarding the GHG emissions associated with the Brazilian green coffee production. Located in the southeast region, Minas Gerais is the largest coffee-growing State in Brazil. It accounts for nearly 55% of the national coffee production. As a part of a pioneer and continuous project initiated in 2009, the objective of this study was to determine the main greenhouse gases (GHG) sources and to measure the carbon footprint of coffee production in the State of Minas Gerais. Fourteen coffee farms located in three typical production regions of Minas Gerais were selected. Data from three farms were evaluated in the biennium 2009-2010 (crop years 2009/10 and 2010/11). The remainder of the information (11 farms) refers to biennium 2010-2011 (crop years 2010/11 and 2011/12). The carbon emission sources considered within the coffee farm were mobile and stationary combustion, nitrogen and organic fertilizers, lime and electricity. The upstream emissions related to the production and transport of agricultural inputs has not been accounted. Total emissions were different in the three different regions. Considering the emissions of all farms evaluated, the GHG intensity of the coffee produced in the State of Minas Gerais ranged from 800 to 2,783 kg CO_{2eq} $tonnes^{-1}$ green coffee. The GHG emissions weighted by cultivated area varied from 0.8 to 4.3 tonnes CO_{2eq} ha^{-1}. Results show that the major source of carbon emissions was nitrogen soil fertilization. Fuel consumption for farm operations and lime application are also shown to be important. Electricity was not significant as most of the energy supply in Brazil comes from low GHG emission sources (e.g. hydroelectric and biomass). Additionally, some mitigation strategies related to nitrogen fertilization were proposed, such as the substitution of urea for lower GHG emission sources (e.g. ammonium nitrate) and the correction of fertilization rates and methods. With these results it will be possible to improve the data on GHG emissions and design management strategies to promote sustainability along the coffee production chain and to add value to the final product.

INTRODUCTION

In recent years, the debate about environmental impacts and the sustainability of agricultural products has increased. Consumers are more and more concerned about the quality of food products and now looking for those with a low environmental impact, with a particular attention to carbon emissions.

Coffee has marked out the route for sustainability of tropical crops already back in the eighties. This tradition has grown inside the coffee market giving life to many sustainability voluntary and corporate standards and in general putting the issue of sustainable coffee production on the table of the entire sector. At recent growth rates and in light of big players' claims, in 2015 market growth is expected to reach 20-25% of the global coffee trade. As a consequence, there will be a saturation of the actual certified coffee production which therefore needs to be expanded to meet the future demand. These sustainability standards have moved from specific issues like social fairness, environmental protection or traceability to embrace the complexity of sustainable agriculture. They are all more recently dealing with the growing importance of climate change. It is indeed of international concern the impact of climate change on agriculture and on how to mitigate and adapt to it. This issue is debated by the civil society, which is extremely concerned about the future food supply. The main indicator for assessing the impact of agriculture on climate change is carbon footprint. Carbon footprint is the measure of the total emissions from a defined system of the greenhouse gases, listed by the IPCC, in CO2-equivalents according to each 100 years global warming potential (GWP100). Coffee urgently needs studies on this topic to anticipate the adoption of future standards and to grow in harmony with the modern concept of a profitable agriculture respecting and preserving the environment as well as the people.

There are few studies regarding the greenhouse gases emissions associated with the Brazilian green coffee production. Located in the southeast region, Minas Gerais is the largest coffee-growing State in Brazil. It accounts for nearly 70% of the national coffee production. The objective of this study has been to determine the main greenhouse gases (GHG) sources and to measure the carbon footprint of coffee production in the State of Minas Gerais.

MATERIALS AND METHODS

We collected data from 14 farms, including three coffee farms from the biennium 2009-2010 (crop 2009/10 and 2010/11) and 11 farms from the biennium 2010-2011 (crop 2010/2011 and 2011/12). The farms were select from the following regions of the Minas Gerais State in Brazil: Cerrado Mineiro (CM), Sul de Minas (SM) and Matas de Minas (MM). In Table 1, we present data on location, production area and yield of each selected farm for both biennium evaluated. The crop yield is the average of the entire farm, from coffee plants in the growing phase, pruned and in full production.

Table 1. Production areas and crop yields of the 14 farms evaluated in 2009-2010 and 2010-2011 biennia.

Region	Farm identification	Planted area (ha)[1]	Harvest area (ha)[1]	Yield (60 kg Bag)[1]	Productivity (Bag/ha)
CM	A1*	750	629	20.044	32
	A2+	336	274	7.679	28
	A3+	1200	815	32.465	40
	A4+	636	487	19.262	40
	A5+	524	489	15.179	31
MM	B1*+	1150	1071	36.000	34
		1150	1112	37.073	33
	B2+	100	92	3.700	40
SM	C1*	261	186	8996	48
	C2+	754	669	29.078	43
	C3+	1071	820	27.706	34
	C4+	276	112	4.687	42
	C5+	520	502	19.813	39
	C6+	580	486	14.786	30

*[1]Data from planted area, harvested area and production were obtained by a somatory of two crops considered at each biennium. *Data related to the biennium 2009-2010. + Data related to the biennium 2010-2011.*

[+] The same farm was evaluated at both biennia.*

The most important greenhouse gases for the agricultural sector - carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) - were assessed in each of the studied farms. The main sources of emissions are listed below (Table 2).

Table 2. Emission sources and greenhouse gases considered in this inventory.

Emission Scope	Emission Source	GHG
Direct		
Mobile combustion	Diesel, biodiesel, ethanol e gasoline	CO_2, CH_4, N_2O
Stationary combustion	Cooking gas, Coffee parchment, Eucalyptus wood and Coffee wood (from pruning)	CO_2, CH_4, N_2O
Fertilizers	Synthetic fertilizer	CO_2 e N_2O
Fertilizers	Organic fertilizer	N_2O
Soil additive	Lime	CO_2
Indirect		
Electricity	Purchased electricity	CO_2

In the case of fossil fuels, we considered the emissions of CO_2, CH_4 and N_2O. For biomass fuels (firewood, ethanol and biodiesel), we considered only the CH_4 and N_2O emissions. The CO_2 was disregarded, assuming the biogenic CO_2 is absorbed by the next crop through the process of photosynthesis.

N_2O from the application of nitrogen fertilizers include direct emissions that occur after application through reactions of nitrification and denitrification, and indirect emissions, where part of the nitrogen is lost through volatilization, leaching or runoff, and is subsequently emitted as N_2O. We also considered the CO_2 emissions arising from the application of agricultural lime and urea. CO_2 emissions derived from generation and distribution of electric energy consumed by these farms were incorporated in the inventories.

The upstream emissions related to the production and transport of agricultural inputs have not been accounted.

The methodology used for estimating GHG emissions was the activity data multiplied by emission factors. The results for N_2O and CH_4 were converted into CO_2 equivalent, considering the concentration and Global Warming Potential (GWP) of each gas in the backdrop of 100 years, following the Fourth Assessment Report (AR4) of the IPCC.

RESULTS AND DISCUSSION

Agricultural crops tend to exhibit great variability of production due to weather conditions, management of soil fertility, pest management and economic conditions. The coffee crop has other peculiarities, especially concerning the production biannuality. Since in Brazil the coffee crop has a high yield in a crop year and a low yield in the following one, GHG emissions were assessed for the production processes carried out in two sequential cropping years, also referred as biennium. Additionally we also consolidated the data assessed from two biennia to provide a robust data set on GHG emissions of coffee production at farm level.

Total GHG emissions and relative participation of emission sources for each producing region evaluated are shown in Fig. 1.

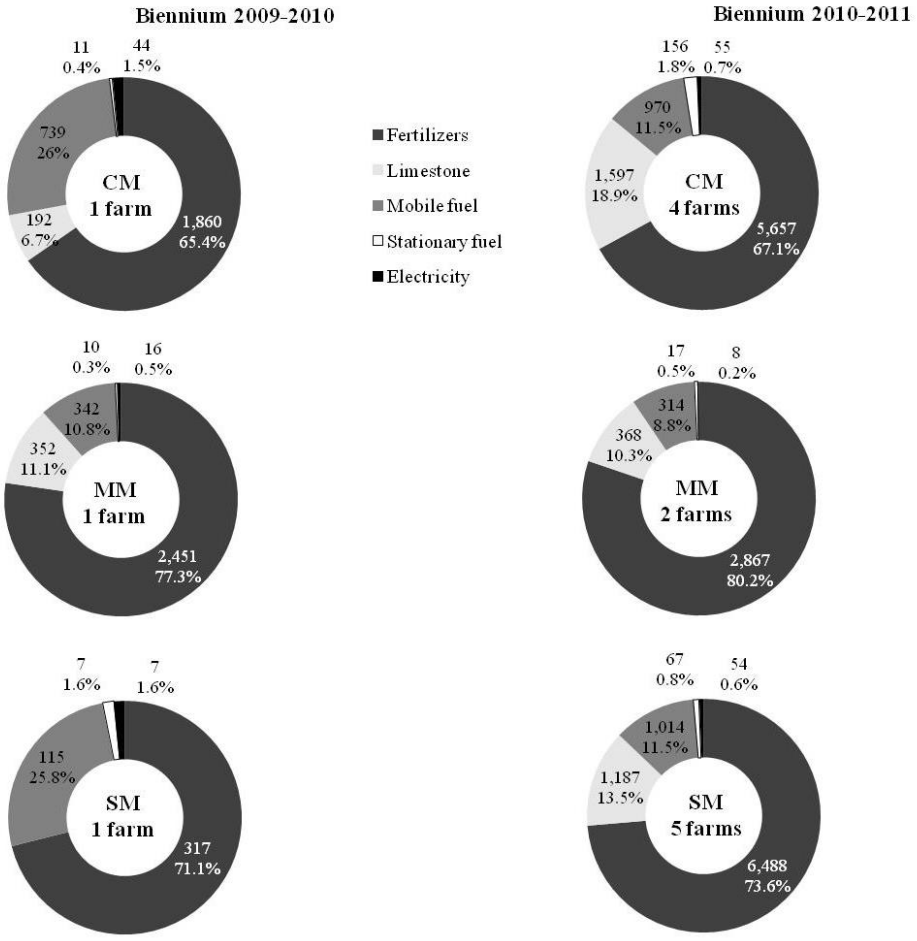

Figure 1. GHG emissions (tCO2e) and relative participation of emission sources in three coffee producing regions (Cerrado Mineiro - CM, Matas de Minas - MM and Sul de Minas - SM) in the state of Minas Gerais for both 2009-2010 and 2010-2011 biennia.

Considering the biennium 2009-2010, the farm located in the south of Minas Gerais (SM), had the lowest total GHG emissions (446 tCO2e) compared to other producing regions of the state. The farm located at Cerrado Mineiro (CM) presented the highest GHG emission (3,171 tCO2e), followed by the farm at Matas de Minas (MM) (2,846 tCO2e).

For the biennium 2010-2011, whose data set was formed by 11 farms, the lowest total GHG emission was presented at MM region (3,574 tCO2e) followed by the farm located at CM and SM regions (8,435 and 8,810 tCO2e respectively). These results, however, should be analyzed with caution since they are directly related to the size and profile of the farms and the average productivity of the crop in each situation. In this case, more important than the total GHG emissions are the relative share (%) of the emission sources in each coffee production region. The majority of GHG emissions was derived from the application of synthetic fertilizer. The high impact on the application of nitrogen fertilizer is due to the N2O emissions after the fertilizer reacts in the soil. The global warming potential of this gas is about 300 times higher than CO2, which enhances the degree of importance of N2O emissions in agricultural systems.

The second largest source of GHG emissions was fossil fuel burning in agricultural operations and transport, followed by the application of lime to correct soil acidity. Electricity consumption in the farms evaluated resulted in relatively low emissions. This is due to the fact that the energy matrix in Brazil is primarily composed of energy sources with low greenhouse gas emissions, such as hydropower and biomass.

Besides the total amount emitted by the farms in a given period, it is important to assess the amount of GHG emitted per unit of final product and production area to avoid comparisons of farms with different sizes. The weighting of emissions per unit of product or production area also allows for monitoring the environmental efficiency of the farm, enabling the establishment of targets to reduce emissions and the carbon footprint.

Taking into account the coffee production of each farm during the period of assessment, we calculated the GHG emissions per area (tCO2e/ha), per bags of coffee (kg CO2e/60 kg bag) and per tonne of grain (tCO2e/ ton) for each biennium evaluated (Table 3).

Table 3. GHG emissions per area and yield (60kg bag and tons) in three producing regions in the state of Minas Gerais in the 2010-2011 biennium.

Region	Farm identification	tCO_2e/ha	$kgCO_2e$/ 60 kg bag	$kgCO_2e$/tonne grain
CM	A1*	3.8	142	2,367
	A2+	2.6	112	1,867
	A3+	3.0	112	1,867
	A4+	3.5	115	1,917
	A5+	3.3	112	1,867
MM	B1*+	2.8	88	1,467
		2.8	88	1,467
	B2+	3.3	88	1,467
SM	C1*	1.7	48	800
	C2+	2.4	61	1,017
	C3+	4.3	167	2,783
	C4+	0.8	49	817
	C5+	2.2	58	967
	C6+	1.8	70	1,167

* *Data related to the biennium 2009-2010. + Data related to the biennium 2010-2011*

*+ *The same farm was evaluated at both biennia.*

In general, farms located at CM region presented the highest intensity of GHG emissions per area, per bag of coffee and per tonne of grain. On the other hand, those farms located at SM region presented the lowest emission coefficients, followed by the results obtained at MM region. It is important to note that the information presented does not necessarily represent the emissions standard of the regions surveyed, but mainly a diagnosis of major emission sources in each locality. Associating the results with the production profile of each region may result in errors, since no information were obtained from a representative number of farms in each region.

Nevertheless, the results indicate that nitrogen fertilizers are the main source of GHG emissions from the production of coffee in the state of Minas Gerais, which allows recommending some alternatives for reducing emissions in the production process.

Mitigation opportunities

As seen, the main source of GHG emissions in three farms evaluated in the 2010-2011 biennium was the application of nitrogen fertilizers.

It is known that the coffee crop requires large amounts of nitrogen in order to achieve optimum levels of productivity, resulting in emissions of large quantities of N_2O into the atmosphere.

Previous studies have reported that 84% of the annual emissions of N_2O from soil occur after application of the nitrogen fertilizer. According Hergoualc'h et al. (2008, 2012) the influence of nitrogen fertilization on N_2O fluxes is more pronounced in the first weeks after fertilizer application.

The coffee agricultural sector can embrace some measures to reduce GHG emissions, considering the supply of coffee's nutritional needs and maintenance of the productivity. Some suggestions are presented below:

Source

The first measure that can be adopted is the substitution of the nitrogen source applied to the soil. Urea, the main fertilizer used in the coffee farms, contributes to the emission of large amounts of GHG into the atmosphere. Because it contains carbon in its composition, after the reaction occurs in the soil this carbon is emitted as CO_2. Therefore, replacing or reducing the use of urea by other nitrogen sources such as ammonium sulfate and organic waste, can contribute to reducing GHG emissions.

Since organic residues are not capable of supplying the quantity of nutrients necessary to maintain high levels of productivity in coffee, we recommend using organic waste from the processing of coffee beans in addition to the synthetic fertilizer applied to the soil.

Application

The surface application of nitrogen in the soil should be avoided because it results in higher N_2O emissions to the atmosphere. The application in furrows, injected or drip can significantly reduce emissions, compared to surface application in the soil.

Nitrogen application rates

Regarding the quantity of nitrogen fertilizer being applied, there is evidence that there is a direct relationship between the quantity of fertilizer added to the soil and N_2O emissions to the atmosphere.

Therefore, application rates that match the nutritional needs of the plantation, without excesses, can contribute to reducing emissions.

Agroforestry systems

Another recommended measure for the reduction of GHG emissions to the atmosphere is the adoption of agroforestry coffee system.

Preliminary studies in Costa Rica (Hergoualc'h et al., 2012) reported that the system conversion from monoculture to agroforestry contributed to the reduction in the balance of GHG emissions to the atmosphere.

In Brazil, coffee has been cultivated mainly in monoculture systems (full sun). In many other producer countries, however, the coffee has been traditionally cultivated under a canopy with different tree species.

These trees provide shade (Moguel and Toledo, 1999) and create microclimate conditions compatible with the ecophysiology of coffee (Da Matta, 2004). Furthermore, the root system of trees protects the soil against erosion and provides a continuous input of organic matter to the soil.

The soil quality in tropical agroecosystems depends largely on the biomass produced, the entry of plant waste (Tian et al., 2007) and the residence time of the litter (Hairiah et al., 2006), that provides protection to the soil and food for soil organisms, which contribute to the improvement of soil structure, moisture retention and soil nutrient supply (Kibblewhite et al., 2008).

Based on successful examples of coffee agroforestry systems, the study by Souza et al. (2012) showed that there is potential to combine coffee agroforestry systems in Brazil with the maintenance of soil quality, biodiversity conservation from the point of view of climate change and contribute to supporting ecosystem services.

CONCLUSION

The analysis of the GHG emissions in the 2009-2010 and 2010-2011 biennia allowed for a more consistent assessment of farms with different agricultural managements and with different crop yields between years.

However, the results are specific to the farms inventoried and do not necessarily reflect the reality of GHG emissions in the regions evaluated. Most of all, this study is a pioneering initiative and represent an initial estimate of GHG emissions of coffee production in Brazil.

Nevertheless, the results indicate that nitrogen fertilizers are the main source of GHG emissions, which allows recommending some alternatives for reducing emissions and the carbon footprint in the production process. Reducing emissions could give these farms greater added value to the coffee produced and even advantage over competitors in the market.

The authors emphasize, finally, the following ways to reduce CO2 emissions:

- Reduce the use of urea and/or replacement by organic fertilizers + other nitrogen sources as ammonium sulfate;
- Incorporation of N fertilizer (in trench, liquid injection or dripping irrigation);
- Use of N and lime based on soil analysis (agronomic recommendation accordingly to the real needs of crop and soil);
- Intercropped use of species that fix nitrogen to soil;
- Use of solar energy for drying coffee instead of mechanical driers.

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Research needs in the Coffee Sector: survey results

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INTRODUCTION

Research in coffee and cocoa is done by a large number of organizations, with different objectives, modes of financing and levels of openness and willingness to share methodologies and results. Much of their research addresses concerns about availability of sufficient volumes of coffee and cocoa of the desired qualities, which in turn leads to concerns about productivity, farmer livelihoods, climate change adaptation, pest and disease control and the like. However, a need to improve the efficiency of the research, as well as the use and dissemination of its results has been signaled. This paper describes setup and selected results of a survey among stakeholders in the coffee and cocoa sectors on finding out whether indeed this 'need' is felt in these sectors. It is part of a research programme, financed by the Dutch Ministry of Economic Affairs through its Topsector programme.

Setup and Roll out of survey

We developed a set of questions regarding background of respondents, their involvement in the coffee and cocoa sectors and in current and future research related to these sectors. Also we asked about their ideas about (lack of) coordination of this research and whether and if so how such coordination could be institutionalized. Majority of the questions was 'closed' and respondents could choose from a limited number of answers. A minority of questions was 'open' and respondents could freely respond. A complete list of the questions can be obtained from the authors.

The survey was rolled out by digitizing the survey and making it available on the internet. Apart from making use of our own network of potential respondents, both ICO and ICCO endorsed our survey and kindly made a link to our survey available on their websites.

Respondents

In total 92 respondents from various backgrounds (Table 1) filled out the survey, with strong representation from respondents based in Europe (Table 2).

Table 1. Background of respondents.

Stakeholder \ Sector	Only Coffee	Only Cocoa	Both	Unknown	Total
Private[1]	4	17	10	2	33
Civil[2]	4	5	11	0	20
Research	3	6	20	0	29
Government	3	4	3	0	10
Total	14	32	44	2	92

[1] includes farmers, members of cooperatives[2] includes not for profit foundations established by private companies

Table 2 Origin of respondents (% of total).

Stakeholder\ Region	Europe	Africa	Americas	Asia	Australia	World	Unknown	Total %	Total #
Private	54.5%	15.2%	18.2%	0.0%	3.0%	0.0%	9.1%	100%	33
Civil	55.0%	25.0%	10.0%	0.0%	0.0%	5.0%	5.0%	100%	20
Research	58.6%	13.8%	13.8%	6.9%	3.4%	0.0%	3.4%	100%	29
Government	40.0%	30.0%	20.0%	10.0%	0.0%	0.0%	0.0%	100%	10
Total %	54.3%	18.5%	15.2%	3.3%	2.2%	1.1%	5.4%	100%	
Total #	50	17	14	3	2	1	5		92

SELECTED RESULTS

Current research

Reasons why to get involved in research are similar for the Cocoa and Coffee sectors (
Figure 1). However, different stakeholders have different preferences. While researchers generally evaluate each issue of about same importance, particularly private sector and government respondents have clear preferences for improvements in quality and supply of cocoa and coffee. NGO's tend to put rather low importance to these issues, while indicating that they like to invest relatively more than the other stakeholders on issues where they think not enough research is being done. It is interesting to see that particularly in coffee the private sector mentions its Corporate Social Responsibility (CSR) strategy as an important driver for getting involved in research.

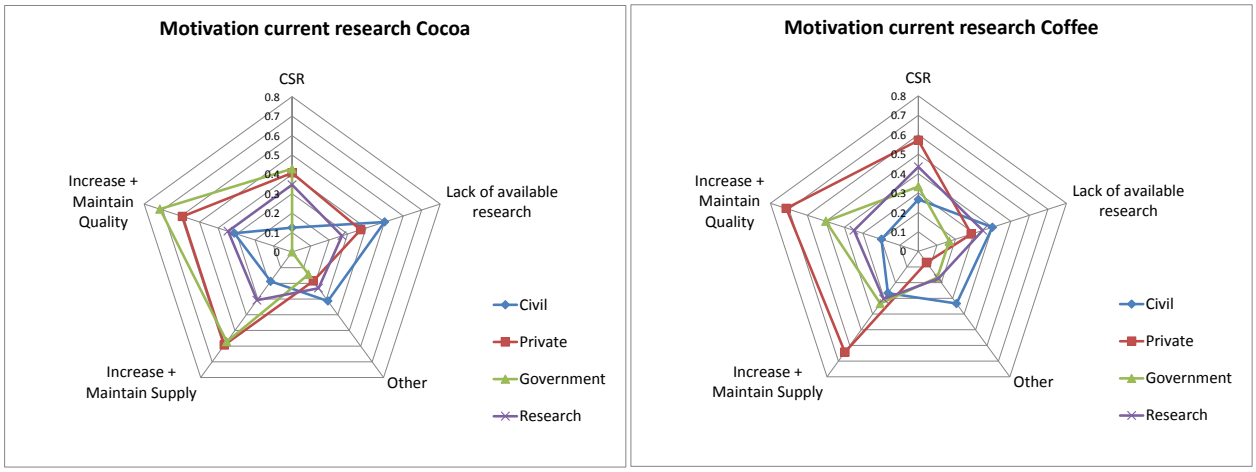

Figure 1. Issues that drive involvement in research. Numbers indicate the fraction of total respondents per type of stakeholder that agreed with each issue.

Regarding the types of research that the stakeholders currently are involved in, it seems that the private sector relatively is less involved that other stakeholders, while the differences between stakeholders is biggest in coffee (
Figure 2). Governmental respondents indicate low involvement in Service delivery / Concerted action, which corresponds with the movement of government out of service provision to farmers in many countries.

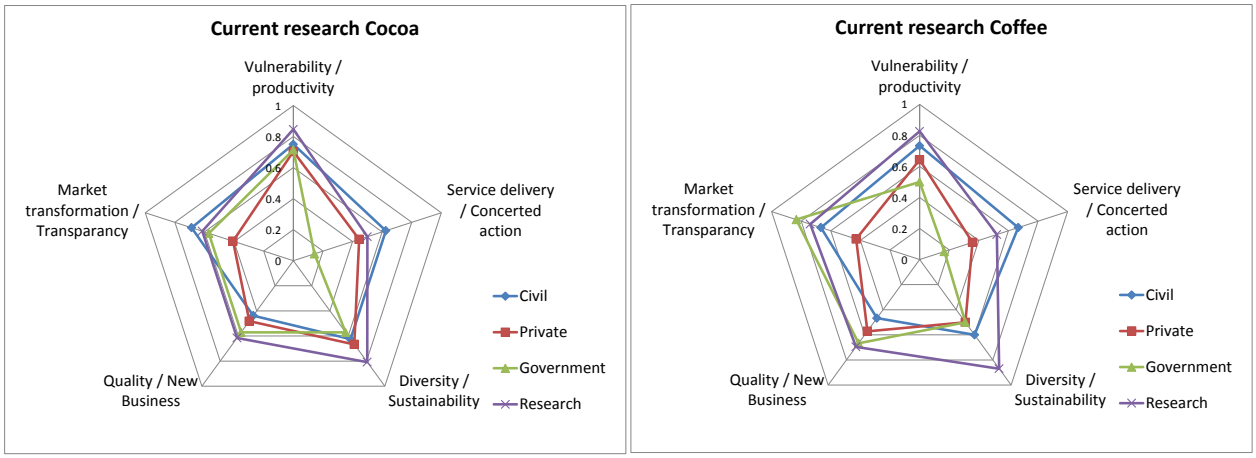

Figure 2. Main topics of current research. Numbers indicate the fraction of total respondents per type of stakeholder that indicate participation per type of research.

Identified knowledge gaps for research to fill

Quite a number of different knowledge gaps were identified, most of which are part of the Agronomy header, with on average well above 1 gap identified per respondent (
Figure 3). Under other headers (Economics, Environment, Processing, Social, Sustainability, Organization and Others) only well below 0.2 gaps per respondent were identified.

Topics under the Agronomy header included Genetics/Breeding, Pest & Disease Control, Climate Change, Productivity, Soil and Fertility, Rehabilitation and Rejuvenation, Diversification and Risk Management and Others. It is interesting to see that the private sector respondents gave most importance to the Genetics/Breeding topic and least to Climate Change compared to their answers on other topics (
Figure 4). In contrast, governmental respondents gave highest importance to Climate Change and much less to Genetics/Breeding. Private sector put also low marks on the Productivity issue while in the question on current research, this issue came out as one of the topics where private sector is doing most research on (
Figure 2). This may reflect the notion within the private sector that it is already well known how productivity can be improved.

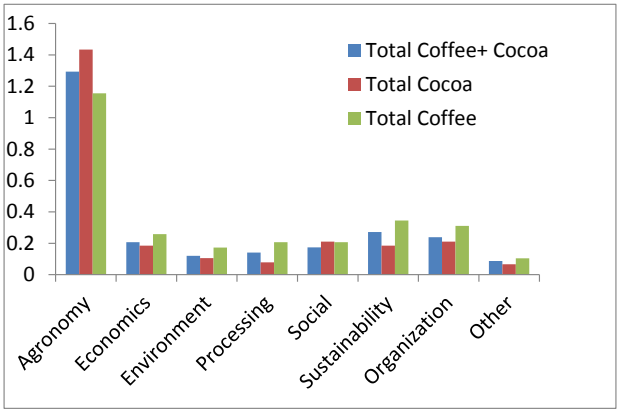

Figure 3. Number of identified gaps in knowledge. Numbers indicate the average gaps identified per respondent (all stakeholders taken together).

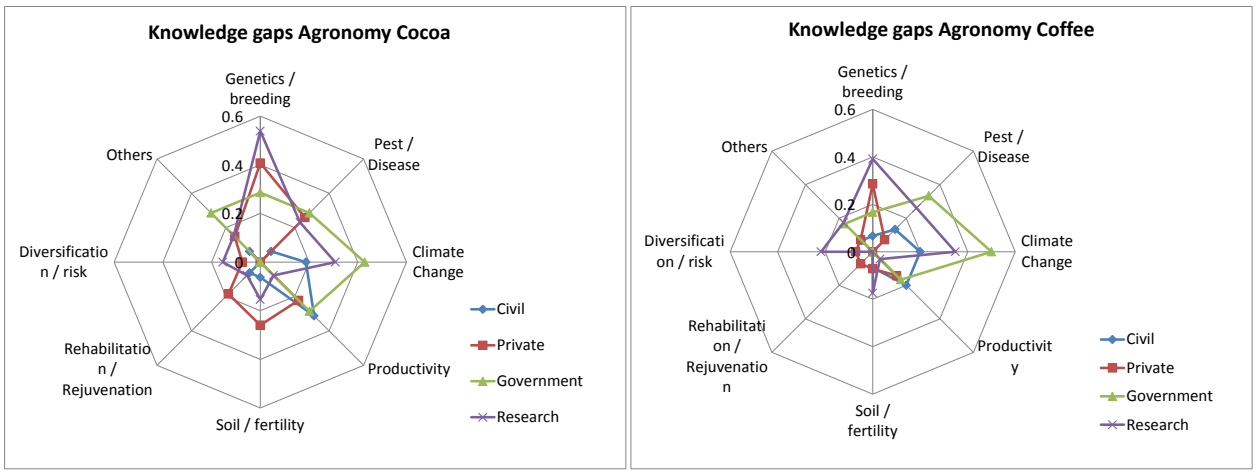

Figure 4. Indicated knowledge gaps per field of work within agronomy. Numbers indicate the average gaps identified per respondent per stakeholder type.

Overlap in Research and Need for coordination

Not all participants replied to the questions related to overlap in research; especially representatives of the private sector and governments refrained from providing an answer (Table 3). Even when the average response is calculated with the total number of respondents, the results indicate that a large fraction of respondents agrees with the proposition that there is such overlap (
Figure 5). A small minority of respondents state that they don't think such overlap is negative, particularly as it facilitates more thorough testing e.g. of varieties or new management practices.

Table 3. Total and relative number of participants without providing an answer on the question whether there is overlap in research compared to the total number of respondents.

	All Cocoa			All Coffee		
Type of stakeholder	**Total # respondents**	**# no answer**	**% no answer**	**Total # respondents**	**# no answer**	**% no answer**
Civil	16	4	25.0%	15	4	26.7%
Private	27	12	44.4%	14	10	71.4%
Government	7	3	42.9%	6	3	50.0%
Research	26	6	23.1%	23	6	26.1%
Total	76	25	32.9%	58	23	39.7%

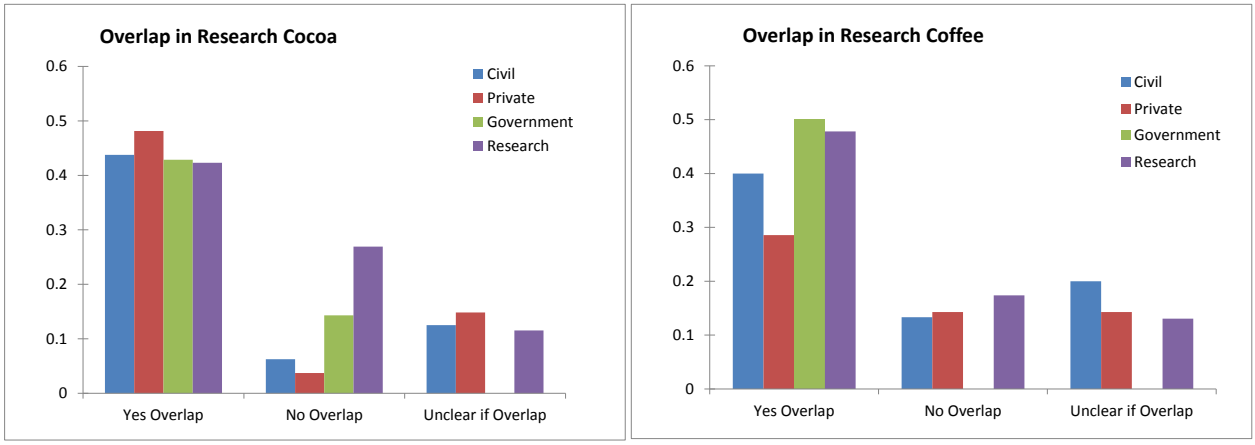

Figure 5. Average number of responses per stakeholder and per answer type regarding overlap in research.

On the question of the need for coordination/collaboration, again private sector and government were less enthusiastic than the two other stakeholder groups in answering positively (
Figure 6). Even then, in Cocoa sector more support to coordination and collaboration is seen than in the Coffee sector.

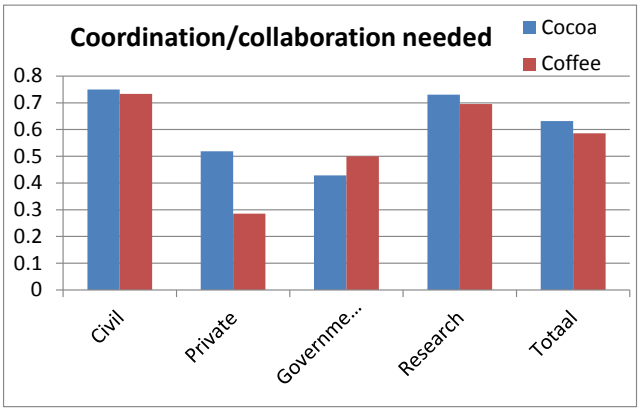

Figure 6. Responses to the question whether coordination of and/or collaboration in reseach in Cocoa and Coffee is needed

DISCUSSION AND CONCLUSION

Even when we take into account that European respondents with a private, civil and research background often work internationally, the dominance of European respondents may have resulted in bias in the outcome of the survey. This seems to be the case regarding the questions whether there is an overlap in research and whether coordination of research is needed, with maybe an exception for the researchers (
Figure 7). We did not check bias in other questions (yet). We think that getting rid of a regional based bias may only be possible when enough respondents from other regions will participate in our survey. Whether this is feasible within the time frame and budget that is allocated to us still has to be seen.

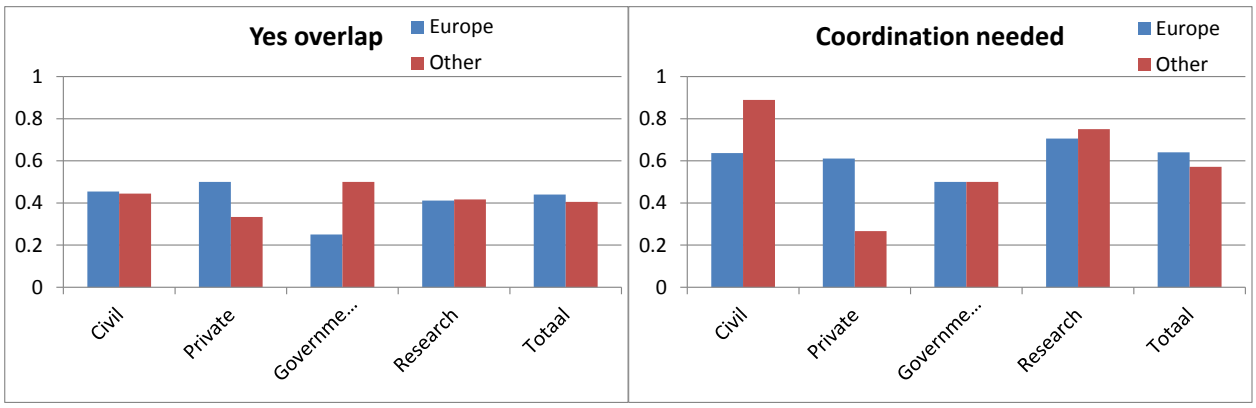

Figure 7. Comparison of responses from European and Other origin. Numbers indicate the fraction of each origin * type of respondent (e.g. Europe * civil) that agreed with the answer indicated in the title of the graphs.

For the most important food crops, a few regions and agroforestry systems, an international consortium exists, that among others integrates the research of its 15 members and coordinates its efforts with that of other, such as the National Agricultural Research Centers (CGIAR-consortium, 2014). This research system has a rather reliable funding mechanism through the CGIAR Fund that is administered by the World Bank and governed by the Fund Council a representative body of fund donors and other stakeholders (http://www.cgiarfund.org/aboutthefund).

For coffee and cocoa, even though being important cash earning crops for many farmers, such a clear coordination structure doesn't exist. Survey results show that a large fraction of respondents feels that more coordination is needed, although governmental and private sector respondents are a bit more hesitant than the research and civil society respondents. Partially this may be the case because quite some collaboration exists between private sector parties, such as in the Caobisco-ECA-FCC Research Working Group. Also other organizations exist, that coordinate specific activities often for a limited group of stakeholders, e.g. the World Coffee Research (WCR) that in contrast to its name mainly is involved in Central America, and specifically on the topic of coffee leaf rust. This proliferation of small scale coordination makes sector wide collaboration and coordination rather difficult. The Chief Sustainable Officer (CSO) of Mars company acknowledges this problem but insists that the cocoa industry and producing countries work together to be able 'to keep chocolate on the shelves' (The Guardian, 2013). Although he was mainly referring to collaboration on reaching directly to farmers, Mars also applies this strategy of collaboration in research. Within coffee, no major coffee roaster has (yet?) taken up such strong position regarding collaboration within the sector.

Whether improved coordination of research in the coffee sector is needed to improve collaboration and efficiency of implementation and whether enough support is available to make such coordination feasible is still unsure. These questions we will try to answer in the follow up of this survey.

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Research and Development of Arabica Coffee in Thailand

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SUMMARY

Arabica coffee has been introduced to Thailand since 1849. The Royal Project, including various public sectors and private organizations are promoting Arabica coffee in highland area as a substitute crop of opium poppy cultivation, reducing soil erosion, creating substantial income and reducing the destruction of forest and habitat. During 1975-76, coffee leaf rust disease, caused by *Hemileia vastatrix* Berk & Br. was found as serious disease affecting Arabica coffee in Thailand. In 1982, His Majesty King Bhumibol Adulyadej of Thailand has inspired the Department of Agriculture (DOA) to improve Arabica coffee cultivar for rust resistance, high yield, good quality and suitable to cultivate in highland area of Thailand. Since 1984, DOA has studied and selected for leaf rust disease resistance in various coffee varieties. Until 2007, Catimor coffee variety was selected and officially release as the recommended coffee variety named Chiang Mai 80. Meanwhile, DOA carried out the Research and Development of Arabica coffee by Hybridization project which granted by the Agricultural Research Development Agency (ARDA, Public Organization) between May, 2005 and Jan., 2013. The objectives were to improve the coffee hybrid plants resistance to coffee leaf rust (*Hemileia vastatrix* Berk & Br.) with high yield, good quality of green bean and drought tolerance. The results were found that 17 promising seedling trees were selected from 4-year old seedlings in the field trials. These coffee seedling trees are selected from 3 groups as follow; 1) Five selected trees with 100% resistance to coffee leaf rust disease, high yield, large bean size and good cup taste quality, 2) Resistance to coffee leaf rust disease 99-99.75%, high yield, large bean size and good cup taste. The third group is resistance to coffee leaf rust disease 98-100% and drought tolerance with medium high yield, big bean size and good cup taste quality. The results from cup taste recorded are similar to the chemical analysis figures with the additional of spicy flavor, wild flower odor, chocolate and wild honey flavors. For the coffee leaf rust genes transfer from maternal parents to hybrid seedlings, it was found that most of rust resistance seedlings come from coffee plants group A type and HDT derivatives group with mainly contained SH2,5 genes which resist to coffee leaf rust Race I and II. Unfortunately, over the past few years the cultivar Catimor has become susceptible to CLR. Recently, rust races not yet earlier found in Thailand were characterized at CIFC: race XXXVII (v2,5,6,7,9) from cultivar Typica, Caturra and SL6, race XXXI (2,5,6,9) from cultivar H528. A new rust race with the virulence genotype (v1,2,5,6,7,9 or v2,4,5,6,7,9) was also detected in Catimor and H420 samples. Meanwhile, DOA and CIFC have increased a cooperative programme to introduce new coffee germplasm with broad spectra of resistance to leaf rust. Guidelines have also been given to prevent or delay the emergence of new rust races.

INTRODUCTION

Coffee growing areas are in Thailand are mainly Robusta in the south and Arabica in the north. Upper Northern Province is a mountainous area with the area of 85,920 km^2 approximately which borders Myanmar to the north and west and Laos to the north and east. Coffee is grown at altitude between 700 and 1400 meters with the average annual temperature between 18 – 28^o C, and an annual rainfall of 1,200 - 1,500 mm/year. Due to production at high altitude, they are mostly categorized as watersheds or conservation areas. In many Arabica coffee planting areas under the coffee promotion program of the Royal Project Foundation, it has been shown that growing Arabica coffee under natural shading forest gives a good return to farmers for long term. Although the farmers have less management work and investment, they still receive some returns.

Arabica coffee is a significant cash crop for the northern highland part of Thailand. Production has dramatically increased recently compared to Robusta which decreased due to growers changing to oil palm and Para-rubber production. Thailand's coffee planting area in 2013/2014 was 54,837 ha. Total production is around 42,394 tons a year. Arabica is about 15% of the total production. The estimated growing area of Arabica coffee for this year is over 1,600 ha with 8,451 tons of coffee green bean production. The average yield is 980-1,010 kg of green bean per ha. The main planting area is restricted to two provinces in the north, Chiang Rai and Chiang Mai. Thailand still imports coffee due to increase domestic consumption demand for both instant and roasted/ground coffee from 2,757 tons in 2006 to 41,334 tons in 2013. Many modern coffee houses have been established in the big cities in recent years.

COFFEE PRODUCTION IN THAILAND

Coffee has been grown in Thailand for more than 100 years. The country officially became a coffee exporter in 1976, selling 850 tons of Robusta coffee. Because of strong world market prices in 1980s, exports thrived culminating was in a peak in 1991-92 of 60,000 tons. The collapse of the International Coffee agreement in July 1989 and the following slump in world market prices hit markets hard. Facing an oversupply, the Thai government initiated the five-year plan in 1992 to encourage coffee farmers to switch crops.

Thailand's Robusta coffee is decreasing in terms of area and production, due to the high cost of production, many growers have turned their attention to other lucrative crops like Para rubber and oil palm. However, Arabica coffee plantations in the north are increasing. For Arabica coffee, there is an increasing area and production because of high consumption and private sector support for the farm growers. The production is expected to be around 8,451 tons this year (Table 1), while demand is 10,000 tons annually. These figures demonstrate that Arabica coffee in Thailand, both in area and production have increased quite rapidly over the past 20 year, considering the country was only producing 500 tons in the early 1990s. The sharp increase of coffee shops lately in all major towns all over the country, has resulted in not enough coffee production to meet the local demand. As far as of Robusta coffee in South Thailand, its supply was certainly lower than the demand, with a production of 40,000 to 50,000 tons against a demand of 75,000 tones with average growth of about 10% a year, which was reported by the National Focal Point Working Group on Coffee, DOA. Per capita consumption of coffee is estimated at about 200 cups per year in Thailand, which is relatively low compared with 500 cups in Japan and 700-800 cups in the United States. The Thai coffee market is worth about 1.03 billion USD but is mainly dominated by instant coffee and three-in-one packs.

Table 1. Arabica Coffee Production in Thailand: 2005-2014.

Year	Harvested Area (ha)	Yield (tons)	Yield/ha (tons)
2005	2,859	2,150	0.75
2006	3,022	2,477	0.82
2007	3,104	2,873	0.93
2008	3,489	3,366	0.96
2009	4,045	3,681	0.91
2010	4,676	4,725	1.01
2011	5,469	5,339	0.98
2012	6,240	6,300	1.01
2013	na	8,270	na
2014	na	8,451	na

Source: Office of Economics, MOAC

From having a coffee plantation area of almost 67,941 ha in 2007, Thailand is estimated to have only 42,176 ha in 2014 (Table 2), down from 47,490 ha last year. The number of coffee households, production volume and the yield in tons per ha is also decreasing. The coffee production in Thailand in 2013/2014 was 38,300 tons. The majority of the production is Robusta coffee in the South with 29,849 tons or 77.93% whereas Arabica coffee in the North has a total production of 8,451 tons or 22.07%. It is reported that 90% of coffee production is for domestic consumption and only 10% for export.

The cost of production is at 50.80 Baht/kg with farm gate price of 69.10 Baht/kg. The main production cost about 60% of the production cost is the labor cost for harvesting coffee and maintenance, and the rest from inputs ie. fertilizers, pesticides, fuel. etc.

Several coffee growers have shifted to other lucrative plants such as rubber and oil palm because of their higher market prices. Thailand's coffee production has dropped over the last six years to an estimated 40,000 tons this year.

Table 2. Coffee Production in Thailand: 2005-2014.

Year	Harvested Area (ha)	Yield (tons)	Yield/ha (tons)
2005	69,535	59,644	0.858
2006	68,780	46,873	0.681
2007	67,941	55,660	0.819
2008	62,186	50,442	0.811
2009	58,454	56,315	0.964
2010	57,518	48,955	0.851
2011	51,663	42,394	0.821
2012	48,978	41,461	0.846
2013	47,490	37,460	0.790
2014	42,176	38,300	0.910

Source: Office of Economics, MOAC.

Table 3. Thailand Coffee Export and Import in 2005-2014.

Year	Bean & Ground Coffee (tons)		Instant Coffee (tons)		Coffee 3 in 1 (tons)	
	Export	Import	Export	Import	Export	Import
2005	16,127	217	11,211	1,020	na	na
2006	25,784	600	2,421	2,175	24,812	7,802
2007	11,239	674	7,798	2,006	17,680	8,578
2008	1,662	14,815	3,391	2,889	26,850	4,215
2009	381	6,503	4,122	2,222	30,898	4,120
2010	534	14,621	8,308	3,017	37,928	4,841
2011	856	34,851	5,263	4,446	33,979	6,261
2012	2,085	29,061	7,260	6,531	40,142	7,650
2013	368	34,907	1,624	6,427	51,548	6,706
2014	16,127	217	11,211	1,020	na	na

Source: Department of Customs, MOC

Table 4. Arabica Coffee Production in northern provinces of Thailand: 2011-2014.

Province	Harvested Area (ha)	Yield (tons)			
	2010/11	2010/11	2011/12	2012/13	2013/14
Chiang Rai	2,323	2,440	2,950	3,980	3,864
Chiang Mai	1,896	1,967	2,340	3,050	3,202
Mae Hong Son	382	341	350	395	387
Nan	323	224	240	325	381
Lampang	206	182	211	280	291
Tak	198	89	100	130	207
Phrae	141	96	110	110	114
Total	**5,469**	**5,339**	**6,300**	**8,270**	**8,451**

Source: Office of Economics, MOAC

ARABICA COFFEE HISTORY

Arabica coffee has been introduced to Thailand since 1849. From 1972-1979, the Thai/UN Crop Replacement and Community Development Project was implemented as a pilot project to explore the viability of replacing opium poppy cultivation with a variety of substitute crops and alternative sources of income. It was found that Arabica coffee is a cash crop that shuld be promoted to replace opium in the long run and can provide high cash incomes, not only to opium poppy growing farmers, but to a larger number of other farmers in the highland as well. The main reasons of this are that land and climate are suitable for coffee growing, transportation and storage of coffee are relatively easy, yield are good and that there is a strong demand for good quality highland coffee. Thus Arabica coffee is very appropriate and viable as a cash crop to replace opium in the highlands of Thailand. Catimor and Sarchimor, the most advanced selections, have been widely distributed in the coffee growing countries, not only in Latin America but also in Africa, Oceania and Asia including Thailand. After local selection for several years, Catimor received regional designation as Chiang Mai 80 in Thailand.

In the early 2000s, coffee prices picked up again due to increased demand for coffee on the world market and have been climbing ever since and lately to record levels. This has had a positive effect on coffee growing internationally but specifically in the northern highland with increasing interest in the crop which culminated in a surge in planting.

The advantage of the coffee boom was the rapid expansion of coffee fields in and around the Doi Chaang village in Chiang Rai province, a small community of mainly Akha and some Lisu people, who demonstrated an incredible drive and enthusiasm to grow the crop and profit from it in a very lucrative way. Once coffee growing was well-established in the village, the Doi Chaang coffee farmers were united into the Doi Chaang Coffee Company (International). Most coffee produced is being exported and Doi Chaang coffee beans received international recognition lately with a cup ranking of 94 for its Pea berry coffee bean by an international panel, making it a renowned specialty coffee par excellence. Currently the total area under Doi Chaang coffee is around 3,000 ha with a total production of over 700 tons. It is the largest single Arabica coffee area in northern Thailand.

RESEARCH AND DEVELOPMENT OF ARABICA COFFEE VARIETY

Coffee extension and development has been conducted since 1957. Many cultivars of Arabica coffee from different parts of the world were introduced. DOA introduced seeds of Arabica coffee, Typica, Boubon, Catuai and Mundo Novo from Brazil. Firstly, the high yield variety Caturra was introduced by the UN pilot project under the patronage of the Royal Project, but Caturra is seriously affected by leaf rust (*Hemileia vastatrix*). Early research and study of various aspects of Arabica coffee was conducted by the Department of Agriculture and the Highland Coffee Research and Development Centre, Faculty of Agriculture, Chiang Mai University. Coffee leaf rust surveys made in 1973 found the infection at experimental stations. In 1974, the Royal Project Foundation received F2 generation seeds of 26 lines of Hibrido de Timor derivative and non-Hibrido de Timor of Arabica coffee, through USDA from CIFC. These hybrid seeds were bred for rust resistance. DOA was appointed by the Royal Project Foundation to study the possibility of growing Arabica coffee to replace opium poppy.

Then, in 1974, the most promising Catimor derivatives were introduced and selected lines were screened on the basis of compact tree size, leaf rust resistance, high yield, vigor, good bean, cup quality and drought tolerance. Though all these combination characteristics are hard to find in one variety, certain selected Catimor lines have shown good potential, both for production and market. Tammakate has elaborated on this selection process and the most promising lines. The DOA released the Catimor variety, Chiang Mai 80 from line CIFC 7963 in 2007.

The selection of F2 generation trees in trial plots started in 1979 by using phenotype of Caturra, a dwarf type productive tree as the model. Seedlings from each selected tree of 26 lines of hybrids were inoculated with uredospores of *H vastatrix* Race II. The inoculated seedlings were incubated in a chamber at a temperature of 22 ± 2 ^{o}C with a relative humidity of about 90 % in darkness. The inoculated seedlings were kept under nursery conditions for four weeks.

In 1983-84, four rust resistant lines of Catimor seeds, CIFC 7958, 7960, 7962, 7963, were introduced from Protugal to Thailand. The Catimor is a cross between Caturra and HDT (Hibrido de Timor). It is a compact growing cultivars and resistant to most races of coffee leaf rust. Since 1985, the Arabica coffee variety, Catimor CIFC 7963, was studied and selected for leaf rust disease resistance. The rust resistant varietal line (F7) CIFC 7963-13-28 was selected and trialed in four locations. This variety showed good performance with average green bean

production up to 1.344 ton/ha compared to Caturra, Bourbon, Typica which normally produced 563-750 kg/ha. The percentage of grade A (≥5.5 mm.) coffee bean 81.3-87.3 %. The cup quality test was between 6.5 to 7 (out of 10) compared to 5.5 for Caturra. The recommended growing area is >700 meters above mean sea level with temperature 18-25^{o} C. Rain distribution was more than 1,500 mm/year. This variety appears to susceptible to drought condition, therefore, it should be planted in the shade of natural forest or intercrop of fruit tree orchards.

COFFEE CULTIVATION

In the early period of the Arabica coffee extension program, the recommended spacing of planting was 2x2 meters in a mono-cropping system, which gives 400 plants per rai (6.25 rai =1 ha). With technical and marketing support from the Highland Development project, about 500 tons of quality Arabica coffee beans were in the market by 1990/91.

Unfortunately, the drop in coffee prices in 1990-1992 had a big impact on the coffee market. The price drop also coincided with the lack of support from the highland Development project, and some of the coffee growers cut down their coffee trees because they could not get any income from coffee during that period. Gradually the system has been changed in favor of incorporating coffee in a mixed multi-cropping system using shade trees and intercropping, based on low external input and sustainable production.

At present, coffee cultivation system in the highlands of Thailand consists of mixed cultivation and an agro-forestry system where the hill-tribe people grow coffee with fruit trees. The trees used do not compete heavily with coffee. This is the most appropriate recommendation for coffee farmer because of its advantages for coffee cultivation in the highlands. The wet processing method is recommended for good quality coffee bean, which is favored by the market and attracts good prices. The process includes pulping, fermentation, soaking, drying, hulling and grading.

CHARACTERISTICS OF COFFEE IN THAILAND

The most serious disease in the northern Thailand's coffee plantation is coffee leaf rust (*Hemileia vastatrix* Berk & Br.) race I (V2,5). The investigation on leaf rust was carried out at Doi Muser Horticulture Experimental Station and the first evidence on leaf rust in Thailand has been recorded. Recently, Catimor became susceptible to CLR. Coffee leaf rust surveys were made and some samples from coffee plantations were characterized at CIFC (Portugal). New rust races were discovered in Catimor which were never characterized before. Thailand has tried to develop new resistant coffee cultivars as well as to implement some strategies to prevent or delay the appearance of new rust races in northern coffee regions. The collaborative research works include selection of resistant varieties, introduction of new coffee genotype and selection of coffee genotypes with a larger spectra of resistance to CLR. DOA carried out the Research and Development of Arabica coffee by Hybridization project which was granted by the Agricultural Research Development Agency (ARDA, Public Organization) between May, 2005 and Jan., 2013. The objectives are to improve the coffee hybrid plants resistance to coffee leaf rust while producing high yields, good quality of green bean and drought tolerance. The results found that 17 promising seedling trees which were selected from 4-year old seedlings in the field trials. These coffee seedling trees are selected from 3 groups as follow; 1) Five selected trees with 100% resistance to coffee leaf rust disease, high yield, large bean size and good cup taste quality 2) Resistance to coffee leaf rust disease 99-99.75%, high yield, large bean size and good cup taste. The third group is resistance to coffee leaf rust disease 98-100% and drought tolerance with medium high yield,

big bean size and good cup taste quality. The results from cup taste recorded are similar to the chemical analysis figures with the additional of spicy flavor, wild flower odor, chocolate and wild honey flavors. For the coffee leaf rust genes transfer from maternal parents to hybrid seedlings, it was found that most of rust resistance seedlings come from coffee plants group A type and HDT derivatives group with mainly contained SH2,5 genes which produce resistance to coffee leaf rust Race I and II. Unfortunately, over the past few years the cultivar Catimor has become susceptible to CLR. Recently, rust races not found earlier in Thailand were characterized at CIFC: race XXXVII (v2,5,6,7,9) from cultivar Typica, Caturra and SL6, race XXXI (2,5,6,9) from cultivar H528. A new rust race with the virulence genotype (v1,2,5,6,7,9 or v2,4,5,6,7,9) was also detected in Catimor and H420 samples. However, these rust samples are under study to confirm if it is they are new rust races. Meanwhile, DOA and CIFC have increased a cooperative programme to introduce new coffee germplasm with broad spectra of resistance to leaf rust. Guidelines have also been given to prevent or delay the emergence of new rust races.

PROSPECTIVE OF COFFEE RESEARCH AND DEVELOPMENT

Although Thailand's coffee production remains low compared with other ASEAN countries such as Vietnam and Indonesia, Thailand has high potential to grow as the regional center of coffee, given the quality of their grown coffee production processes and the favorable geographical location. Thai coffee makers may blend indigenous coffee with imported coffees but its quality, odor and flavor need unique development to make it a Thai coffee formula.
The government policies for coffee are the formation of the Coffee Committee under the Ministry of Agriculture and Cooperatives, and the Sub Committee of Horticulture under the Executive Committee of Agriculture and Cooperatives. The strategic plan for coffee in Thailand (2014-2017) includes the increase of production efficiency, decrease of production cost, increase value of coffee products, increase GAP coffee farmers/GMP/GI/International certification, promote specialty coffee, and development of a sustainable coffee sector with environment assurance and food safety.

Coffee Research and Development in Thailand for Arabica coffee includes increase in yield by improving rust resistant varieties, increase yield per area by production technology, improve bean quality by pre- and post-harvest technology and promote coffee communities to have value addition.

In conclusion, although coffee is one of the economics commodities which gives a significant national incomes, its low productivity and high cost of production still needs to be addressed. Good Agricultural Practices (GAP) are needed to increase the number of farms in order to reduce the cost of production and improve quality and productivity. Diversification of coffee products and value added would provide more income and sustainability for coffee farmers, processors and exporters. The cooperation between coffee producing countries especially among ASEAN countries are useful for coffee industry by sharing through research collaboration, germplasm exchange and trade agreement. DOA and CIFC have increased a cooperative programme to introduce new coffee germplasm with broad spectra of resistance to leaf rust. Guidelines have been given to prevent or delay the emergence of new rust races.

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Impact Assessment of a Decade of Coffee Research and Technology Transfer in Tanzania: Lessons Learned

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SUMMARY

The major achievements of Tanzania coffee research institute (TaCRI) is the release of 19 Arabica hybrid coffee varieties that are high yielding, resistant to coffee berry disease (CBD) and coffee leaf rust (CLR) and with good beverage quality. Also release of four Robusta hybrids coffee varieties that are high yielding, resistant to coffee wilt disease (CWD) and with good bevarage quality. Another achievement is packaging & promoting eight and 10 commandments of coffee productivity and quality improvement. The institute has also disseminated technologies on good agricultural practices (GAP) through village based training, documentaries, and demonstration plots. TaCRI developed and perfected techniques for accelerated multiplication and distribution of improved coffee hybrids through clonal propagation, grafting techniques, tissue culture and hybrid seeds production by hand pollination. The study was conducted to assess the impact of TaCRI's technologies transfer and training had positive impact to farmers' incomes and livelihoods using stractured questionnaires. The questionnaires was administered to 300 respondents selected randomly from two villages in four coffee growing zones to assess the impact improved coffee varieties and adoption of on livelihood improvements and challenges. The study also involved a review of secondary data. Primary data were analysed using Statistical Package for Social Science (SPSS) to compute means and frequencies. Results revealed that TaCRI's technologies transfer and training on GAPs, seedlings multiplication and distribution and farmer training, have positive impact to farmers' adoption of improved coffee varieties, improved income security and improved livelihood in the study area. Also the study noted that farmers are adopting GAPs and improved varieties in large numbers and planting them in their farms. The emphasis required now is to (i) Developing and strengthening coffee seedlings multiplication and distribution system and (ii) Strengthening coffee extension or technologies transfer and training.

INTRODUCTION

The world coffee crisis of 1990s resulted low production (Osorio, 2002). Most of the coffee growers neglected or uprooted coffee trees because of low economic returns (Hella, 2005). Low coffee production was also contributed by the widely grown disease susceptible commercial varieties in Tanzania. Tanzania Coffee Research Institute (TaCRI) was established in 2001 with objective of rejuvenating the Tanzanian coffee industry, placing new emphasis on stakeholder-led, demand driven research for development. The purpose of TaCRI is to develop and to disseminate appropriate coffee technologies so as to increase productivity, improve quality, reduce cost of production, improve competitiveness of Tanzania's coffee in the world market, improve income security and livelihoods of coffee growers and increase the contribution of the coffee sub-sector to the GDP. The major achievements of TaCRI since 2001 include release of 19 Arabica hybrid coffee varieties that are high yielding, resistant to coffee berry disease (CBD) and coffee leaf rust (CLR) and with

good beverage quality. Also release of four Robusta hybrids coffee varieties that are high yielding, resistant to coffee wilt disease (CWD) and with good bean size and bevarage quality (Kilambo *et al.*, 2013 and Teri *et al*., 2011). Another achievement is packaging & promoting eight and 10 commandments of coffee productivity and quality improvement respectivelly. In addtion TaCRI has been using a number of approaches (village based training, agricultural shows, open days, demonstration plots, backstopping programme and publications) to promote and disseminate GAPs to coffee farmers. Likewise TaCRI perfects different methods (clonal propagation, grafting techniques, tissue culture and hybrid seeds production by hand pollination) for multiplication and distribution of improved hybrid seedlings. The achivement noted from this effort include training of 377,496 farmers in all coffee growing zones, establishment of 18,000 clonal mother gardens with total 330,368 mother plants owned by 600 farmer groups, 20 estates, 80 co-operatives unions, and 15 individuals farmers and establishment of 1,632 on-farm demonstration plots in all coffee growing zones owning and managing nurseries/ vegetative propagation unit (VPU) which contribute to over 46,000,000 clonal hybrid seedlings multiplied and distributed to coffee growers. were the major sucess under technology trainfer and training. This study aims to assess the impact of coffee research and technology transfer on coffee productivity and profitability, improving household income and livelihoods, and to assess challenges in adopting coffee technologies in four coffee growing Districts in Tanzania.

MATERIALS AND METHODS

Structured questionnaires were used to collect information on the level of adoption of improved coffee varieties and their impacts, adoption of GAPs and impact on productivity, systems of technology transfer and training, and seedling multiplication and dissemination. Complimentary data were on socio-economic characteristics (gender, age, level of education, position in the household, household size) and farm details (size, number of coffee trees and varieties, average production). Additionally respondents were requested to give an account of contribution of TaCRI's technologies on coffee productivity, the contribution of improved technologies in livelihood improvements and the challenges in adopting improved coffee varieties. A total of 80 respondents were selected randomly from each zone as representative of coffee growers in areas, making a total of 320 respondents. Representative areas include: Mbozi district (Southern Highlands), Mbinga District (Southern Highlands), Tarime District (Lake Zone) and Hai District (Northern Zone) (Fig. 1). Data were processed and analyzed using Statistical Package for Social Science (SPSS) version 16 (SPSS Inc, 2007) to compute means and frequencies of socio-economic characteristics and farm details. Microsoft excel was used to compute gross margin whereas focus group discussions with farmer groups and personal observations by taking photos were used to capture livelihood improvements and the challenges in adopting improved coffee varieties.

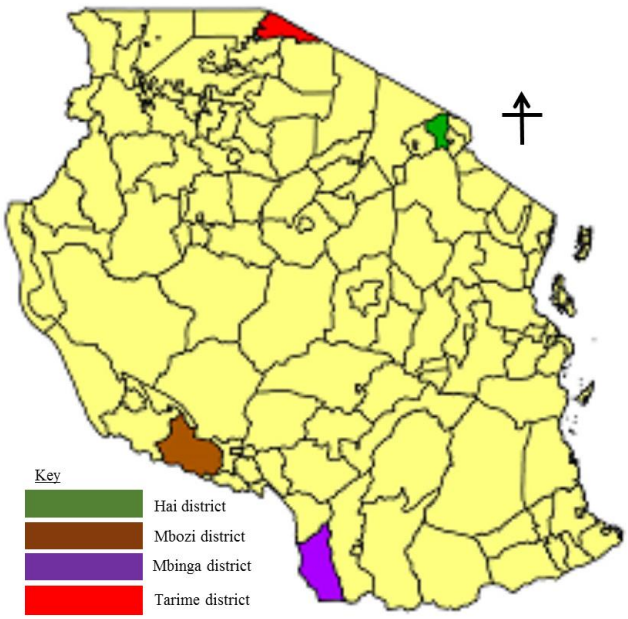

Figure 1. Map of Tanzania indicating area where data was collected.

RESULTS AND DISCUSSION

The results in Table 1 indicates the socio-economic characteristics of respondents in the study area of which 60% of respondents had age above 60 years old which implies that, youth are not involved much in coffee farming. This may have implications on the adoption of new varieties, and GAPs application. Cruz, (1978) reported that age has significant influence on farmers' adoption of technologies. On basic education: 75% of respondent's attained primary education, 17% secondary education, and 6% college education. The study conducted by Riddell (2012) indicated that, education has casual impact on measures of technologies but does not influence the use of technologies associated with routine tasks. Majority of farmers have primary education and are more experienced in coffee farming. It was found that 90% of respondents were males but most of farming activities in the study area are performed by female.

Table 1. Socio-economic characteristic of respondents in the study area.

Socio-economic Characteristics		Percentage of respondents				
		Tarime	Hai	Mbozi	Mbinga	Average
Age	18-45	15	10	12	14	13
	46-60	30	28	29	28	29
	>60	55	62	59	58	59
Education	No formal education	8	0	0	5	3
	Primary	76	71	80	71	75
	Secondary	13	18	17	18	17
	College	3	11	3	6	6
Sex	Male	93	97	93	77	90
	Female	7	3	7	23	10
Marital Status	Single	0	3	0	7	3
	Married	93	93	97	93	94
	Widowed	7	3	3	0	3

The farming system in the study area

The stuy also found that, 77% of the respondents in the study area intercrop coffee x banana, sometimes with beans, while 23% practice pure stand coffee farming. Other agricultural activities practised by farmers in the study area include maize, horticultural crops and livestock keeping (cows, goats, and poultry). Parrish (2005) observed that, farmers adopt different farming systems in order to diversify risks. The average land size owned by respondents in the study area under coffee is 0.8 ha which implies that, the land for coffee cultivation is small hence need to emphasis adoption of improved coffee varieties so as to gain more yield from the available land. Overall, farmers do not apply fertilizer and other inputs to control pest and disease because of high price of inputs. According to Hammond (2010), high price of inputs limits increase in productivity, quality and hence affects profitability.

Adoption of improved coffee varieties and good agricultural practices

From this study 47% of 320 respondents had full adopted improved varieties and implementation of good agricultural practices. This is an effort for a eight years period since TaCRI's first official release of nine improved Arabica hybrids. The percentage distribution of coffee growers with new coffee varieties and implementation of good agricultural practices is presented in Fig. 2a and b.

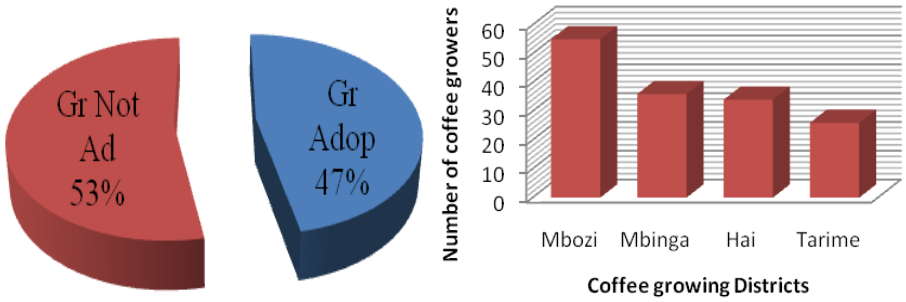

Figure 2. (2a. left) Distribution of respondents (out of 320) with and without improved coffee varieties and implementation of good agricultural practices. (2b. right).

Results from this study Fig. 3 indicates that, respondents in the study area access coffee seedlings for planting from different sources including TaCRI 44%, farmer group nurseries 35% and District nurseries 21%. This implies that, involving different stakeholders in seedlings multiplication speed-up access of improved coffee varieties in easy way.

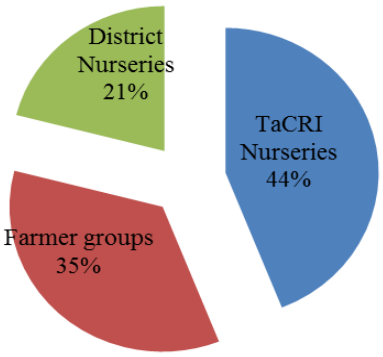

Figure 3. Main source of improved coffee seedlings.

This is part of the efforts in contributing a total of 18 million coffee seedlings per year TaCRI is producing. Target is to produce 20 million seedlings per year. But of importance in this particular case is involvement of other stakeholders in seedlings multiplication.

The impact of adoption of improved coffee varieties and GAPs on productivity

Fig. 4 indicates the average production gained by respondents with improved coffee varieties as opposed to traditional varieties. Kilambo *et al.* (2011) the potential yield of improved varieties can go up to 3 kg/tree of clean coffee under good agricultural practices.

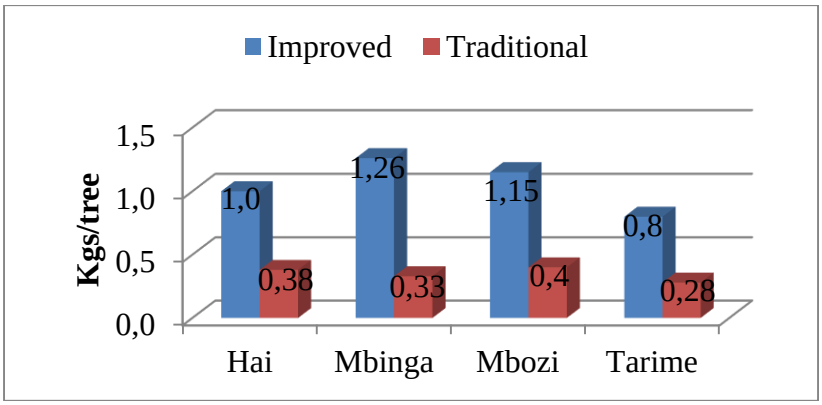

Figure 4. Average yield of improved against traditional varieties.

Fig. 5 shows coffee yields before and after the adoption of the new coffee varieties and implementation of good agricultural practices. Yields have more than doubled from 100% of traditional varieties to 329% of improved coffee varieties. This implies more income gain by respondents with improved varieties as opposed to those with traditional varieties. Positive comments received from respondents with traditional coffee varieties: "*We are replacing the traditional coffee varieties with improved coffee varieties from TaCRI because of realized economic benefits".*

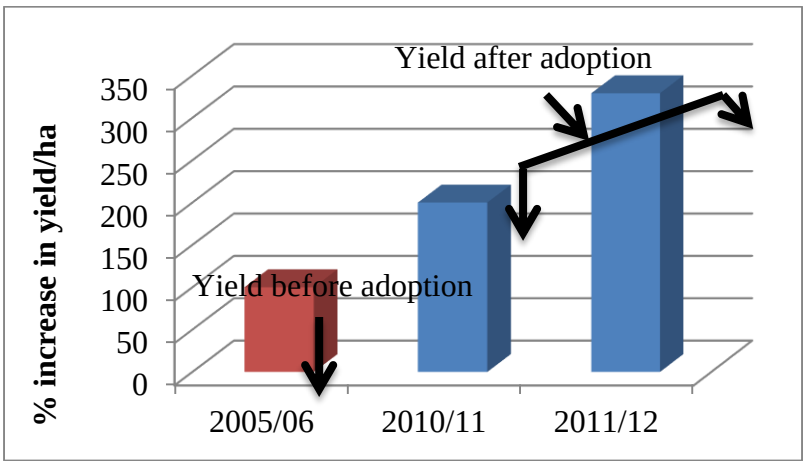

Figure 5. Yield before and after adoption of improved coffee verities.

The contribution of coffee in improving income security and livelihoods

From the study it was found out that, coffee grower's uptake of GAPs is high. Growers from the study areas use a wide range of training materials prepared by TaCRI. There are males and females coffee farmers actively participating in technology adoptions and transfer and

becoming farmer promoters. This approach has assisted TaCRI to diffuse their technologies in a wide range of coffee areas, improve productivity and therefore the livelihoods of the coffee farmers. Fig. 6 indicates different sources of household income gained by respondents in 2011/12 production season.

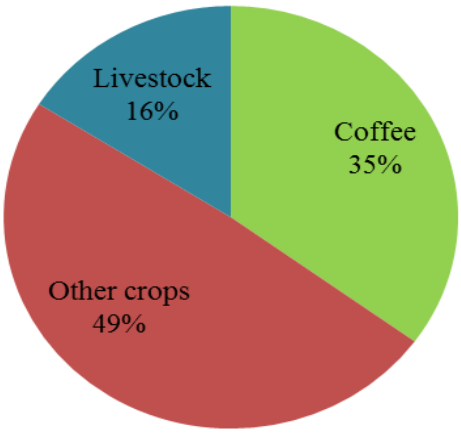

Figure 6. Composition of Household Income.

Cost of production & gross margin of improved visa traditional coffee varieties

It was found from this survey that the average cost of producing 1 Kg of parchment coffee of improved coffee varieties including implementation of GAPs is reduced to 50% of traditional varieties (Fig. 7) due to 80% reduction fungicides use with improved coffee varieties. In this case the gross margin gained by farmers increased. Cardino (2014) reported an increase of Arabica productivity from 250 to 300 Kg of green beans per hectare in most of the coffee growing areas in Tanzania. Partly, this has been contributed by the adoption of improved Arabica varieties and GAPs.

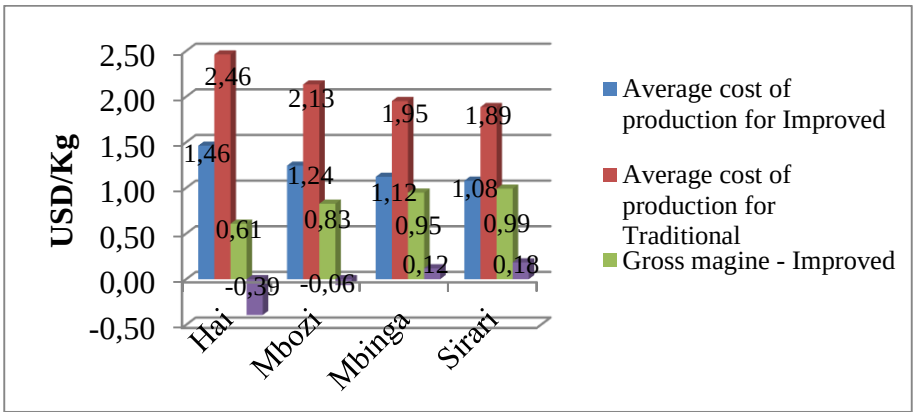

Figure 7. Cost of production and Gross margin for improved and traditional varieties.

All respondents who adopted improved coffee varieties and implementing GAPs are reported to have increased purchasing power of household basic needs, paying school fee and affording access to health services. Growers' capacity to purchase valuable assets increased as a result of coffee farming. Assets bought such as maize milling machine, motorcycle and livestock (photo below), television sets, radios and construction of modern houses.

Figure 8. Some of photos indicating improved livelihoods in the study area.

Impacts of improved coffee varieties have been observed in the study areas in land utilization. In Hai district, long time abandoned coffee estates are now being rehabilitated and planting with new coffee varieties. In Mbinga, smallholder coffee growers are establishing new coffee plants in farms used to be cultivated with maize. Simon (2012) reported that, farmers take risks to invest where there is economic returns. Other notable impact of TaCRI technologies is the established strong platform that links researchers, extension officers, policy makers and farmers to share ideas.

Challenges to adoption of technologies

Table 2 summarizes challenges mentioned by respondents in coffee growing areas of the four visited districts. Low price of coffee at farm gate is ranked challenge number one in limiting adoption of improved coffee varieties and the GAPs. This was also mentioned as prime in a baseline survey by Hella (2005). It is suggested that policies should be supportive for the coffee farming to be productive.

Table 2: Challenges to adoption of improved coffee varieties and GAPS

Chalenges	**% of respondents**
Low price of coffee in the market	89
Weak of extesnion servises	75
Aged coffee producer	62
Low supply of improved seedlings	59
Delayed payment	53
Gender mainstriming	48
Prolonged drought	42

Lesson learnt in the study area

TaCRI's technologies transfer and training on GAPs, seedlings multiplication and distribution and farmer training, have positive impact to farmers' adoption of improved coffee varieties, improved income security and improved livelihood in the study area. Also the study noted that farmers are adopting GAPs and improved varieties in large numbers and planting them in their farms. Similar lesson were noted by CARDNO (2014).

CONCLUSION

It can be deduced from the study that TaCRI's technologies transfer and training had positive impact to farmers' incomes and livelihoods. These technologies can impact coffee productivity, and therefore economic returns. However, for the technologies to have positive impact to majority of farmers there should be supportive policies that will emphasis

development and strengthening coffee seedlings multiplication and distribution system and strengthening coffee extension system.

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Relationship Coffees in the Specialty Coffee Sector: What Benefits for Indonesian Smallholders?

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SUMMARY

Direct relationships between international specialty coffee roasters and smallholder coffee farmers appear to offer exciting opportunities for livelihood improvements in otherwise marginalised rural communities in the developing world. This paper reports on an action-research activity on the Indonesian island of Sulawesi, where such a relationship has been developed over a four-year period. This particular experience found that superior prices were obtained by a local cooperative, and that these prices were generally passed on to individual farm households, who also benefitted from labour savings under a cooperative-managed centralised processing unit. However, a majority of local farmers still prefer to sell into traditional collector networks, and the arrangement does not appear to have had a significant impact on farmer welfare and livelihoods in the community. This is due to the diversified character of local risk-averse livelihood strategies, institutional limitations that result in delayed payments and low levels of membership in the cooperative, and the perceived unfavourable trade-offs associated with compliance to stringent quality standards. More effective institutional design for such relationships, which is sensitive to the livelihood aspirations of rural households, will be required if such coffees are to generate meaningful development outcomes in rural Indonesia.

INTRODUCTION

International demand for specialty coffee is growing, providing an opportunity for smallholder farmers in Indonesia to upgrade the quality of the coffee they produce and to attain premiums within high-value supply chains. At the same time, specialty roasters in international markets are eager to demonstrate to consumers their superior knowledge of, and intimate commercial links to, origin communities. These conditions have coalesced to stimulate a trend towards 'relationship coffees', which are defined in this article as coffee presented to consumers that is procured through a relationship between a coffee roaster and producers that involves personal interaction, mutual trust, price transparency, a commitment to quality improvement beyond a single harvest, and an intention to improving the lives of producers. 'Direct Trade' then may be considered a sub-set of 'relationship coffees', but where the latter does not necessarily negate the potential commercial role for middlemen.

While proponents of this model, including the roasters themselves, believe that relationship coffees are improving the lives of their supplier farmers, there has been very little independent assessment of the farm-level impacts of these relationships in smallholder communities. This paper presents the results of an action-research study on the Indonesian island of Sulawesi, where a relationship between a smallholders farmer cooperative (*Koperasi Pertanian*) and an international coffee roaster has been facilitated and monitored over a four-year period. The research sought to understand to what extent, and under what conditions, relationship coffees

present opportunities for improved livelihoods within smallholder coffee-growing communities.

In development theory, relationship coffees can be considered to be a subset of a broader set of development interventions, generally referred to as value chain interventions for development. The last decade has seen the widespread adoption by international development agencies of value chains for development as a framework for improving the lives of producers located in developing countries. One popular variant of this approach is to assist producers, such as smallholder farmers, to develop tighter commercial relationships with downstream buyers, known as lead firms, which govern strategic nodes within the global value chain. The underlying rationale was strongly inspired by analyses of the so-called 'East Asian Miracle' and the industrial upgrading of supplier firms in Asia, where firms developed important skills, technologies and learning from their initial engagement as key input suppliers with lead firms in sectors such as electronics and garments. This process was described by Gary Gereffi: "Participation in global commodity chains is a necessary step for industrial upgrading because it puts firms and economies on potentially dynamic learning curves." (p. 39).

Similarly, engagement with international specialty coffee roasters, as lead firms, may provide the right conditions to allow smallholder producers to engage in product and process upgrading, by moving into high quality market chains and to produce coffee products more efficiently and therefore more profitably. The evidence for poverty-reduction benefits associated with value chain interventions in the coffee sector and elsewhere, however, remains limited.

METHODS

This research applied an action-research intervention whereby the research team provided assistance to the cooperative in 2010 to develop a direct relationship with the international specialty coffee market. This assistance was generally technical in nature, with researchers acting as brokers and generating mutual trust between commercial actors, although minor infrastructure support (drying beds) was also provided to the cooperative. Despite this minimal support, commercial and technical decisions about processing activities and the relationship were made entirely by the cooperative and the commercial partners, and the research team observed and monitored these decisions and their outcomes.

A relationship with an Australian-based roasting firm was initially developed through the introductions of an international green bean trader, but after the first two seasons, the roasting firm began to deal directly with the cooperative through an Indonesian-based exporter, claiming that the international trader was not sufficiently committed to the relationship. In 2012, with technical guidance and standard operating procedures provided by the international partners, the cooperative shifted from purchasing semi-dried parchment coffee from farmers to purchasing cherries and processing these centrally at a wet mill in an attempt to assert greater quality control. Coffee processed in this way became known locally as kopi spesial and a strict policy of only purchasing fresh, fully-ripe cherries was implemented with the cooperative collecting fresh cherries directly from local farmer groups on motorbike. The cooperative incurred increased processing costs for the kopi spesial program relative to trade in conventional parchment coffee. Eight farmer groups (with 184 listed farmers) were invited by the cooperative to participate in the kopi spesial program, which the cooperative implemented alongside a continued trade in conventional coffee (purchasing parchment coffee from farmers and selling green beans). Specialty coffee exports have been undertaken through a Makassar-based exporting firm, which is paid a service fee by the roaster for final sorting, bagging, port handling, and arranging export documentation. In both 2012 and 2013,

prices however, were negotiated directly between the roasting firm and the cooperative, and in 2013, ten tonnes of green coffee was shipped to the international roaster.

The relationship was monitored each year through multiple field visits to the Sulawesi cooperative, interviews with the roasting firm, and field reports from a field assistant working for the roasting firm. The cooperative shared its financial accounts, which showed all costs and income generated throughout the period. In July 2014, a household survey was undertaken across the eight farmer groups, all of which had at least some members who sold fresh cherries to the cooperative in 2013. There were 184 farmers listed within these eight groups, and we randomly selected a sample of 98 respondents for the survey (ie. 53% of all farmers in these groups). These surveys collected background farm and livelihoods data, along with farmer attitudes and actions regarding coffee marketing during the 2013 and 2014 coffee harvests, the latter of which was just commencing at the time of the field survey.

THE CASE-STUDY SITE: THE SULAWESI HIGHLANDS OF INDONESIA

The case-study villages for this research are located in the Enrekang District of South Sulawesi province, a district that produces approximately 1500 tonnes of smallholder-grown Arabica coffee annually. This coffee is commonly sold under the trade name 'Kalosi' at a premium price above international terminal markets. Coffee has conventionally been sold at the farm-gate to collectors as semi-dried parchment coffee, which is then wet-hulled at privately-owned mills, and sun-dried as green beans prior to export.

The case-study villages are located at an altitude of 1400-1500 metres above sea level approximately seven hours drive from the container port of Ujung Pandang (Makassar). Table 1 presents the general characteristics of the coffee farming community.

Table 1. General farm characteristics across the case-study site

Indicator	**Value**
Average farm size (coffee and other crops combined)	1.1ha
Average coffee income (2013, gross)	$590
Average non-coffee agricultural income (2013, gross)	$710
Coffee farmers with some off-farm income	17%
Average off-farm income from those with off-farm income (2013, gross)	$1600
Local farm labour costs (8 hour day with meals)	$5/day
Average age of farmer	44

Data source: Author Survey 2014

Coffee is generally grown under a diversified shade canopy and integrated with livestock, (75% of respondents maintained goats or cattle). In 2013, coffee provided an average annual income of $590 per household, which farmers estimated constituted (on average) 50 percent of their total income. 70% of coffee farmers also devote significant land and labour to horticulture, especially tomatoes, shallots and cabbage, as suggested by the high levels of gross non-coffee agricultural income shown in Table 1 (this is somewhat exaggerated, however, as production costs for horticulture are also considerably higher than for coffee). Many respondent households generated off-farm income, and it is clear that these households were generally better off financially as a result.

It needs to be emphasised that these are peasant smallholders cultivating tiny plots with a relatively small degree of internal social differentiation (ie. no respondents owned more than

five hectares of land, and there are unlikely to be any large landowners in the entire district owning more than ten hectares of coffee). No farmers were linked to formal credit markets, all adopted relatively low-input cultivation techniques, and almost all farm labour is supplied from within the farm household and through community-based labour-sharing schemes, and this is only occasionally supplemented with paid labour from outside the household unit.

A village-based farmers' cooperative was established in one of the villages a decade ago with local government support that included the provision of coffee processing machinery (depulper, huller, warehouse and a mechanical dryer) and an initial injection of funds as operational capital. Cooperative membership is currently restricted to active farmers, and the cooperative has a young, educated and capable leader, who was also the administrative village head for ten years, and who is passionate about selling local coffee into specialty markets.

RESULTS AND DISCUSSION

The centralised wet-mill was operational in the 2012 coffee season, and the cooperative sold five tonnes of *kopi spesial* to the Australian roaster that year, which increased to ten tonnes during the 2013 season. In both years, the coffee was sold at a premium price (Table 2) above the local market for conventional coffee (which, as already mentioned, was already higher than international markets). The roasting firm was generally pleased with the improvements in quality made by the cooperative through the engagement, although heavy rains and limited drying capacity during the 2013 harvest meant that the quality of some beans were negatively affected, highlighting both the quality risk absorbed by the cooperative and the risks to the roaster related to a long-term relationship. Local coffee prices increased significantly during the 2014 harvest and, at the time of writing, the cooperative leadership and farmers were satisfied selling conventional coffee at these high prices without the need to produce *kopi spesial*, and the Australian roasting firm was also not pressing them for supply. It is unclear, however, if this means the end of the relationship.

Table 2. Cooperative Sales of *Kopi Spesial*

Year	Tonnes	Cooperative Price (USD/kg)	ICO 'Other Milds': June-September average (USD/kg)
2012	5	5.5	3.9
2013	10	6.3	2.9

As indicated in Table 2, the *kopi spesial* relationship resulted in coffee sale prices for the cooperative that were well above international benchmarks. The cooperative reported making a profit in these years, although this was tempered somewhat by their relatively high processing costs of approximately 1.2USD/kg in 2013 (for bank interest, labour costs, transport and various operational expenses, in declining importance).

Apart from assessing the short-term business success of the cooperative, it is necessary to ascertain whether individual farm households received benefits from the relationship. Farm-level benefits could potentially occur through a number of pathways, including: 1) dividend payments based on membership of a profitable trading cooperative; 2) increased income due to elevated farm-gate prices for *kopi spesial*; 3) farm-level labour-savings by selling red cherries rather than parchment coffee; 4) improved access to skills, knowledge, technical supports and finance; and 5) employment opportunities through local value adding activities.

It is important to make the distinction between the first and second pathway above, because not all farmers in the case-study villages are formally members of the cooperative (which had a relatively limited and stable membership of only 41 individuals). As a result, *ceteris paribus*, it makes very little material difference to most households if their coffee is sold to a traditional collector or to the cooperative, and it is possible for the cooperative to be a business success, but with few benefits flowing to farm households. Moreover, all profits generated by the cooperative have, to date, been reinvested as operating capital for coffee trading the following year rather than shared with members as dividend payments. While it is possible that building up the cooperative over time will eventually lead to benefits amongst members, these benefits have not been realised so far, and expanding membership would pose new management challenges.

The cooperative paid prices for good quality red cherries to farmers that were, on average, 20% higher than local equivalent prices for parchment coffee, and this was confirmed by 82% of survey respondents. At least some of the higher selling price attained by the cooperative was, therefore, being passed on to farmers. However, only 10% of those farmers who reported selling cherries to the cooperative in 2013 identified the price premium as the main factor determining this choice of marketing option. Instead, 86% of these farmers identified the labour savings achieved by selling fresh cherries rather than parchment coffee as the most important reason they sold to the cooperative.

The cooperative has a weak financial capacity and to facilitate red cherry purchasing, it takes out a series of loans from a commercial bank (these loans are actually made in the name of individual cooperative members who possess land certificates as collateral, as the cooperative itself is ineligible for commercial loans). The interest on these loans is substantial and constituted 62% of total operating costs (excluding actual coffee-buying) during the 2012 harvest. During the 2013 harvest, the international roasting firm provided an upfront payment to the cooperative to facilitate red cherry purchasing, thus allowing considerable savings (in interest payments) for the cooperative. The improved access to finance for the cooperative facilitated by the relationship, however, did not significantly affect access to finance at the household-level as the payment was not used for micro-credit. Moreover, 77% of households claimed that the cooperative payments were delayed in 2013 compared to local collectors, and 27% stated that this was the primary reason they did not intend selling to the cooperative in 2014 (Table 3).

All households surveyed had the opportunity to sell red coffee cherries to the cooperative, but their marketing decisions (Table 3) suggest that many chose not to. Despite the acknowledged price premiums and the benefits arising from being relieved of the task of wet-processing, it is clear that most farmers still prefer selling parchment coffee to local collectors. Their reasons for this are informative. While it might have been expected that farmers would prioritise immediate payments and the convenience of local collectors, to whom they are accustomed with dealing, a significant number of households identify onerous quality requirements as the primary reason for not being involved in the *kopi spesial* program. This was especially true during the 2014 harvest, when high infestations of coffee berry borer combined with poor weather had badly affected the crop. It also challenges the assumption that quality upgrading and integration into specialty markets will necessarily be a desirable livelihood strategy for smallholders.

Table 3. Coffee marketing and marketing intentions.

2013 Harvest	**% of Respondents**
Parchment coffee sold to local collectors	53%
Parchment coffee sold to cooperative	4%
Fresh cherries sold to cooperative	30%
Some parchment sold to collectors and some cherries sold to cooperative	13%
Reasons for selling to cooperative	
- Less effort related to processing (labour savings)	86%
- Better price	10%
- Other reason	4%
2014 Harvest intentions	
Intend to sell to cooperative	26%
Not intending to sell to cooperative	74%
Reasons for not selling cherries to cooperative:	
- Payments are frequent delayed	27%
- Quality demands are too onerous	37%
- Prefer the services offered by collectors	26%
- The cooperative buying station is too far away	7%
- Other reasons	3%

Data source: Author survey 2014

The establishment of a successful village-based business enterprise contributed towards off-farm rural employment opportunities in the community. In 2013, the cooperative employed casual labour at the processing unit equivalent to a total of 560 labour days over the four months of the harvest, for which eleven individuals were recruited. In addition, the cooperative management (Head, Secretary and Treasurer) also received salaries. Of the 98 farmer household respondents, however, only one reported having worked at the processing unit. Most farm households were instead relieved at not having to be involved in processing activities. Despite a long history of state-support for the cooperative movement within Indonesia, there have been relatively few success stories in the agricultural sector. During Suharto's New Order regime, state patronage for cooperatives allowed many to function relatively effectively, but few were able to develop genuine economic viability and few have been sustainable. It is worth highlighting that there is nothing inevitable about the role of cooperatives in smallholder-based relationship coffees, and indeed small private-sector businesses may offer more sustainable alternative models.

The relationship with the roaster resulted in the successful transfer of skills to some individual members of the cooperative regarding the quality demands of the specialty coffee market, factors resulting in quality degradation, and some basic cupping skills. The buyer also provided limited support for processing infrastructure (drying beds, moisture meter, and sample roaster) and technical advice on establishing a wet-processing unit. The relationship can thus be considered to have facilitated a new conduit for knowledge exchange, particularly for cooperative management structure. During the 2014 harvest, various new buyers from Java began visiting and dealing directly with the cooperative, such that the cooperative's experience and knowledge regarding the specialty coffee market proved valuable.

The effective management of risk is the most challenging, and critical, aspect of developing successful relationship coffees with smallholders, and indeed many institutional arrangements within the conventional trade have evolved to minimise risk exposure. The shift towards higher quality coffee and centralised processing in Sulawesi involved a significant risk for the cooperative. If coffee quality deteriorated due to poor weather, drying difficulties or other logistical constraints, then the cooperative could be forced to sell *kopi spesial* into the local market at standard prices after incurring inflated processing costs. There are no hedging tools available to the cooperative. Similarly, the time required (up to 2 months) for the cooperative to purchase enough coffee to fill a container meant that (in addition to the risks of quality decline), the cooperative is exposed to price fluctuation unless a selling price is fixed at the outset. Evidence from elsewhere in the Indonesia coffee industry suggests that high risk exposure to price fluctuations is a primary cause of bankruptcy amongst village-based processing units.

It should be recognised that the roaster is also exposed to substantial risk in the relationship, particularly when pre-shipment finance is provided to the producer, as in the Sulawesi case-study. Formal contracts are rare in the Indonesian smallholder environment and would be extremely difficult to enforce, such that mutual trust is paramount. Even with a genuine commitment from the cooperative, quality deterioration can occur due to circumstances beyond the control of cooperative management, and this presents a dilemma for the buyer who is forced to either accept receipt of sub-standard quality coffee or to insist that the financial loss is incurred by the cooperative.

CONCLUSION

The results of this research suggest a complicated portrait of livelihood improvement associated with the development of relationship coffees in Indonesia. Some clear economic benefits have accrued to those individuals involved in key quality-control nodes in the local processing system, local off-farm employment has been generated, and the relationship has allowed a transfer of knowledge, skills, and price certainty along the value chain. The overall impact on farmer livelihoods, however, does not appear to have been as significant as is claimed by proponents of the model.

Compared to producers in Central and South America, very few successful, long-term relationship coffees have been established in Indonesia. While this is partly due to the inherent challenges of managing relationships with relatively unorganised peasant smallholders, it also reflects the unwillingness of roasters to genuinely engage with the long-term support required to develop institutional mechanisms that generate appropriate incentives and address issues of risk exposure. This then raises questions about the long-term viability of relationship coffees as a sustainable business model within the specialty coffee sector (at least as currently applied in Indonesia). Despite an apparent trend in the specialty sector where international roasters are exploring tighter upstream linkages, this may not be sustainable in the long-term due to the high costs and investments required to make such relationships work. Alternatively, it may be that greater locally-adaptive experimentation is required to develop institutional innovations appropriate to the localised smallholder context. Either way, it does not appear that relationship coffees are likely to solve the so-called ‘coffee paradox’, whereby a specialty coffee boom in consuming countries occurs concurrently with a coffee crisis in producing countries.

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Adaptation to Climate Change: a Systematic, Science-based, Comprehensive and Practical Response for Coffee Farmers

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SUMMARY

Climate-related events are now starting to have substantial effects on regional coffee production, including three recent major episodes: the Colombian wet period (2008-2010), the great Latin American rust outbreak (2012-2013) and the Minas Gerais drought (2014).

However there has been no coordinated response by the coffee industry to tackle this problem. The present paper describes *the initiative for coffee & climate* (c&c; www.coffeeandclimate.org) a scientific, systematic, comprehensive and practical way to supply this need.

This paper summarizes principal features of the c&c approach; c&c is a public private partnership consisting of 12 roasters, traders and public sector organisations.

INTRODUCTION

A recent systematic review of adaptation actions showed generally little evidence of all forms of adaptation occurring in developing countries. The study concluded that considerable research on adaptation has been conducted yet the majority of studies report on vulnerability assessments and natural systems, or intentions to act. Where adaptation actions had occurred, they were more frequently reported in developed nations, with middle income countries underrepresented and low-income regions dominated by reports from a small number of countries. A subsequent review of adaptation policy noted an 'implementation deficit' caused by weak funding and institutions.

The coffee sector is no exception to this general situation. For this reason a group of public and private entities agreed to set up a practical approach to fill the implementation deficit. Founder members of the group include BMZ, GIZ, Hanns R. Neumann Stiftung (HRNS), Jo Johansson, Lavazza, Löfbergs, Paulig, Tchibo. Subsequent members now include ECOM, Frank d.d. (Croatia), Sustainable Coffee Program, SIDA and Tim Hortons. The initiative is currently being implemented by HRNS and CABI.

MATERIALS AND METHODS

The main principles upon which subsequent fieldwork has been based can be stated thus:

- A large amount of information and knowledge about climate change adaptation (CCA) already exists but is not in a form readily accessible or understandable by end users;
- Both coffee growing and climate change impacts vary from zone to zone, making generic advice problematic and necessitating a site-specific approach.

- A science-based and systematic approach to analysing and testing CCA tools can materially assist farmers in their efforts to adapt.
- An assembled body of knowledge (including economic evaluations) is an essential first step to formulate a convincing theory of change with which to influence the global coffee industry.
- The initiative was undertaken in four localities Sul de Minas (Brazil), Central Highlands (Vietnam), Mbeya (Tanzania), Trifinio (Central America).

RESULTS AND DISCUSSION

A wide range of tools (*sensu lato*, any physical method, analytical or thought tool that could be of benefit to farmers, extensionists, decision-makers) have been assembled. To date more than 30 have been identified. These can be categorized as:

- Analysing tools (e.g. simulation, risk and cost benefit analysis)
- Adaptation tools for farms (e.g. soil stabilization and protection
- Adaptation tools at the community, landscape, watershed level (a focus for phase 2)
- Mitigation tools (not prioritized because of insufficient income for farmers)
- Enabling tools (credits, insurance)
- Climate change database (Distribution & zoning maps, Risk maps, country reports
- Scientific studies)

To date it has not been possible to action and test all possible tools. Prioritization for action has depended on local needs with the initiative discovering that there was a range of pressing issues.

The framework: from an early stage in the work, it became clear that a framework tool is essential to guide the development of a fieldwork programme. The framework developed was based on that developed by the United Kingdom Climate Impacts Programme. Important features of the framework (Figure 1) are that it is:

- cyclical and iterative: adaptation is not a one-off activity, but should become part of standard practice for coffee institutes and support agencies, including cooperatives.
- pragmatic and flexible: if progress stalls, it encourages going back to a previous stage for a re-think
- adaptable: quantitative data is preferred but it also allows for and supplies tools for qualitative and semi-quantitative approaches.

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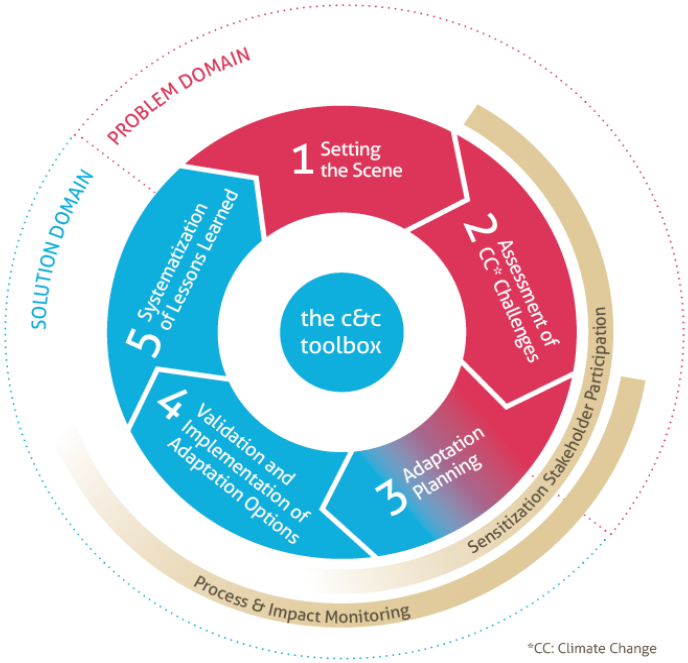

Figure 1. The c&c framework tool.

There are five basic elements of the framework:

Setting the Scene

- *Purpose*: identification of problem and objectives.
- *Principles*: mostly locations already known to be experiencing CC related problems should be selected, though increasingly most coffee zones are now reporting difficulties. Appropriate local partners are sought and a local oversight committee set up.

Assessment of CC challenges

- Purpose: risk assessment of recent and current climate-related difficulties faced by farmers in a specific zone.
- Principles: it is essential to identify real climate-related problems affecting coffee production; these are the ones to start working on with farmers. Climate change affects different localities in different ways; the most pressing aspects should be prioritised.

The identification process should be timely in order to set up adaptation trials and get results within the life-time of a project cycle. A triangulation method is used: farmers are asked to talk about their production problems which are then ranked. Climate change is not raised with them at this stage. Extensionists and other local experts are then interviewed for their views. Then the available scientific data is accessed as the third corner of the triangle to corroborate field interviews. In practice farmers and extensionists agree quite closely on the main production difficulties and climate impacts. The scientific data is often not in a format easy to assimilate by stakeholders and specific consultancy work is required.

Adaptation planning

- *Purpose*: to identify a) candidate adaptation options for testing, b) farmers, local extensionists and other stakeholders with whom to collaborate, c) agree on a programme of work.
- Principles: there are a number of adaptation options that can be tested and modified with farmers on demo and field school type plots.

Validation

- *Purpose*: the above activities should provide a short list of priorities for action and the likely tools to use. These must be tested however, because costs, effectiveness and acceptability will depend on local conditions
- Principles: farmers carry out most of the fieldwork advised by technicians. Equipment and consumables are provided by c&c. Where possible a wide range of tools and variants should be tried, especially including tools already being used by farmers.

Synthesis

- *Purpose*: collecting results and experiences to formulate decisions on a range of issues: which adaptations work, which should be discontinued, which can be up-scaled, which need further study.
- Principles: M&E should be carried out through out stages 2 to 4 so that synthesis should not reveal unhappy surprises. Assessment and learning should be continuously carried

Principal features are

- *A cyclical and step-by-step approach*: consists of five steps, which build upon each other and allow for a systematic implementation process.
- *Location-specific*: there is no "one fits all" solution for climate change.
- *Participatory*: c&c engages stakeholders and farming households in the identification of local problems and potential solutions.
- *Practical*: practical, hands-on and applicable tools and training material.
- *Complementary*: the c&c approach is suitable as an add-on to training or capacity building programs.
- *Science based and farm oriented*: the c&c approach combines climate change science with proven farming methods and local expertise to reach the best possible solution.
- *Learning network*: serves to establish a network (local, regional and potentially global) that actively exchanges information, lessons learned and experience on climate change.
- *Difficulties*: working directly with farmers means that to a great extent they set the agenda. They will not implement things that they perceive as being costly or of little immediate use. This has meant that field activities have focused more on 'coping' i.e. short term adaptation, rather than long-term transformational change. Some farms probably only have a limited future in coffee, but it is currently difficult to advise on alternatives and a time-frame for change. Wildly fluctuating coffee prices can mean that a farmer could decide to exit from coffee just as a global shortage manifests itself.

A principal focus of future activities will look at how to offer decision-support services in the face of mounting uncertainty.

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Use of Polychoric Indexes to Measure the Impact of Seven Sustainability Programs on Coffee Growers' Livelihood in Colombia

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SUMMARY

By promoting standards of better social, environmental and economic practices at the farm, sustainability initiatives expect to get an improvement in farmer's livelihood over time. Until now a lack of tools and methodologies to measure this impact has made it difficult to demonstrate the impact of these programs and available studies are commonly focused on analyzing separated simple indicators. The multidimensional nature of sustainability is not still represented in impact assessment.

This study is the first to analyze the effects of coffee sustainability programs -Voluntary Sustainability Standards VSS- on coffee farmers' livelihood in Colombia by incorporating the multidimensional scope of the initiatives. The work is based on COSA approach, adapted by CRECE to Colombia, which consists of the development and application of an internationally-recognized methodology and data gathering process to measure the outcomes of the adoption of sustainability initiatives. A set of sustainable practices in the social, environmental and economic dimensions is used to test for aggregate effects of the programs on farmers' livelihood. To consider differential effects, contrasts by farm size and multi-certification status are also considered.

The data come from a longitudinal and panel data set on a probabilistic sample of 3.372 coffee farmers participating in seven VSS. Four certifications (Fairtrade, Organic, Rainforest Alliance and UTZ Certified) and three codes of conduct (Nespresso AAA, 4C and Starbuck's C.A.F.E. Practices) as well as a group of conventional farmers operating as a control were followed-up during three years. Principal Component Analysis (PCA) for ordered categories -polychoric correlations-, was implemented to summarize the performance of the farms in sustainability dimensions. The method guarantees comparability since the rating obtained by one specific condition will be the same for all the certifications, all the locations and all the years.

The performance of aggregated indexes is positive during the years of observation for the seven sustainability initiatives analyzed. There is an average improvement in the social, environmental and economic conditions of farmers participating in sustainable schemes. On average, farmers have better socioeconomic and environmental conditions compared with their conventional counterparts. However, the difference of scores between sustainability initiatives and control groups tend to decrease over time and the aggregate indexes decrease for smaller farms.

METHODS

This study examines the use of polychoric principal component analysis for the measure of farms' sustainability. This method allows to synthetize multiple variables in one aggregate index that ranges from 0 to 100. Through the polychoric analysis, three indexes are calculated: the Economic, Social and Environmental Indexes. At the same time, the sustainability index is calculated as the average of a linear function of the three indexes, as it is shown in the Function 1.

$$SU = \frac{1}{3}E + \frac{1}{3}S + \frac{1}{3}A \quad (1)$$

Function 1 results in an index that summarizes the three sustainability sub-indexes by assigning them the same weight, considering that each one of these categories has the same relevance inside the sustainability concept.

Each one of the sub-indexes are calculated by using scores that result from performing polychoric principal component analysis over a series of variables that are theoretically considered to be related with economic, social and environmental conditions of the farms.

The polychoric procedure uses discrete variables and calculates what would be their correlation as if they were on a continuous scale (Uebersax, 2006). In the case in which there are two variables (X_1 and X_2) that represent the binary discretized form (this discretization is defined by thresholds) of two continuous variables (Y_1 and Y_2), the polychoric correlation of both variables would suppose that there exists a common continuous latent trait (T) defined by the interaction of both variables. This relation is explained by Uebersax (2006) as follows:

$$Y_1 = \beta_1 T + u_1 + e_1 \quad (2)$$
$$Y_2 = \beta_2 T + u_2 + e_2 \quad (3)$$

In which *u* represents unique components of each variable and *e* represents the errors. As β_1 and β_2 are the correlations between the variables and the latent trait, they can be considered as one (β). The polychoric correlation between both variables would be:

$$r^* = \beta^2 \quad (4)$$

The polychoric correlations can be used for the calculus of a set of vectors (equal to the number of variables), in which each vector is a lineal combination of the variables[1]. The first vector is the one that represents the higher variance, so it is the one that is used for calculating the indexes (Kolenikov & Angeles, 2004).

The variables used for the indexes are dichotomous, categorical and discrete. Some of them are transformations of continuous numerical variables into categorical variables.

The Economic sub-index includes yield of the farm, net income that comes from the coffee production (the variables were categorized in an ascending order), the level of knowledge that the producer has on the market and the access to it, technical use of inputs, perception of the economic situation of the farm, affection by diseases and pests and the percentage of coffee sold as low quality.

[1] This formulation explains the tetrachoric correlations. For a further explanation that includes the underlying distribution assumptions see Ekström (2008).

The Social sub-index includes indicators of farm's resilience, working conditions inside the farm, producer's perception of their household quality of life and their relationships with their employees.

The Environmental sub-index includes information about the recycling programs in the farm, conservation practices, training in environmental topics and subjective perception of the environment conditions of the farm and the village.

Previous to the polychoric analysis, the association of the variables inside each index was tested by the non-parametric test Kendall's τ_b. This test showed that almost all the variables were positively and significantly correlated which suggests that they can be represented by a single latent variable.

For the Polychoric Principal Component Analysis, the Stata module polychoric.ado was used. By using the polychoric correlations, the variables of each index can be transformed into a new linear combination of components in which each component represents a proportion of the total variance. The first component is the one that captures the highest variance so the weights of each variable given by this component can be used as a proxy for the common information contained by the variables that correspond to each one of the sub-indices (OECD, 2012). A score is estimated by every single farm according to the weights that resulted from this analysis. The scores were standardized so they could be represented in a scale from 0 to 100.

The sample was divided into certified and non-certified farms. Then Target and Control farms were selected by using Propensity Score Matching and Difference in Differences method was added in order to control for factors other than the program over time. Despite the methods used, it has to be said that part of the difference could be due to unobservable factors coming from the selection criteria used by the organizations. The group of certified farms is composed by Organic, UTZ Certified, FLO and Rainforest Alliance; the group of verified farms is made up of farms Nespresso AAA, 4C and C.A.F.E. Practices in 2008, 2009 and 2011[2].

RESULTS

The upward trend for the calculated indexes between the first and the fourth year (Figure 1) indicates an improvement over time in the social, environmental and economic conditions of the producers in both groups -except for the economic in conventional producers-. The scores show that producers[3] participating in a sustainability initiative tend to have better overall conditions in the three dimensions of sustainability. The difference between target and control groups in the first year –around ten points in every dimension- reveals certain selection bias originated in the way the organizations chose the participating farmers. In fact, in most cases it was known that farmers that had participated in other programs were preferably engaged.

[2] For Nespresso AAA the first and the fourth years correspond to 2009 and 2012, while for Organic they correspond to 2009 and 2011.

[3] The mean showed values that were very similar to the median.

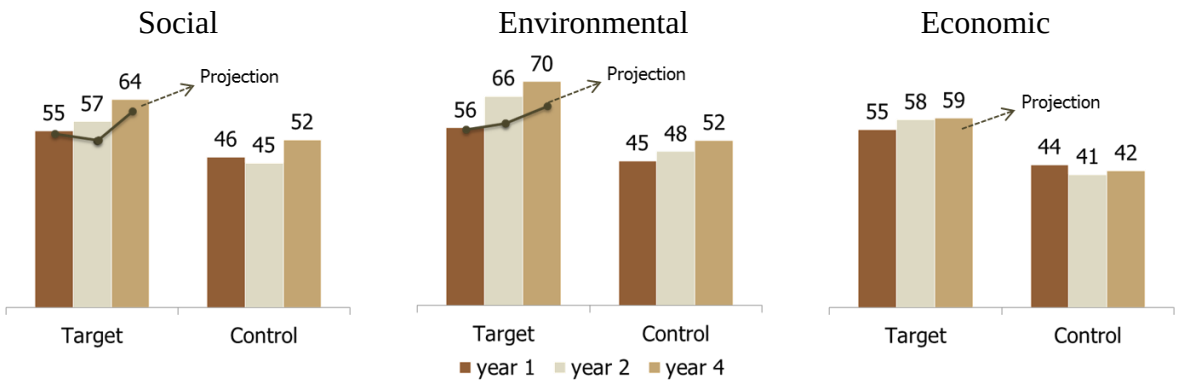

Figure 1. Sustainability indexes by year. Target and Control.

In order to control this bias as much as possible to quantify the impact of the VSS programs, PSM + DID methods were implemented, making clear that some bias could even persist after due to the potential influence of unobservable factors. In line with the methods, the projection line in every graph depicts the trend that the target group should have had if the baseline difference target to control were imposed during the period of evaluation. In other words, it is a counterfactual situation that shows what would be the average scores of the producers if they had not been part of a sustainability program. Therefore, the impact of the initiatives over the corresponding index is the difference between the score of the target group and the projection line.

As a result, the impact of VSS is positive for the three dimensions. The highest score corresponds to the environmental dimension, which shows that 8.1 points in average of the index (70-62) can be attributed to the effect of the VSS as a group[4], compared to the situation in absence of the program. The impact for the social and the economic indexes were also positive and equal to 4.3 and 5.4 respectively.

In the social index, the variable worker's living conditions drove much of the positive effect of the VSS. A share of around 30% of the producers were found in the highest category of the variable in the first year, while in the last year this proportion was near 60%. However, three variables remained almost constant during the period: Farm crop production for family consumption, Revenues from sales of other farm crops and Possession of household assets.

All the environmental variables improved over time for the group of VSS, being the most representative Soil conservation practices: in the first year around 35% of the producers under environmental-focused initiatives used more than five soil conservation practices, while this achieved around 65% in the last year.

The component variables of the economic index that explain its increase for the group of economic-focused initiatives are mainly training in marketing topics and perception of better business opportunities. However, variables such as yield and percentage of the coffee sold as low quality decreased or keep stable. This impeded the initiatives to have a larger impact on the economic index. On the first year around 55% of the producers had a yield higher than 80 @/ha[5], while in the last year, this proportion was around 50%. This result is highly influenced by the conditions of the context in the country, mainly characterized by weather variability,

[4] This result corresponds to the seven programs grouped, so the significance is not presented. The programs differ in the amount of the scores and not all of them have achieved statistically significant impacts.
[5] The arroba @ is equivalent to 12.5 kg.

presence of plagues and diseases such as coffee rust and Coffee Berry Borer, as well as high coffee trees renovation rates that kept some of the crops temporarily without production.

To face the question if VSS have differential effects on small producers, we opened the indexes in three farm sizes: less than one hectare, one to five and greater than five hectares. When compared by coffee area sizes it can be seen that, as expected, the higher the size of the farm the higher the score of the index in the dimensions of sustainability (Figure 2). At the baseline, the status of sustainability differs among the farm sizes: the difference between the biggest and the smallest farms scored 20 points for the social index, 9 for the environmental and 7 for the economic.

Looking at the differences achieved over time, it is observed that the impacts of the VSS on sustainability tend to be concentrated on the small and medium size farms and in the environmental conditions. However, the average differences in the impact are quite low among the groups, scoring no more than two points.

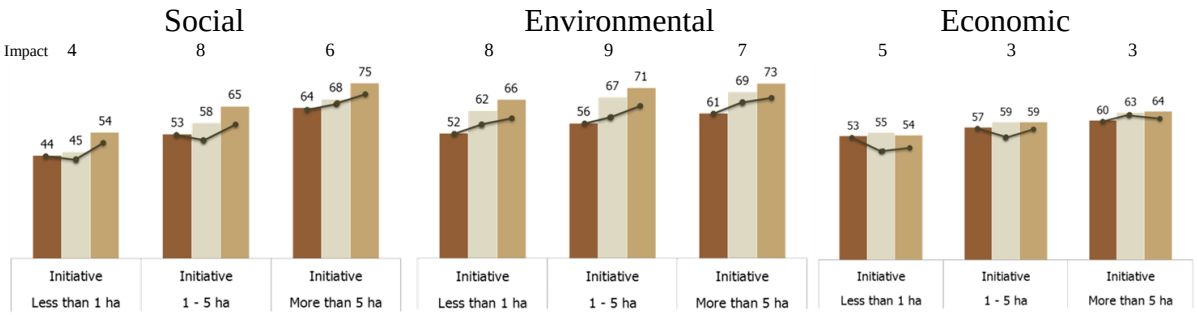

Figure 2. Sustainability indexes by farm size.

To evaluate the effect of participating in one VSS against two or more at the same time, the sample was divided into these two groups. It was expected to find a higher effect when the farmer is multi-certified, mainly under the assumption that this condition allows them access to better market opportunities and by this way getting a better price. In that sense, the economic index would perform better for multi-certified farmers. As can be seen from the next figure, the sustainability index shows better results when the farmers participate in more programs.

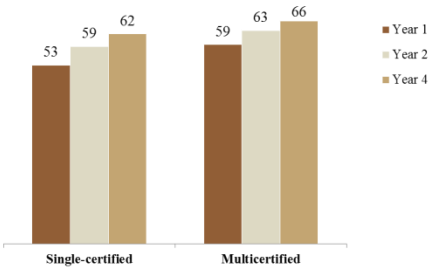

Figure 3. Economic index by Single-certified and Multi-certified

However, the initial difference of six points for the economic index between multi-certified and single-certified farmers has been slightly reduced over time to just four points, both in the second and in the fourth year. This suggests that the effect of multi-certification could be positive but decreasing.

DISCUSSION

The Polychoric PCA method allows score the aggregated impact of sustainability programs through synthetic indexes of the social, environmental and economic dimensions. The indexes show to be a proper tool for measuring sustainability since they can integrate a large number of variables for representing a multidimensional concept. They also consent comparing groups of producers over time under different conditions which also allows performing impact assessment of sustainability initiatives.

The results show that the producers who participate in sustainability initiatives have higher indexes than conventional coffee producers. All the indexes also showed to be related to the coffee area of the farms. It was also found that for every coffee area range, the producers under sustainable initiatives presented higher indexes. Producers with a larger number of initiatives tend to obtain higher scores, which suggest that the fact of having multiple certifications can contribute to sustainability conditions.

Even if the indexes showed to represent the sustainability conditions of the farms, there are some aspects that can be taken into account for improving their performance. Namely, variables that were included such as the different perceptions of the producers on their conditions could be reconsidered in order to obtain a more objective measure and improve the formulation.

Some influencing factors make complex the interpretation of the results: the starting point of the farmers and the regional conditions, farm size, multi-certification and number of years in the certification program. More investigation is needed to gain better understanding of the complexity of the impact of sustainable initiatives in coffee.

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Towards a Better Understanding of the Coffea Arabica Genome Structure

The Arabica Coffee Genome Consortium

An international consortium was initiated in 2012 with the goal to perform the sequencing of the *Coffea arabica* genome. This consortium includes 34 researchers, engineers and technicians coming from 13 institutions in six different countries.

In December 2013 the first draft genome of a *Coffea* species was published in Science (Denoeud et al. 2014), it is the genome of the *C. canephora* species, a diploid and the second mainly cultivated species after *C. arabica*. This later species is the only tetraploid of the genus resulting from a recent spontaneous hybridization (± 0,5 Mya) between *C. eugenioides*, a wild species from East Africa, and *C. canephora*, whose genome was sequenced (Lashermes et al. 1999).

Et39 di-haploid (n=22)

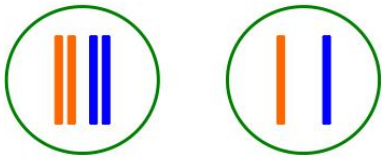

Figure 1. Et 39 dihaploid.

The genome of *C. arabica*, an allotetraploid (2n=4x=44), is about 1.3 gigabase in size (Cros et al. 1995). Its two parental genomes are closely related and their genomes have a high degree of identity. This fact makes a separated assembly of the two sub-genomes quite difficult. In order to overcome in part this difficulty, we chose to sequence a dihaploid plant produced by IRD issued from the Et39 accession.

A dihaploid has only one set of chromosomes from each subgenome (Fig. 1) in this case the sequenced *C. arabica* plant has only 22 chromosomes. This situation allows not to be

embarrassed by the polymorphism that may exist within each subgenome. Choosing this genotype should facilitate the assembly of such a tetraploid genome and differentiate more easily the two subgenomes.

SEQUENCING STRATEGIES

Two main whole genome sequencing (WGS) strategies were used. Both adopted a shut gun approach; using the short reads provided by the Illumina technology for the first one, or taking advantage of the long reads provided by the Pacific Bioscience SMRT sequencing platform for the second one. A very small amount of sequencing was also performed with the Roche 454 chemistry. The final coverage obtained with short reads reached about 164x (Table 1).

Table 1. Sequencing results for short reads.

Technology	Insert Size	Length (bp)	Coverage
Illumina paired-end	400 bp	100 x 2	80 x
Illumina mate pair	3 kb	100 x 2	45 x
Illumina mate pair	8 kb	100 x 2	20 x
Illumina MiSeq	500 bp	~450	17 x
454	500 bp	~393	2 x
Total coverage	-	-	164 x

The long reads from Pacific Bioscience, with two different chemistries (P4C2 and P5C3), gave a total coverage of about 56x , with the longest reads reaching 40,445 bp.

GENOME ASSEMBLY

An assembly was performed on the Pacific Bioscience long reads using the Falcon assembler. The final assembly, made exclusively by contigs, covers almost 80% of the estimated *C. arabica* genome size, i.e. 1.042 Gb out of 1.3 Gb. An independent assembly was also conducted with the Illumina short reads. It appears that, at the contig level, the total proportion of the assembled genome is roughly identical to that obtained with the long reads, but, as expected, the general results are of lower quality (Table 2).

Table 2. Assembly results for short and long reads, independently.

	Short Read	PacBio
Total assembly size (bp)	1,031,405,416	1,042,371,195
# of sequences	119,185	9,840
Longest Sequence	55,056	4,837,614
N50 (bp)	8,810	267,399

GENETIC MAPPING:

A segregation population of 138 F2 plants issued from a cross between two wild Ethiopian genotypes collected by IRD (de Kochko et al. 2010) was used to build a genetic map, which contains 613 SSR markers and covers 4946 cM. Some gaps still remain in the map, these are gradually removed by transposing SSR markers detected in the *C. canephora* genome (Figure 2). This genetic map is under completion using SNP markers and will be used to anchor the genome sequence.

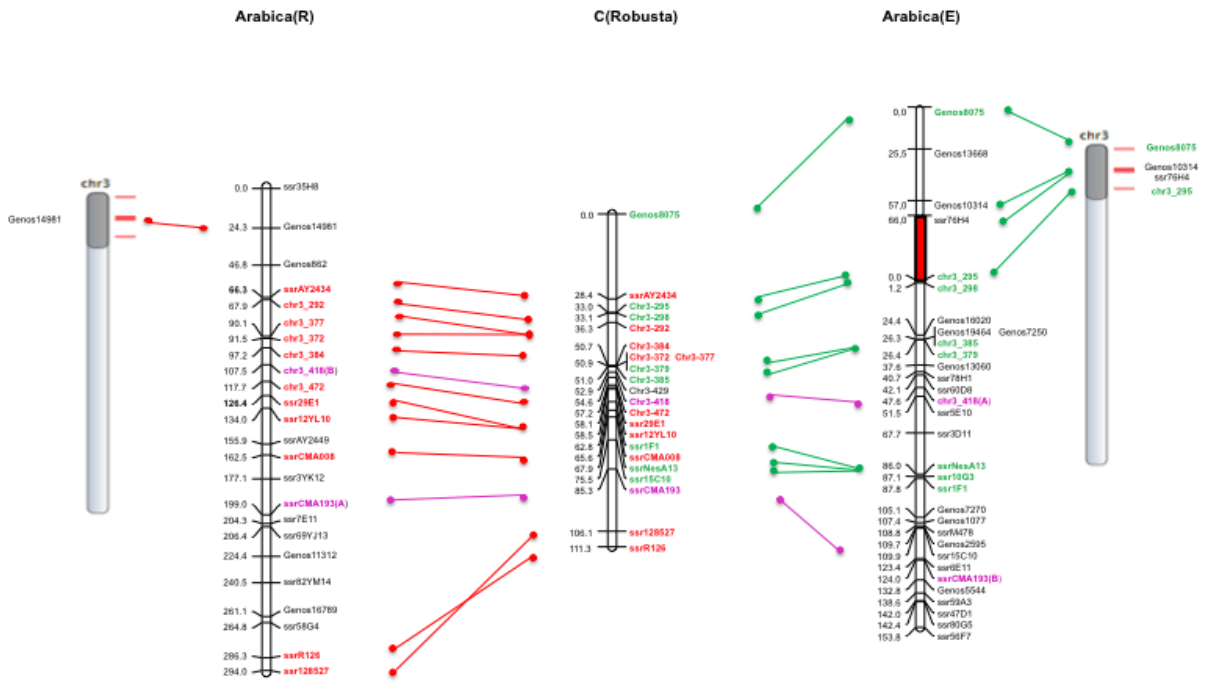

Figure 2. Linkage group C (3) from the *C. arabica* genetic map showing both sub-genomes and compared with the same LG from the *C. canephora* map. Some markers were also positioned on the *C. canephora* physical map.

ORIGIN OF *C. ARABICA*

Previous study based on SSR and GBS analyses of *C. canephora*, *C. eugenioides* and *C. arabica* germplasm indicated that among the genetic diversity groups of *C. canephora*, the most probable *C. canephora* ancestor was originating from the Ugandan genetic group (Poncet et al. personal communication). In the frame of our project, a *C. canephora* genotype from this Ugandan group is also sequenced together with a selected *C. eugenioides* plant.

Comparison between the genomic data from these three *Coffea* species, should give new and precious insights on the exact origin of the tetraploidization leading to the emergence of *C. arabica*.

C. ARABICA RESEQUENCING AND DIVERSITY STUDY

Thirty *C. arabica* accessions, wild and cultivated, selected based upon their genetic diversity and representative of the entire species, were chosen to be re-sequenced using the Illumina short reads technology. This study should reveal eventual neo-diversification emerging in the cultivated varieties vs. their wild relatives.

The goal of this sequencing project is to produce a high quality genome and develop tools that will make the finished genome accessible and useful to breeders and researchers. Results of these efforts will be published and publicly available on a specific website and in the MoccaDB database (http://moccadb.mpl.ird.fr).

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QTL Management for Biochemical, Sensory and Yield Characters in *Coffea canephora*: Comparison of Three Models

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SUMMARY

Coffea canephora is competiting with other crops such as rubber tree and oil palm, especially for farmer sustainability and also for consumer requirements. Coffee breeding programs have to focus on specific traits linked to these two key targets, such as quality character, largely depending on the bean's biochemical composition and field yield.

Two segregating populations A and B, from crosses between a hybrid (Congolese x Guinean) FRT58 parental clone and a Congolese FRT51 genotype, and between two Congolese parents FRT67 and FRT51, respectively, were used to characterize the quantitative trait loci (QTLs) involved in agronomic and biochemical traits.

A consensus genetic map was established using 249 SSRs covering 1201 cM. Three QTL detection models per population with MapQTL (Model I) and MCQTL (Model II) followed by a connected population approach with MCQTL (Model III) were compared based on their efficiency, precision for QTL detection and their genetic effect assessment (additive, dominance and parental-favorable allele). The analysis detected a total of 143 QTLs, 60 of which were shared between the three models, 28 found with two models and 2, 13 and 40 specific from Models I, II and III, respectively. The last Model III based on connected populations is much more efficient in detecting QTLs with low variance explained and led to the genetic characterization of favorable allele.

The present quantitative genetic study will be used to accelerate and guide breeding programs dedicated to managing complex agronomic or qualitative traits.

INTRODUCTION

Robusta represents approximately 38% of world coffee production and is used for most soluble coffee, which is increasingly consumed throughout the world.

Coffea canephora is strictly an outcrossing diploid species and presents a wide natural distribution area which extends west to east from Guinea to Uganda, and north to south from Guinea to Angola. This wide genetic diversity of Robusta including seven genetic and geographic *C. canephora* groups is an important resource for breeding programs.

Currently, coffee growing is in strong competition with other raw materials such as palm oil or rubber which offer better profits to farmers. One of the main issues for breeding is to develop new varieties with higher yields to sustain coffee farming. Coffee yield is measured by weight of fresh cherries and can be predicted by other yield-related traits such as the

number of productive branches, the number of nodes per branch and the number of cherries per node. Coffee quality is also a major selection criterion for coffee improvement. Robusta is known as a lower quality coffee than Arabica due to its higher caffeine content and bitterness. By focusing on the biochemical compound content related to cup quality (caffeine, chlorogenic acids, lipids, proteins and sucrose), Robusta quality can be enhanced. Considering the complexity of traits such as yield potential and coffee quality, using molecular markers associated with quantitative trait loci (QTL) is expected to improve the efficiency of Robusta breeding.

Recently, an approach by crossing designs composed of bi-parental populations that are connected by common parents was developed to increase genetic diversity under QTL studies. Studies on maize and on ryegrass reported a higher number of QTLs detected with this connected model than in single-population analyses. The multi-parent approach also made QTL positioning more precise for flowering time QTLs in three connected populations of *Medicago truncatula*. However, it has been reported in different studies that some QTLs detected in single-population analyses were not found in the multi-population analyses. The small population size or the dilution of low genetic effect on the whole design explained the loss of QTLs in the connected model.

Some QTL studies on coffee have already been conducted but only two studies reported QTLs related to yield and quality traits for coffee. These last two studies involved bi-parental populations and used single marker analysis (ANOVA) or MapQTL as the QTL detection model. Therefore, the use of multi-population connected analysis would seem to be a promising tool for *C. canephora* breeding, combining the advantage of wide diversity and powerful QTL detection.

The purposes of this study were (1) to compare three QTL detection methods: the model per population of MapQTL and MCQTL, and the multi-population connected analysis of MCQTL, (2) to identify QTLs for yield and quality traits for genetic improvement of Robusta and (3) to learn about our study for new insights into Robusta breeding using marker-assisted selection.

MATERIALS AND METHODS

Plant material

Two progenies were created by crossing three parental clones of *Coffea canephora*. The two parents FRT67 and FRT51 belong to the Congolese genetic group while the parent FRT58 is a hybrid between Congolese and Guinean genetic groups.

The two progenies derived from the crosses were planted in 2005 in an experimental coffee station located in Ecuador. The number of trees is 191 for Progeny A and 178 for Progeny B.

Observation of phenotypic traits

The coffee trees were individually observed for seven years (2006 to 2012) for different characteristics including:

- Eight field traits such as the number of primary branches (Br), internode length (In), stem diameter (St), number of cherries per node (Cn), number of nodes on primaries (No), yield (Yi), weight of 100 beans (Bw) and percentage of peaberries (Pe).

- Six biochemicals and one sensory traits using Near Infrared (NIR) assessment for caffeine bean content (Ca), trigonelline (Tr), lipid (Li), protein (Pr), sucrose (Su), chlorogenic acid (Ch) and bitterness (Bi).

Near-infrared (NIR) analysis

The biochemical composition and the bitterness sensory trait were predicted by near-infrared spectroscopy using calibration models previously developed for Robusta green. Spectral data in reflectance mode for each coffee sample was recorded at ambient temperature using the Thermo Electrons FT-NIR Antaris II spectrometer.

SSR genotyping

Total DNA of the two population progenies (A and B) were purified from adult leaves using Dneasy®96 plant kit, QIAGEN. A total of 249 SSRs were used to build the two genetic maps and the consensus map. The SSR genotyping was performed according to [13].

Genetic map construction

The two Genetic Linkage Maps A (FRT58 x FRT51) and B (FRT67 x FRT51) were built using JoinMap® 4. The parameters used for the analysis are described in Mérot-L'Anthoëne et al. (2014). Finally, the linkage maps of the two crosses were integrated into a unique consensus linkage map of the multi-cross design using the "Combine maps" function in JoinMap software.

QTL Analysis

An initial QTL analysis of each F_1 segregating population was performed with MapQTL V.6.0. The interval mapping method was used to test for the presence of a putative QTL. The analysis parameters are described in Mérot-L'Anthoëne et al. (2014). Allelic effects were estimated as described in Ben Sadok et al. (2013).

A second method for detecting QTL for each F_1 population was performed using MCQTL v.5.2.4 with the linear marker regression model. Either the additive allelic effect alone (additive model) or the additive and dominance allelic effects (dominance model) were used as the QTL model. The analysis parameters are described in Mérot-L'Anthoëne et al. (2014).

Finally, a QTL connected analysis for both two F_1 populations together was performed with MCQTL v.5.2.4. The QTL model was a connected model assuming that the QTL allelic effects were identical within the two F_1 populations for the common parent FRT51. All the other features of this QTL connected analysis were identical to the per F_1 population MCQTL analysis.

Shared QTL between the three detection models were defined trait by trait as QTL which have confidence regions that cover each other and similar genetic effects. We mapped the QTLs detected using models per population on the consensus map before comparing the QTL confidence regions using Biomercator v.4.2 software. A heatmap of the QTLs detected with the three models was created using R.

RESULTS AND DISCUSSION

Comparison of the three QTL detection models

A total of 143 QTLs (Fig. 1) were detected in our study from which 60 were common to the three models. Moreover, 28 QTLs were shared between two models and 55 were specific to a given model with 40 found exclusively in the connected analysis (Model III).

The analyses per population using MapQTL and MCQTL detected nearly the same set of core QTLs. However, the power of MCQTL was found superior, with an increase of 21% for the number of QTLs detected. The two software applications do not have the same multiple QTL model. They both begin with a forward search of cofactors, then MapQTL conducts a MQM mapping, while MCQTL uses an iQTLm algorithm. This could explain part of the difference between the results since the iQTLm algorithm analyses more multiple QTL models than MQM. Moreover, they do not use the same modeling of the putative QTL. MapQTL uses a mixture model while MCQTL uses a regression model. The mixture model is slightly more powerful with major QTL with sparse mapping and small sample sizes, but the two models were proved to be asymptotically equivalent in other cases. A large part of the power superiority of MCQTL is probably due to the use of an additive QTL effect model, while MapQTL only proposes an additive and dominance. In our analyses, 48% of the supplementary QTL detected by MCQTL and not with MaqQTL were detected with the additive model alone.

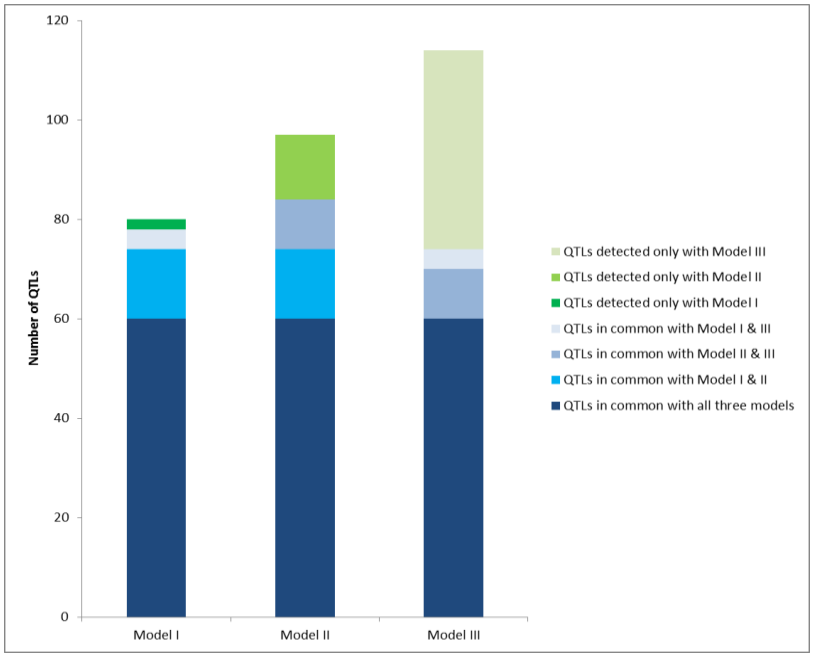

Figure 1. Comparison of the three QTL detection models. Number of QTLs for all the traits detected specifically in single analyses with MapQTL (Model I), single analyses with MCQTL (Model II), multipopulation connected analyses (Model III) or in common with different models.

The gain in power of the multi-population QTL mapping was clear in our study with an increase of 43% and of 18% for the number of QTLs detected in comparison to the per

population analyses with MapQTL and MCQTL respectively. However, the risk of dilution of small QTL effects that was highlighted by simulations and observed in an oil palm factorial design with five parents was also present in our design despite the two large connected populations which should have limited this risk. Only 11.3% of the QTLs detected by the model MCQTL per population were shared between the two progenies. This observation could threaten to produce a strong dilution effect in the connected model. However, 72% of the QTLs detected by this model per population were found by the connected model. And, as expected, QTLs shared between the per population and the multi-population analyses had higher average percentages of explained variance (Fig. 2) and smaller confidence regions than those detected by the per population analyses alone.

Other advantages of QTL mapping with connected populations have regularly been discussed. They concern the possibility of ranking and studying more than two parental alleles and the reduction of the confidence. For example, in our study, significant different parental allele effects for the chlorogenic acid content in beans were detected from the three parental FRT clones. These results allowed the breeder to select among the most relevant parental alleles to be used in the new breeding cycle for the trait targeted. The decrease in the confidence region length was small (10% on average) for connected populations when comparing the shared QTLs from single-population analyses with MCQTL. However, looking closer at the confidence regions, three QTL appeared to have a much larger confidence region with the multi-population analyses. They were resolved in two close QTL by a multi-QTL search beginning with two cofactors on the linkage group. When these three QTLs were discarded, the decrease in the confidence region length jumped to a level of 20%.

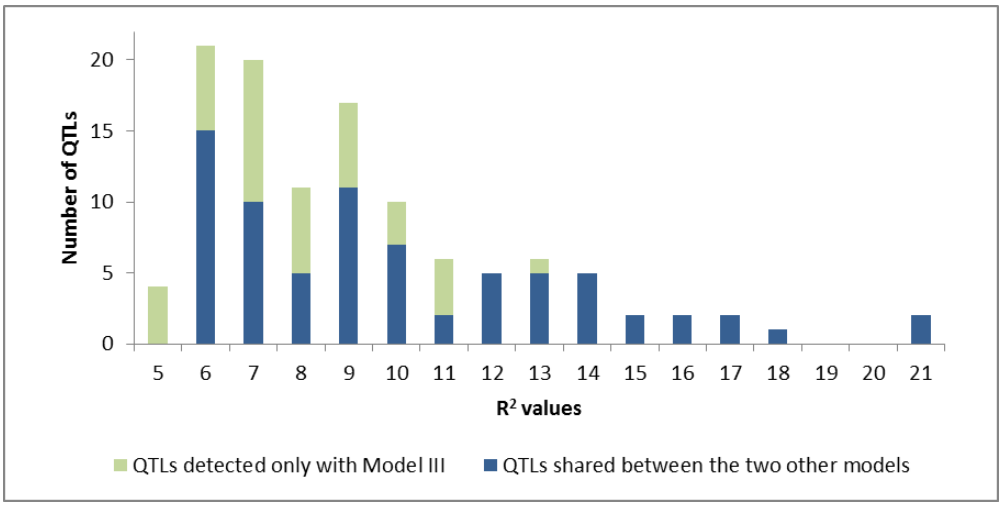

Figure 2. Distribution of R^2 values of the 114 QTLs detected with the model III dissociating the QTLs only detected with this model and the QTLs shared between the other two models.

Overview of QTL analyses

Fig. 3 shows the result of the overview of QTL detection charted on the *Coffea canephora* genetic map. QTLs involved in bean biochemical composition were distributed across all LGs except LG H, but six major genomic regions (LGs A, B, E, G, H and J) with high R^2 values (>12%) were highlighted. These six QTL hotspots were also involved in the bitterness trait. This result suggests either pleiotropic effect from single gene or the presence of different genes in the same genome area. For field traits, the QTLs were located across all the LGs with

co-locations on LGs A, B, F and J. However they generally had medium-range R^2 values (<12%) except on LG F for its 100-bean weight. None of the yield QTLs showed higher R^2 values than 10% and these QTLs were not consistently detected over the seven years of the analysis. But secondary yield component traits such as the 100-bean weight showed reliable detection in the two years of data recording with high R^2 values.

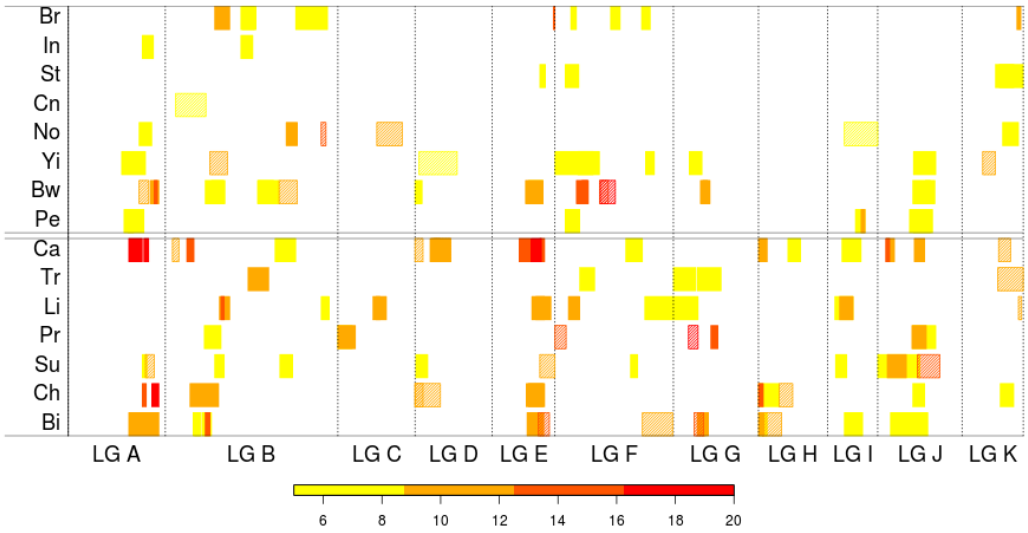

Figure 3. Heat map of the detected QTLs with the three QTL detection models. The chromosomes and the traits are represented in columns and in rows respectively. The QTLs detected by the model connected, were represented on the map with the characteristics (confidence regions and R^2 value) of this model by an opaque rectangle. When the QTLs were detected by the per population models only, the QTL with the tightest confidence region was kept to be represented by a hatched rectangle. A color state is used to indicate the percentage of variance explained by the QTL (R^2).

QTL detection on coffee in the literature

Previous QTL studies related to biochemical traits for coffee have already been performed. In 2013, Ky et al., using an interspecific cross (*C. pseudozanguebariae* x *C. liberica* var. *dewevrei*) identified two QTLs involved in the caffeine bean content on a genetic map with AFLP and RFLP markers. However, the lack of common markers with our genetic maps built only with SSR markers disallows making a comparison between the two studies.

However, seven QTLs related to yield and quality (LGs A, B, I, J and K) detected in our study also appear to be mapped in areas where Robusta QTLs had already been identified by Leroy et al. in 2011. Indeed, common markers between these two studies offer the possibility to compare these two analyses. These QTLs are involved in traits of bean biochemical composition such as the caffeine and chlorogenic acid content, bitterness and yield. The co-localization of QTLs for the same traits identified under different environments and with different genetic backgrounds support the interest in a candidate gene approach. To date, the availability of a complete genome sequence of Robusta will allow us to identity genes more precisely in relation to the QTLs detected in these studies.

Marker-assisted Selection on quality and yield for Robusta

Up to now, all Robusta QTL analyses linked to key economic traits such as the quality and field yield have been performed on Guinean and Congolese genotypes. The first breeding

programs used these two origins for reciprocal recurrent selection. Results of these intergroup hybrid trials indicated high yield and vigor, demonstrating the efficiency of this approach. But recent molecular studies based on SSR markers demonstrated that the Robusta genetic diversity is much more important with at least seven groups including Guinean and Congolese. Taking this new classification into consideration, breeders will be able to use the genetic diversity available to create mating schemes for determining the heterotic performance of hybrids by assessing marker-based parental groups. This type of experimental design could be of interest for field traits such as yield and disease resistance. However, quality traits could also be managed by this type of study since the biochemical composition throughout the genetic groups is highly variable, especially for caffeine and chlorogenic acid.

The introgression of QTLs using the marker-assisted selection will required a further step of validation in the different genetic backgrounds in order to keep the most robust and reproducible ones. Once the linked markers to these QTL have been identified, they can be used to predict a quantitative trait and could consequently at the origin of a MAS program. It will be quicker and more efficient to genotype a population than to phenotype it for complex traits such as cup quality or disease resistance which require mature plants from bean harvest and sensory analyses or intensive pathological studies. Another key advantage of a MAS program is that the screening phase can be performed at the seed level in order to transplant only coffee trees with the desirable characters in the field.

Ideally in the end, MAS can also be used to pyramid genes from multiple genetic parental populations. The subsequent step in coffee breeding will be to focus on validating QTL assessment throughout the various genetic groups characterized in Robusta. This step is certainly important for any efficient breeding program wishing to take advantage of the wide genetic diversity available.

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Environmental Effects on Coffee Seed Biochemical Composition and Quality Attributes: a Genomic Perspective

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SUMMARY

Reserve compounds and secondary metabolites that accumulate in mature coffee seeds contribute to a large extent– directly or through roasting-induced chemical reactions – to the broad spectrum of aromas and flavours of the coffee brew. Although cultivation of coffee trees under shade or at high elevation is known to favorably affect coffee quality, quantitative data describing the influence of climatic conditions on the chemical composition of the seed are still scarce. After a review of the relationships between coffee quality, seed chemical composition and environmental effects, this report will focus on the last advances in the understanding of environmental regulations of coffee seed metabolic pathways. Using multienvironment designs displaying broad climatic variations, and parallel monitoring of gene expression levels and metabolite accumulation profiles, we showed how growth conditions - such as mean air temperature - influence, in a predictable way, fatty acid, sugar and chlorogenic acid metabolisms and alter the chemical composition of the mature seed through subtle transcriptional regulations during seed development. Progress in this area could ultimately help in developing robust genomics/metabolomics fingerprints of coffee bean origin and quality, and help in assisting breeding programs dedicated to coffee quality.

INTRODUCTION

The flavour of a cup of coffee is the final expression and perceptible result of a long chain of chemical transformations from the seed to the cup. The reserve compounds and secondary metabolites that accumulate during seed development contribute to a large extent– directly or through roasting-induced chemical reactions – to the broad spectrum of aromas and flavours of the coffee brew. In addition to common seed storage proteins (11S globulin), sugars (mainly sucrose) and lipids (triacylglycerols), coffee seeds also synthesize spectacular amounts of peculiar compounds not found in the seeds of model plants, such as galactomannans, chlorogenic acids, caffeine and diterpenes. Each of these chemical classes plays a crucial role in the complex roasting chemistry (Flament, 2002). For example, proteins and amino acids are essential for the conversion of reducing sugars into aroma precursors through Maillard reactions. Reducing sugars themselves result from the degradation of sucrose and cell-wall polysaccharides. In addition, triacylglycerols are the major carriers of aromas in the roasted bean. Their fatty acid composition determines the generation of thermally-induced oxidation products, in particular aldehydes, which react readily with Maillard intermediates giving rise to additional aroma compounds. Finally, chlorogenic acids and caffeine are responsible for bitterness. Therefore, during the last decade, much research has been devoted to elucidating the numerous factors that influence the chemical composition of the seed. The seed content in aroma precursors may vary with genetic traits (Leroy *et al.*, 2006), agricultural practices (Vaast *et al.*, 2006), post-harvest techniques (Selmar *et al.*, 2006), as well as climatic conditions. The first part of the present review aims at summarizing

our knowledge on this latter aspect: the impact of the environment on the coffee seed chemistry.

Aroma precursors are synthesized and/or transported in the endosperm (a triploid tissue that constitutes most of the coffee seed volume) during seed development and maturation. Therefore, a better understanding of the developmental processes that govern the accumulation of these compounds, *i.e*. the identification of their biosynthetic genes, enzymes and the characterization of the regulatory processes involved, may provide novel targets and strategies for coffee quality breeding. Deciphering metabolic pathways that occur during coffee seed development has benefited from several technical advances in transcriptome analysis: real-time RT-PCR (Salmona *et al.*, 2008), oligonucleotide microarrays (Privat *et al.*, 2011) and RNAseq (Denoeud *et al.*, 2014). Using these techniques, the characterization of gene expression profiles during seed development enabled to identify key quality-related genes (for a review, see de Castro & Marraccini, 2006; Joët *et al.*, 2012). In order to better understand the influence of the environment on the chemical composition of the coffee seed, these transcriptomic approaches have recently been employed in multilocation trials. The second part of the present review provides the most relevant findings arising from these investigations.

ENVIRONMENTAL EFFECTS ON COFFEE SEED CHEMISTRY

Although environmental factors such as shade and altitude are empirically known to have beneficial effects on coffee quality, the impact on the environment has hardly been documented before the 2000s (Guyot *et al.*, 1996). Macro-diagnostic surveys led at the regional scale in Honduras, Costa Rica or Brazil, first established the relationships between coffee quality and environmental factors (Decazy *et al.*, 2003; Avelino *et al.*, 2005; Barbosa *et al.*, 2012). These studies showed significant correlations between coffee quality and geographic/topographic parameters such as latitude, altitude or slope exposure. However, contradictory results were observed regarding the influence of the environment on the chemical composition of green beans. These discrepancies may be due to the fact that these surveys were not performed using experimental sites that were strictly designed to study environmental effects: agricultural management and post-harvest treatments were not controlled.

Further studies therefore focused on the effect of individual environmental factors using dedicated experimental designs. Since elevation is the environmental factor most frequently mentioned with respect to quality, and coffee grown at high elevations fetches a higher price than that grown in lowland regions, this environmental parameter has received much attention. It was thus demonstrated that coffee from higher elevations exhibits better beverage quality (Bertrand *et al.*, 2006). Elevation also impacts the chemical content of green beans, for instance their lipid content and composition (Villareal et *al.*, 2009). The percentages of the two major fatty acids, namely linoleic and palmitic acids (30–45% each), increased with altitude and were negatively correlated with environmental temperature, while oleic and stearic acids (5-10% each) were favored in warmer conditions. Shade is another environmental factor frequently mentioned with respect to coffee quality. For instance, agroforestry is empirically known to provide cooler conditions that delay berry ripening and positively affect coffee quality. Several reports documented these effects using shade houses specifically built to adjust growth irradiance at a fixed fraction of the global irradiance. This approach was first described by Vaast and colleagues in Costa Rica (Vaast *et al.*, 2006; Geromel *et al.*, 2008; Somporn *et al.*, 2012). Shade was shown to increase seed size, to positively affect coffee quality, as well as to influence chemical composition such as reducing

sugar content (Geromel *et al.*, 2008). Finally, the influence of rainfall was assessed in various locations in Brazil through controlled water irrigation (Da Silva *et al.*, 2005). However, the influence of water availability was found to be rather limited.

QUANTITATIVE EFFECTS OF CLIMATIC FACTORS ON SEED CHEMICAL COMPOSITION

A better understanding of the metabolic status of the seed at harvest in relation with climatic factors requires an accurate measurement of the main climatic variables (temperature, rainfall, irradiance, and evapotranspiration potential) experienced during seed development and a comprehensive analysis of the quantitative relationships between these climatic variables and coffee quality and chemical content. It also necessitates a large multilocation trial that maximizes climatic gradients but lowers other sources of variations such as agricultural practices.

Reunion Island, which associates a rich homogeneous volcanic soil, strong altitudinal and rainfall gradients over very short distances, and a high density network of meteorological stations, has recently been shown as an adequate area to address these issues (Joët *et al.*, 2010). In order to accurately assess climatic effects, coffee sensory quality and chemical composition were analyzed from green coffee samples collected from 16 Arabica coffee plots located throughout Reunion Island and encompassing a wide range of tropical climatic conditions. All plots were planted the same year with the same cultivar and underwent identical agricultural management and post-harvest treatments.

Of the climatic factors recorded, temperature played a paramount role on bean quality since it was correlated with six (of the eight) sensory attributes measured. Aroma, acidity, fruitiness and overall quality were all favored by cool climates, whilst the undesirable earthy and green tastes were increasingly present as the temperature increased (Bertrand *et al.*, 2012). The other climatic variables played a minor role but it is worth noting that rainfall and potential evapotranspiration were correlated with bitterness and green taste, respectively. Using the same experimental plots, changes in lipid, chlorogenic acid, sugar and caffeine contents were monitored throughout seed development (Joët *et al.*, 2010). Surprisingly, none of the environmental factors studied significantly influenced the accumulation of the four main classes of storage compounds. Indeed, total cell-wall polysaccharides, total lipids, total free sugars and total chlorogenic acids showed no significant correlation with any of the climatic variables measured. In contrast, within a given chemical class (e.g. chlorogenic acids), several compounds may be significantly influenced by the environment. Half of the 28 metabolites analyzed by conventional analytical chemistry methods were significantly correlated with the average air temperature during the last five months of seed development – i.e. the period when storage compounds accumulate in the seed (Joët *et al.*, 2009). However, no significant correlation was found between rainfall or potential evapotranspiration and any of the compounds studied and only weak correlations were found with solar irradiance.

The slope of regression lines may also differ among the compounds of a given chemical class. For instance, within caffeoylquinic acids (CQAs), 3- and 4-CQA contents were positively correlated with temperature while the reverse trend was observed for the major chlorogenic acid, 5-CQA (Fig 1A). Interestingly, the same phenomenon was found for dicaffeoylquinic acids (di-CQAs): *i.e.* di3.4-CQA and di-4.5-CQA were positively influenced by temperature while a negative correlation was observed for di3.5-CQA. These results suggest that temperature directly acts on routing towards the different branches within the chlorogenic acid metabolic pathway without affecting the total seed chlorogenic acid content. A similar regulation of routing was observed within the fatty acid biosynthetic pathway, as already

reported by Villareal *et al.* (2009). The positive relationship observed between temperature and stearic, oleic, arachidic and behenic acids showed that acyl chain elongation increased with temperature. In contrast, linoleic and linolenic acid contents were negatively correlated to temperature, showing that acyl desaturation increased at low temperatures (Villareal *et al.*, 2009; Joët *et al.*, 2010). Finally, a similar trend was observed for free soluble sugars with opposite temperature effects for glucose and stachyose.

In addition to major seed storage compounds, attention was paid to minor components that may play a direct role on coffee aroma. Of the 44 volatile compounds quantified in green coffee beans by gas-chromatography coupled to mass spectrometry, 21 displayed significant variations among locations. The mean air temperature appeared again to be the predominant causal factor since it was correlated with 16 volatile compounds (Bertrand *et al.*, 2012). Among the volatiles detected, most of alcohols, aldehydes, hydrocarbons and ketones were positively correlated with temperature and solar radiation. These volatile compounds are therefore possible indicators of unpleasant sensory attributes. For example, two alcohols (butan-1,3-diol and butan-2,3-diol) were negatively correlated with aroma and acidity, and positively correlated with earthy and green flavors.

CHARACTERIZATION OF ENVIRONMENTALLY-INDUCED VARIATIONS DURING SEED DEVELOPMENT AND USE OF THESE VARIATIONS TO DECIPHER TRANSCRIPTIONAL REGULATORY PROCESSES OF METABOLIC PATHWAYS

So far, combining the use of multilocation trials, gene expression monitoring and metabolite profiling constitutes the most advanced strategy to understand how environmental factors and developmental programmes interplay to affect coffee seed quality. The metabolism of chlorogenic acids and that of galactomannans are described in the present review to exemplify the potential of this novel systems biology approach.

To investigate chlorogenic acid biosynthesis, the expression of selected phenylpropanoid biosynthetic genes, together with the accumulation profile of chlorogenic acid isomers, was monitored throughout seed development using the 16 locations in Reunion Island described above, which maximize climatic variation. Environmental temperature was shown to have a direct impact on the time-window for chlorogenic acid biosynthetic activity through subtle transcriptional regulations (Joët *et al.*, 2010b). The first steps of chlorogenic acid biosynthesis involve the well-characterized key enzymes of the ‘core phenylpropanoid pathway’, namely phenylammonialyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate CoA ligase (4CL). Transcript profiling revealed the modulation of *PAL* and *C4H* gene expression by temperature during endosperm development (Fig 1B). High temperatures first induced over-accumulation of mRNA encoding these two enzymes in early endosperm developmental stage. The reverse situation (a negative correlation between the level of *PAL* and *C4H* expression and temperature) was observed later, indicating a delay in the activation of phenylpropanoid genes under cool climates. This finding provides a sound explanation for the delay in the accumulation of 5-CQA observed at low temperatures. Moreover, the variability in seed chlorogenic acid composition induced by environmental temperature constituted a valuable system to test whether this accumulation is modulated at the transcriptional level and, if so, to detect rate-limiting transcriptional steps for chlorogenic acid biosynthesis. Final amount of 5-CQA, the major chlorogenic acid, was quantitatively correlated with early expression of *4CL* gene, but also with that of *HQT*, which encodes the enzyme thought to catalyze the last step of 5-CQA biosynthesis (Fig 1C). These two genes thus are rate-limiting transcriptional steps for chlorogenic acid biosynthesis and are referred as Quantitative Trait Transcripts (QTTs) for chlorogenic accumulation (Joët *et al.*, 2010b).

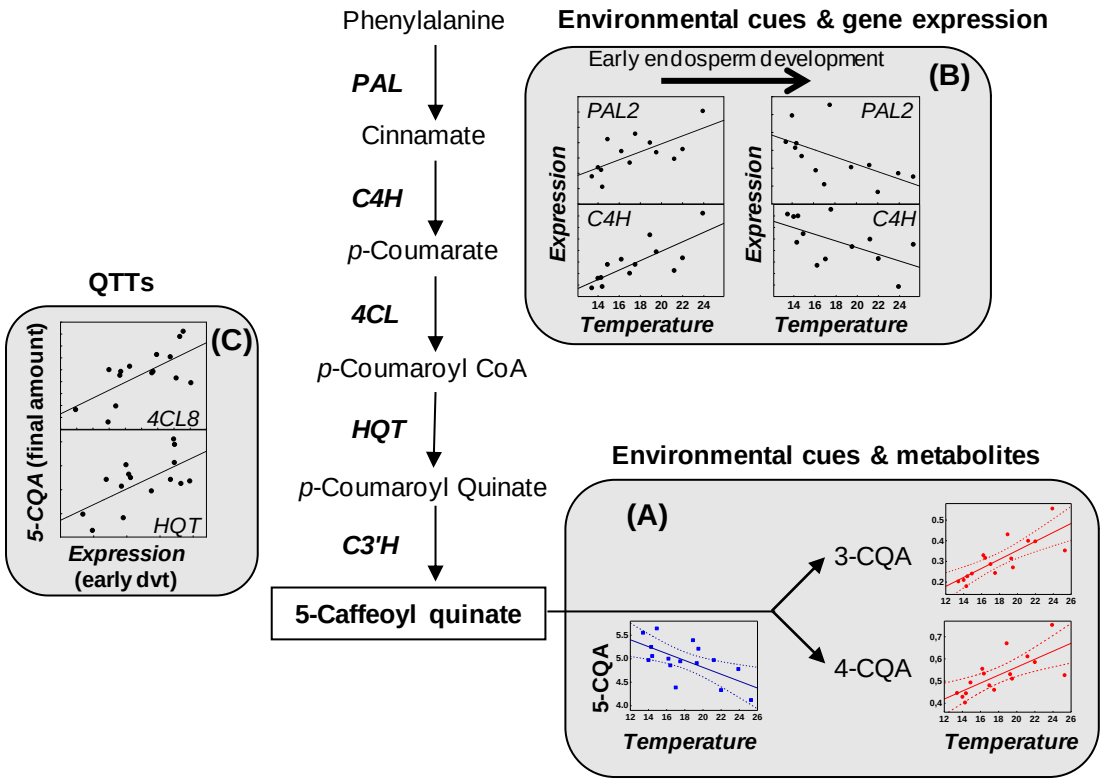

Figure 1. System analysis of chlorogenic acid metabolism in the developing coffee seed. The different boxes highlight highly significant correlations obtained between climatic, genomic, and metabolite datasets. Abbreviations: C3'H, p-coumaroyl CoA 3-hydroxylase; C4H, trans-cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; HQT, hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase; QTT, quantitative trait transcripts; PAL, phenylalanine ammonia lyase. Adapted from Joët et al. (2010b).

A similar strategy was chosen to elucidate the biosynthesis of galactomannans, which are the major cell-wall storage polysaccharide in coffee (Joët *et al.*, 2014). The expression profile of 28 genes related to carbohydrate metabolism and galactomannan biosynthesis was measured during seed development. This enabled to detect two types of environmental effect on transcriptional activity. First, a modulation of transcript accumulation by temperature was revealed for several genes involved in the synthesis of activated nucleotide sugars, i.e. GDP-mannose and UDP-galactose, which primarily serve as building blocks for galactomannan synthesis. For example, the expression level of *MGT* (mannose 1P-guanyl transferase), and *UG4E* (UDP-glucose 4' epimerase) was quantitatively affected by environmental temperature. Second, the environmental temperature impacted the timing of transcription for several key genes encoding galactomannan biosynthetic enzymes, such as galactomannan-galactosyl transferase (*GMGT*). The variability induced by the environment was also evidenced to be a useful tool to detect significant transcript-transcript quantitative correlations, hence enabling the characterization of gene transcriptional modules (quantitatively co-expressed genes). A dense module of nine genes was detected at the onset of galactomannan accumulation. This module included the five genes of the 'core galactomannan synthetic machinery, which encode the enzymes needed to assemble the mannan backbone (e.g. ManS), introduce the galactosyl side chains (e.g. GMGT), modulate the post-depositional degree of galactose substitution (e.g. α-Gal). This module also included the sucrose synthase gene *SUSY1*, stressing the tight transcriptional coordination between the sucrolytic activity required for nucleotide sugar production and galactomannan assembly.

Finally, the module contained two other genes, *GolS3* (galactinol synthase) and *SDH* (sorbitol dehydrogenase), revealing a link between galactomannan synthesis and raffinose oligosaccharide and sorbitol metabolisms, suggesting these metabolites play a role as transient carbohydrate reservoirs during peak galactomannan synthesis (Joët *et al.*, 2014).

PERSPECTIVES

For now, only a few recent studies have coupled transcriptomics and metabolomics to investigate the metabolism of developing coffee seeds. These new system biology approaches, using multienvironment designs, offer outstanding opportunities to unravel the regulation of the coffee seed biosynthetic processes and to revisit the effects of the environment, such as those of shade and drought. Coupled transcriptome-metabolome surveys in larger experimental designs may also be helpful for a better prediction of climate change impact on seed quality. Furthermore, using multigenotype designs, such approaches could also enable the fine characterization of Genotype X Environment interactions. Progress in these areas will ultimately help in developing robust genomics/metabolomics fingerprints of coffee bean origin (Bertrand *et al.*, 2008) and quality, and help in assisting breeding programs dedicated to coffee quality.

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Breeding for Rust Resistance in Arabica – Where we are and What Next?

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SUMMARY

The recent epidemic of coffee leaf rust, affecting all coffee growing countries of the Central American region, with forecasts of significant crop losses, has attracted the attention of the breeders yet again. Coffee leaf rust (CLR) caused by the obligate parasitic fungus *Hemileia vastatrix* Berk & Br., continue to be the principal production constraint for arabica coffee, since its occurrence on epidemic scale in Sri Lanka, India and Indonesia in late 1860s. Timely sprays of prophylactic and systemic fungicides have proved effective for disease control. Nevertheless, the coffee growers across the continents prefer the cultivation of tolerant varieties considering the economic and environmental sustainability as well as ensured livelihood security. Hence, breeding for rust resistance has been the major focus of Arabica improvement world over that lead to the development of several resistant varieties for commercial cultivation. Considering the durable nature of resistance genes of diploid origin compared to that of Arabica, introgressive breeding strategy has been successfully exploited for transfer of diploid genes and spontaneous tetraploid inter-specific hybrids like Hibrido de Timor (HDT), S.26 and Devamachy as primary donors of resistance genes in arabica breeding. However, breakdown in resistance has been observed in the commercial strains on cultivation in large areas due to the evolution of new virulent races of pathogen with improved adaptive ability. Moreover, the changing climate in different coffee growing regions is posing new challenges for durability of resistance in improved varieties of arabica. In the back drop of the existing sources of resistance genes, increased pathogen variability and impact of changing climate, the sustainable options available for the breeders are limited to ensure the durability of resistance.

With a long history of breeding for rust resistance, India has been trying to exploit all available options for achieving durable and long lasting resistance in the commercial arabicas. Various tetraploid inter-specific hybrid derivatives and the resistance donor plants (HDT collections as well as other spontaneous inter-specific hybrids) have been continuously monitored over the years for durability of resistance under field conditions. In the present communication we present the current status of rust resistance breeding in India, highlighting a detailed account on history of leaf rust in India, strategies being followed for efficient management of the disease, response of the resistance donor plants under varying disease pressures, the best donor for resistance breeding in terms of durable resistance, identification of alternative sources resistance and its exploitation in breeding, marker assisted selection for integrating the S_H3 gene with other resistance genes, adaptation of the virulent races on resistant genotypes and current focus of breeding for rust resistance. The potential implications of the Indian experience in leaf rust management in formulating a holistic approach for arabica coffee breeding towards maximizing the durability of rust resistance in coffee are detailed.

INTRODUCTION

Popularly referred as 'Brown Gold', coffee is one of the economically important crops in tropics and sub-tropics as exports earnings from this commodity constitute an important source of foreign exchange earnings for producer countries. Coffee trade attracts global attention because its production is confined to developing countries but largely consumed in developed countries. India is one of the coffee growing countries with a cultivated area of 0.4 million ha and produces around 0.31 million MT annually with 4.5% share in Global production. Coffee is predominantly a small holder enterprise with 99% of coffee farmers owning holdings of less than 10 ha and primarily depends on coffee for their livelihood. Indian coffee is acclaimed world over because it is grown under two-tier shade canopy in most eco-friendly manner. Further, coffee plantations forms distinct multi cropping ecosystems, as they are enriched with suitable inter crops like pepper, Areca, orange etc that also provide additional income to the growers. On the other hand, coffee growing areas in India are exposed to harsh environmental conditions compared to other coffee growing countries, with a continuous long dry period of 150 to 160 days. Incidentally, the climatic conditions prevailing in Indian coffee tracts are congenial for flare up of certain important diseases and pests of which coffee leaf rust (*Hemileia vastatrix*) and white stem borer (*Xylotrechus quadripes*) which are the major challenges for arabica coffee cultivation. In this context, maintenance of adequate shade and integrated management practices are critical for minimizing the crop losses.

Coffee leaf rust (CLR) is certainly the earliest of the major tropical plant diseases reported so far. This classical disease is considered to have originated on wild coffee in Ethiopia but was first reported in Sri Lanka in 1869 on cultivated coffee. In subsequent years the disease spread to neighbouring India and Indonesia. By the later part of the 19^{th} century, leaf rust gradually appeared in majority of the coffee growing countries including that of South and Central Americas causing enormous financial losses. Timely sprays of prophylactic and systemic fungicides are proved effective for disease control. Nevertheless, considering the economic and environmental sustainability as well as ensured livelihood security, cultivation of tolerant varieties is the most preferred option. Hence, breeding for rust resistance has been the major focus of Arabica improvement world over, that lead to the development of several resistant varieties for commercial cultivation. However, breakdown in resistance has been commonly observed from time to time due to the evolution of new virulent races of CLR pathogen. Nevertheless, this phenomenon has been more pronounced in India because of the favourable climatic conditions for rust disease build up and selection pressure on the pathogen due to the spread of several rust tolerant cultivars in field. Therefore, enhancing the durability of resistance in commercial cultivars by utilizing the diverse sources of resistance is the current focus, in the Indian context. The recent epidemic of coffee leaf rust affecting all coffee growing countries in Central American region, with forecasts of significant crop losses has attracted the attention of the coffee community and there is an urgent need to relook into the resistance breeding as well as the better ways and means of resistance management. In the backdrop of these developments, in the present paper we report a detailed account on India`s approaches to manage rust, past achievements and current status of rust resistance breeding in India and the options available for achieving a broad spectrum of rust resistance in commercial coffee cultivars.

COFFEE LEAF RUST – INDIAN PERSPECTIVE

Since the reported introduction of coffee into India sometime during 1600 AD by a Muslim pilgrim Baba Budan, coffee plants were confined to the back yards until the late 1820s when British entrepreneurs started coffee cultivation on commercial scale in South India [5]**.** Coffee

cultivation progressed rapidly during the next 40 years that reached its peak during the early 1860s. At this juncture, coffee cultivation witnessed the ravages of pests and diseases mainly the "coffee white stem-borer" *(Xylotrechus quadripes)* and coffee leaf rust (*Hemileia vastatrix* B. & Br.) that posed a major threat to Indian Coffee. The leaf rust disease was first observed in an epidemic scale in 1869 and concurrently, the coffee cultivation in neighbouring countries like Sri Lanka and Indonesia also suffered with leaf rust epidemic that lead to the replacement of coffee with tea in Sri Lanka and introduction of robusta coffee in Java.

In order overcome the onslaughts of rust disease, some of the enterprising planters in South India made frantic efforts to tackle the leaf rust disease by following simple selection of disease tolerant plants in the existing populations. The varieties like 'Chicks', `Coorgs` and 'Kents' were some of the popular varieties developed by the planters. At that time, fungicides for the control of this pathogen had not yet been invented and the knowledge on Coffee-Rust interactions was also little known. The 'Kents' variety became very popular and was the largely cultivated variety in early 19th century because of its production potential, disease tolerance and superior bean quality characters. To overcome the leaf rust disease, Robusta and *C. liberica* coffees were also introduced from Java around 1900 AD and robusta variety adapted well to the lower elevations. On establishment of Coffee Experimental Station at Balehonnur in Chikmagalur District of Karnataka, during 1925, currently known as Central Coffee Research Institute (CCRI), systematic work on coffee breeding for rust resistance was undertaken by utilizing the available genetic resources.

Thus, breeding for rust resistance was given high priority in India since 1920s while in other countries, initial breeding efforts in arabica coffee were aimed at yield and quality improvement and adaptability to local conditions. These early breeding programmes carried out between 1920 – 1940, resulted in considerable success in developing vigorous and productive cultivars that suggested somewhat larger degree of genetic variability of the base populations [7]. Several of these varieties are still under commercial cultivation. The varieties like `Mundo Novo`, `Caturra` and `Catuai` from Brazil, `Kents` and S.288 from India, `Blue Mountain` from Jamaica, are some examples of these better-known cultivars.

PIONEERING WORK ON COFFEE LEAF RUST IN INDIA

The findings of Wilson W.Mayne, the first Scientific Officer at Coffee Experiment Station, on existence of physiological races of rust pathogen is a classical work on CLR. This pioneering work in India, resulted in identification of four races of the rust fungus, viz. race I & race II race III and race I. These developments paved the way for focused research on disease periodicity, resistance breeding, race diversity, host-pathogen interaction and disease management. Simultaneously, use of fungicides for disease control was also pursued and spraying of Bordeaux mixture (Solution prepared by mixing Copper sulphate and spray lime) was introduced to tackle leaf rust disease way back in 1930's. The basic work in India contributed towards establishment of the Coffee Rusts Research Centre (CIFC) at Oeiras, Portugal, in 1955.

INDIA'S APPROACH FOR MANAGING CLR

Keeping in view the economics of disease management and also to discourage the chemical control measures, development of disease tolerant cultivars is considered as the most preferred and viable option for sustainable coffee cultivation. In India, since beginning, primary focus was on breeding varieties for leaf rust resistance/tolerance. Further, importance was also given for adoption of integrated disease management strategy involving cultural

methods and need based use of fungicides at estate level. Thus, resistance management and cultural management are being effectively integrated for efficient disease management.

RESISTANCE MANAGEMENT - GROWING TOLERANT CULTIVARS

Cultivation of tolerant cultivars has been a practice since the rust epidemics in late 18th century. Chicks, Coorgs, Kents the disease free selections of the enterprising planters were the largely grown varieties during 1920s, at the time when organized research started in India. Subsequently, Central Coffee Research Institute evolved 13 arabica cultivars that manifest a broad spectrum of resistance to CLR by utilizing the available genetic resources and was given for commercial cultivation. Of these cultivars, S.795, Sln.5A, Sln.5B, Sln.6, Sln.9 are the tall phenotypes and popular among the grower community. 'Chandragiri', the latest variety manifest high field tolerance and show wider adaptability. Released for commercial cultivation during 2007, this Sarchimor derivative is at present very popular among the grower sector with a seed demand of about 5000 kg, each year.

MANAGEMENT STRATEGIES FOR LEAF RUST

Maintenance of a two-tier mixed shade canopy, post harvest pruning & post monsoon handling of the bushes to minimize the initial inoculums, balanced nutrition for maintenance of plant vigour, prophylactic spray of 0.5% Bordeaux mixture before build up of disease once before onset of monsoon and again after cessation of monsoon are the cultural management strategies being followed on field. Need based use of systemic fungicides, in combination with Bordeaux sprays is practiced especially for susceptible varieties depending on the disease build up. Thus, rust management strategies have been integrated with routine cultivation practices in India and never considered as contingency measures after the disease development. Both the strategies of growing tolerant cultivars and cultural management practices helped the growers to keep the disease incidence in control.

BREEDING FOR LEAF RUST RESISTANCE: INDIAN EXPERIENCE

Breeding for rust resistance was given high priority only in India, since 1920s. The breeding programme undertaken in India can be grouped under three important phases, 1920s-1950s; 1950s-1980s and 1980s-2000s. In early coffee breeding programmes undertaken between 1920s to 1940s, S.26 a putative natural hybrid between *C. arabica* and *C. liberica,* a diploid coffee species, was used as main source for rust resistance in evolving the early Indian selections S.288 and S.795 of which S.795 is the country's most popular strain with wider adaptability and yield potential of 1200 to 1500 kg/ha. The populations of S.795 are under commercial cultivation since 1947 and manifest high levels of field tolerance to leaf rust races I and II prevalent in Indian coffee tracts. These early Indian selections carry S_H3 resistance factor for coffee rust, introgressed from *Coffea liberica,* through the spontaneous hybrid S.26. This S_H3 resistance gene is responsible for imparting durability of resistance in S.795 for several years in field. So far, nine resistance genes (S_H1 to S_H9) that impart resistance to rust were identified of which S_H3 is an important gene for durable host resistance if present in homozygous condition (S_H3S_H3). Thus, in the first phase of breeding, *C. liberica* introgressed lines were exploited as donors of resistance. Although some virulent rust races like VIII, XVI are able to infect S.795 populations, timely sprays of two rounds of 0.5% Bordeaux mixture keeps the disease under check and rarely requires integration of one round prophylactic and one round of systemic fungicides. Because of the established production potential and well known superior quality traits with respect to bean grades of above 60% `A` grade and fine cup quality attributes, S.795 is still a preferred variety for traditional arabica areas of Karnataka. Hence, a programme for maintenance breeding S.795 variety has been followed by

identification of S.795 plants, homozygous to S_H3 using SCAR marker assays (Sequence characterized amplified polymorphism) linked to S_H3 gene and establishment of clonal seed plots.

In the second phase of breeding between 1950s-1980s, exploitation of spontaneous hybrids of *C. arabica* and *C. canephora* like Devamachy from India and Hibrido de Timor from Timor island introduced from CIFC, Portugal (CIFC 1343) were extensively used in development of several tetraploid interspecific hybrids like Sln.5A, Sln.5B, Sln.8 and Sln.9 (Robusta introgressed lines). All these varieties have been extensively evaluated and have been recommended for commercial cultivation depending on their location specific suitability. From the field performance and feed back from the growers, Sln.5B and Sln.9 are the widely adaptable varieties like S.795. These tetraplod inter-specific hybrid derivatives manifest high field tolerance to rust with a production potential of 1200 to 1600 kg/ha depending on the management. The beans are round, bold in size ranging from 55 to 65% `A` grade with FAQ to above liquor quality. Prompted by the success with introgressive breeding, synthetic variety, Sln.6 was developed by crossing Robusta and Arabica followed by recurrent back crossing to arabica parent. This variety was developed with an objective of developing arabica type of plant with resistance and bearing characters of robusta. Being a robusta x arabica hybrid, the bushes of Sln.6 are tall, vigorous with profuse branching pattern with yield potential of 1200 – 1500 kg/ha. As 'Kents' was used as recurrent parent, 15-20% of the population in Sln.6 manifests rust susceptibility while the remaining 80% of plants manifest high field tolerance to rust. The performance of Sln.6 is found promising in transitional altitudes from 900 m to 1100 m.

In the third phase from 1980s to 2000s, the focus was on exploitation of the varieties derived from crosses between dwarf mutants (San Ramon, Caturra, Villasarchi) and Timor hybrids (832/1 & 832/2). These efforts lead to the development of dwarf/semi-dwarf varieties like San Ramon hybrids, Catimor and Sarchimor. The San Ramon hybrids were developed from multiple crosses involving `San Ramon`, a dwarf mutant and other tall arabicas like S.795, Agaro and Hibrido de Timor (HDT). The original `San Ramon` mutants were very compact in branching habit with closer internodes, deep rooted but highly susceptible to rust. The dwarfs in San Ramon hybrids are suitable for high density planting (4ft x 4ft, 5ft x 4ft.) with yield potential of over 1500kg/ha but with annual variations for production. Sln.7.3 is a late ripener, drought tolerant and suitable for marginal areas. The Catimor variety a derivative of CIFC HW 26 that was given for commercial cultivation in India as `Cauvery` during 1980s. Soon the variety became popular with the coffee farming community, on account of its high yielding potential (1500-2000 kg/ha) and resistance to leaf rust in initial years. However, after few years, break down in leaf rust resistance has been reported due to the evolution five new rust races, indicating the less durable nature of resistance donor parent, HDT 832/1. Rust disease management became critical for realizing economic yields and hence cultivation of this variety is now limited to high elevations. Subsequently, `Chandragiri` a derivative of Sarchimor (CIFC 361/4) was given for cultivation during 2007. This variety is promising with yield potential of 1500 to 1800 kg/ha and high field tolerance to leaf rust. Further, the variety has definite advantage with respect to superior grade percentages as over 70% of the beans belong to `A` grade on average, of which 25-30% belong to AA grade with superior bean density. The variety has been gaining popularity among the arabica coffee growers of the country with large scale replanting.

RESISTANCE MANAGEMENT: CONSEQUENCES

Cultivation of diverse rust resistant cultivars exerted selection pressure on rust pathogen forcing the development of new virulent races. Furthermore, the ideal weather conditions

prevailing in Indian coffee tracts facilitated the hastening up of mutations in rust pathogen. Out of 45 races isolated so far from across the world, ~ 35 races (24 designated + 11 new races), are known to be distributed in India. The new virulent races showed ability to overcome the resistance of the improved varieties in favorable conditions but at the cost of fitness penalty. Thus although new rust races with virulent gene combinations of eight virulence genes have been isolated, only few races are prevalent in field situations depending on their spread of the host material and survival ability.

PATHOGEN DIVERSITY VS HDT LINES, THE DONORS OF RESISTANCE

Continuous monitoring of different types of HDT lines under increased pathogen diversity revealed that on HDT - CIFC 832/1, a few pustules were observed in field during 2007 & 2008. These spores, characterized as new race showed progressive adaptation on the genotype. As regards to HDT - CIFC 832/2, few non-sporulating pustules were observed during 2008. Subsequently, the disease could not be reestablished under controlled conditions. Even under natural conditions, poor adaptation of the pathogen has been observed suggesting the complex nature of resistance in this genotype. With respect to HDT - CIFC 1343, all the existing 9 plants remained resistant since 1961. The four selfed progenies manifested susceptibility ranging from 2 – 6% of the population. Among the new HDT introductions from CIFC Portugal during 2009, viz. CIFC 4106, CIFC 19758, 19759, CIFC 6530 few clones manifested mild susceptibility.

CLIMATE CHANGE - IMPLICATIONS FOR COFFEE LEAF RUST

For the last few years, the changes in climatic conditions such as increase in temperatures, rain fall pattern and humidity levels in coffee growing areas are posing new challenges for arabica coffee cultivation. Rust disease flare ups in Colombia few years back and recent rust outbreaks in Central America are the typical consequences of climate change effects. In the Indian context, although the impact of increase in temperatures is not felt due to shade grown conditions, there is a change in distribution pattern of rain fall for the last couple of years leading to problems in timely adoption of rust management practices.

NEW CHALLENGES

The diversity of the pathogen vs durability of Resistance, limited sources of resistance that ensure long lasting resistance in diverse field situations/disease pressures, disease flare ups due to change in climatic conditions in growing areas are the new challenges for coffee breeding.

BREEDING FOR RUST RESISTANCE IN INDIA – CURRENT FOCUS

Although, there has been a gradual increase in adaptive capacity of the *H. vastatrix* with an ability to overcome host resistances, it has been observed that the resistance genes introgressed from the diploid species such as the S_H3 gene from *C. liberica* as well as S_H6 to S_H9 genes from *C.canephora* are found to be more durable in providing long - lasting protection to arabica under field conditions, as compared to the genes identified in tetraploid *C. arabica*. Hence, combined use of resistance genes of diploid species in a selected arabica genotype or using them in composite varieties are some of the promising approaches to achieve durable rust resistance.

The current focus of arabica coffee breeding is towards the development and evaluation of reciprocal hybrids of *C. liberica* introgressed lines and robusta introgressed lines, derived

from HDT 1343 and HDT 832/2, to ensure durable and long lasting resistance to leaf rust. Further search for new sources of resistance especially the fertile spontaneous inter-specific hybrids in natural populations have been given utmost priority. In this direction, development and evaluation of several hybrid progenies between Catimor and liberica introgressed lines resulted in selection of two promising progenies (S.4814 and S.4817) that recorded superior performance for yield, field tolerance to rust, bean and liquor quality traits. Based on field performance data, nine F_2 and eight F_3 populations were derived and established in different locations for further monitoring and selection. Similarly, four progenies (S.5083 to S.5086) were established from the crosses between Sarchimor and *C. liberica* introgressed lines and two progenies (S.5091 & S.5092) were established from crosses involving tall phenotypes (S.2790 x S.2724). Marker assisted selection with SCAR markers linked to S_H3 gene has been routinely used for tracking S_H3 gene in donor parents as well as hybrid lines. Field performance of these genotypes is found promising for all agronomical traits and resistance to leaf rust that provides good scope for exercising further selection and commercial exploitation.

With respect to new sources of resistance, an interesting spontaneous hybrid of *C. arabica* x *C. excelsa* (*C. liberica* var.dewevrei) with high fertility, good production potential and host resistance identified in natural population has been exploited in breeding. The reciprocal hybrids (S.5081 & S.5082) developed from crosses between Sarchimor x spontaneous hybrid exhibited vigorous phenotype, resistance to leaf rust and tolerance to coffee white stem borer. Initial results from the bioassays against the pest are very encouraging.

CONCLUSIONS AND PERSPECITVES

The approaches followed for management of leaf rust in India have proved effective in keeping the disease under control even in susceptible cultivars, inspite of the fact that the favourable climatic conditions of alternate wet and dry periods prevail in coffee growing regions. However, considering the adaptive ability of the pathogen, breeding for rust resistance is a continuous process and on monitoring the field performance of various tetraploid inter-specific hybrid derivatives, the *C.liberica* introgressed lines and robusta introgressed lines, the current breeding strategy of integrating the resistance genes of diploid origin (S_H3 and S_H6 to S_H9) in tetraploid arabicas looks promising. Some of the salient inferences from the Indian experience of rust management and future perspectives with respect to resistance breeding are as follows.

- HDT 1343 & HDT 832/2 continue to be the better candidates as donors for rust resistance breeding
- Need to monitor other HDT genotypes that have not been used in breeding, for combining ability and resistance manifestation and to exploit as new donors
- There is a need to explore for new resistance sources and re-exploration of Timor island is one of the option to observe the current status of variability in HDT genotypes.
- As the Catimor and Sarchimor derivatives incorporated with S_H3 are showing promising performance, pyramiding of S_H3 gene with genes of robusta origin appears to be the best strategy available at present for achieving durable rust resistance
- The SCAR markers for S_H3 are very efficient in tracking the gene in breeding populations and for marker assisted selection.
- The spontaneous hybrid of *C.arabica* x *C. excelsa* (*C. liberica* var. dewevrei) spotted in India looks to be a new potential donor for broad spectrum of resistance for diseases and pests

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Identification of Pathogenesis-Related Proteins in the Coffee Rust, *Hemileia vastatrix*

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SUMMARY

Coffee Leaf Rust (CLR) has re-emerged in the last few years in Latin American as a major limitation for coffee producing countries, causing severe losses in supply quantity and quality, and increasing the cost of production due to the strict management measures that must be taken to control the disease. Understanding the genetics of the causal agent *Hemileia vastatrix* is a critical element in the retrospective analysis of the CLR onset and in the implementation of forthcoming strategies to reduce the risk and minimize the effect of the disease. Next-gen sequencing technologies have emerged as powerful tools for the characterization of plant-pathogens, particularly in the case of biotrophic organisms that cannot be cultivated *in-vitro*, and for the elucidation of mechanisms involved in plant-pathogen interactions, where pathogenesis-related genes play a critical role, and are now commonly predicted through transcriptome analysis. We performed RNA-sequencing of three isolates of *H. vastatrix* in order to identify putative secreted proteins. RNA-seq datasets of the fungus were assembled with the software Trinity and mapped to a reference genome assembly of *H. vastatrix*. Comparisons against public databases resulted in the prediction of 3,921 gene families. Several pathogenesis related genes such as DNA damage repair, DNA photolyases, protein kinases and transcription regulation genes were annotated by their similarity to other plant pathogen genes. We predicted 483 secreted proteins using a novel bioinformatics approach, with 28 of those proteins being exhaustively annotated due to their display of putative functions related to pathogenesis. Only five of these sequences did not share any homolog with other Pucciniales fungi, showing the apparent conservation of these genes in this group of plant pathogens. Six sequences had homologs already identified as secreted proteins in the *Populus* leaf rust, *Melampsora-larici populina*. The identification and annotation of this set of pathogenesis related genes have significant implications for our understanding of *H. vastatrix* pathogenesis during its infection on coffee plants. These results will support the molecular comprehension of increased aggressiveness and break of resistance events that affect resistance durability and disease management success. Besides, the sequence information collected can be used to design molecular markers to distinguish isolates and races of the pathogen worldwide, and can orientate the decision making process in countries where CLR is causing serious concerns.

BACKGROUND

Recently, secreted proteins have been linked to the pathogenesis of plant-pathogenic fungi. Putative secreted proteins, very likely involved in the pathogenesis of rusts to their hosts, have been identified in *Melampsora* spp., *P. striiformis*, *P. graminis* and *H. vastatrix*. These secreted proteins have been classified in families of candidate effectors. Thus, the discovery

of the secreted protein gene clusters and the functional demonstration of their decisive role in the infection process illuminate previously unknown mechanisms of pathogenicity that operate in biotrophic fungi.

The first *H. vastatrix* genome data were recently published. A total of 6,763 genomic sequences were assigned to the *H. vastatrix* transcriptome and a set of secreted proteins possibly involved in the *H. vastatrix* infection processes of coffee tissues was predicted. We have obtained transcriptome sequences, and these data will allow the identification of potential molecular markers for the study of the rust races. Additionally, the knowledge of the *H. vastatrix* secretome is a starting point for understanding the mechanisms used by the fungus during the colonization of coffee tissues and the similarities to other plant rust processes of pathogenesis.

MATERIALS AND METHODS

Sample preparation for RNA-seq and transcriptome assembly

H. vastatrix urediniospore samples were scraped from infected coffee leaves taking care to sample very young pustules with no evidence of the presence of the hyperparasitic fungus *Lecanicillium lecanii*.

RNA was extracted from urediniospores using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Normalized library construction was performed at Evrogen, Moscow, Russia using Kamchatka crab duplex-specific nuclease. Illumina sequencing was performed at BGI, China. RNA-seq data were filtered before assembly. The quality of the transcripts was measured using FastX for each line of Illumina sequenced. Reads were trimmed by quality and duplicates were removed.

Gene Prediction

For the prediction of gene models, we followed the 'align then assemble' approach. We mapped RNA-seq short reads to the genome using TopHat, and we identified putative transcriptional units using Augustus. Protein sequences were computationally deduced from the transcriptional units. Gene families from predicted proteins larger than 70 amino acids were identified with OrthoMCL using a default MCL inflation value of 1.5 and a blastp e-value of $10e^{-5}$.

Secreted Proteins

The *H. vastatrix* predicted proteins were classified into secreted and non-secreted proteins. For this task, the programs SignalP 4.0 and PProwler were used to predict putatively secreted proteins. A 0.9 probability cut-off was used for PProwler predictions. A set of secreted proteins predicted previously for *H. vastatrix* by Fernández et al. (2012) was used for comparison with our predictions. Briefly, a Blastp was performed between our set of *H. vastatrix* proteins and the Fernández et al. (2012) predictions. Finally, a set of proteins that showed similarity (Blastp e-value=$1e^{-5}$) with the secreted proteins predicted by Fernández et al. (2012) was obtained. Multiple comparisons of the three sets of secreted proteins were performed (SignalP, PProwler and Fernández-Blastp) to establish the proteins shared by the three predictions.

Comparative Genomics

For genome annotation, we used custom Perl scripts and basic bioinformatics software such as BLAST; BLAST similarities were visualized using a BLAST Output Visualization Tool. The databases we used for comparisons corresponded to 67,118 Pucciniales proteins comprising 16,694 amino acid sequences from *Melampsora larici-populina*, 22,815 *Puccinia striiformis* f. sp. *tritici* sequences, 15,979 *P. graminis* f. sp. *tritici* sequences, and 11,630 *P. triticina* sequences.

We also aligned the *H. vastatrix* genome contigs against the genomes of *P. graminis*, *M. laricis-populina* and *U. maydis* using Mauve. The Low Collinear Block (LCB) values were set through visual inspection by searching the best block size for each pair of alignments (largest coverage of both genomes). Finally, values used for LCB were as follows: *P. graminis* 12,154, *M. laricis-populina* 10,409 and *U. maydis* 1,203.

RESULTS

Transcriptome Assemblies

RNA-seq clean reads were assembled using Trinity. The smallest number of assembled contigs was obtained with the normalized library. We mapped the *H. vastatrix* RNA-seq datasets to the hybrid 454-Illumina assembly of *H. vastatrix*. We compared the RNA-seq datasets by using the program BLASTn with an e-value of 1e-30 and we also compared the transcriptomes against the NR database to identify plant, bacterial and other contaminant sequences and visualized the results using MEGAN. We carried out homology annotation with BLASTp against rust protein datasets described above.

Predicting protein-coding genes

We used TopHat to map the RNA-seq data sets against the HvHybrid genome assembly, and we identified a total of 21,345 contigs that matched the RNA-seqs. This set of contigs was selected for gene prediction using Augustus [26]. For annotation of *H. vastatrix* proteins, we used the program already trained with *Saccharomyces cerevisiae* sequences. A total of 18,234 sequences were predicted that code for proteins with 70 or more amino acids. The average length of the predicted gene models was 1,047 bp. We filtered this set of sequences to remove transposon sequences identified in RepBase Release 17.01, and this final set contained 14,445 predicted protein-coding gene sequences. This set of predicted proteins was used to perform BLASTp (e-value=1e-3) sequence similarity searches against Pucciniales sequences (67,118 seqs), and we identified 13,796 hits (73.86%).

Secretome Annotation

We predicted 659 secreted proteins using PProwler and 775 secreted proteins with the SignalP algorithm. A total of 180 proteins in our *H. vastatrix* set presented with similarities to the secreted proteins predicted by Fernández et al. (2012). The SignalP and PProwler methods shared 483 proteins, and 44 proteins were shared with the Fernández et al. (2012) dataset. We filtered this set of sequences and removed those that mapped in the same genome contig and belonged to the same gene family; we ended up with a final set of 28 predicted proteins and they were annotated with Blast using swissprot, RefSeq and UniGene100 databases. All 28-protein sequences were mapped with tblastx to at least one contig from each of the individual assemblies and they were functionally annotated with blastp against swissprot, RefSeq,

Uniref100 and the non-redundant protein sequences databases. Only five sequences did not have a homolog sequence with other Pucciniales fungi. Six sequences had a homolog already identified as a secreted protein in MLP.

CONCLUSION

Our functional analysis of the *H. vastatrix* predicted secretome shows that secreted proteins are well conserved among plant rusts and that they include functions most likely involved in the pathogenesis of the fungus.

The draft genome sequence of *H. vastatrix* will serve as a template for future assemblies of isolates of this fungus and to understand the molecular mechanisms used by this fungus to attack the coffee plant, to study the diversity of this species and for the development of molecular markers to distinguish races/isolates.

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Demonstration of Enzymatic Control of Acrylamide Levels in Coffee

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SUMMARY

Acrylamide is formed in food products after Maillard type reactions. The formation is promoted by low water content and high temperatures. Due to the known toxicity of acrylamide several efforts have been made to reduce acrylamide in food products. The present report represents investigation into enzymatic reduction of the major acrylamide precursor, asparagine, in green coffee beans and subsequent acrylamide mitigation in roasted coffee. Up to 82% reduction of asparagine in green coffee beans was obtained by application of the asparaginase enzyme product, Acrylaway. The acrylamide content was reduced correspondingly at levels of 57% up to 72 % in roasted coffee. The study also presents mechanistic understanding for the enzymatic hydrolysis of asparagine in green coffee beans. Asparagine is shown to continuously leach out from green coffee beans during enzymatic treatment allowing for a significant reduction in asparagine.

INTRODUCTION

In 2002 Swedish researchers discovered high concentrations of acrylamide in carbohydrate rich foods produced under relatively high temperatures (Tareke et al., 2002). Acrylamide is known to be carcinogenic in rodents and has been classified as a "probable human carcinogen" by the International Agency for Research on Cancer (IARC, 1994). Because of the known toxicity of acrylamide in laboratory animals and the status as a possible human carcinogen several investigations have been performed in order to better understand the mechanism for formation of acrylamide in foods and possible means to reduce the acrylamide level as well as assessing the health risk associated with acrylamide intake.

Acrylamide is formed in several food products after Maillard type reaction between asparagine and reducing sugars (Becalski et al., 2002;Stadler et al., 2002;Yaylayan et al., 2005;Zhang and Zhang, 2007;Zyzak et al., 2003). Formation of acrylamide is affected critically by processing parameters such as temperature, water content, and pH as well as the content of reducing sugars and asparagine. High temperatures and low water content promote formation of acrylamide, while low pH reduces formation. A review of acrylamide formation and the various parameters affecting the formation as been described in the review by Zhang and Zhang (2007).

The generally accepted most important mechanism for formation of acrylamide is the reaction between a carbonyl compound (such as a reducing sugar) and asparagine under the formation of a decarboxylated Shiff base through the conjugate *N*-glycosylasparagine. Subsequent deamination or hydrolysis of the decarboxylated Schiff base results in liberation of acrylamide and ammonia (Stadler et al., 2002;Yaylayan et al., 2005;Zyzak et al., 2003).

Great global variations exit in the daily intake of acrylamide according to local eating and cooking habits. A wide range of heat-processed food products contribute to a daily intake of acrylamide. These include bread, fried potato products, potato crisps, biscuits, crisp breads and coffee (CIAA, 2011). Even though acrylamide levels in coffee are relatively low, ranging from 79-975 μg/kg with a mean value of 286 μg/kg (Seal et al., 2008), coffee may contribute to significant intake of acrylamide due to a high consumption of coffee. Especially in the Northern countries (Norway, Sweden and Denmark) where daily coffee consumption is high, coffee may contribute to up to 40% of acrylamide exposure (Friedman and Levin, 2008;Seal et al., 2008).

Commercial coffee production is mainly based on two types of coffee: Robusta coffee (from the plant *Coffea canephora*) and Arabica coffee (from the plant *Coffea arabica*). Studies have also shown differences in acrylamide level for the two types of coffee, where higher levels of acrylamide has been reported for Robusta type coffee (Alves et al., 2010; Bagdonaite et al., 2008; Guenther et al., 2007; Lantz et al., 2006; Summa et al., 2007). Commercially available coffees may be a blend of both Arabica and Robusta, and the acrylamide content in a coffee blends is therefore highly depended on the ratio between the two types of coffee used to make the blend.

Based on the present toxicity data available the Joint FAO/WHO Expert Committee on Food Additives (JECFA) recommends efforts to be made on developing and implementing methods reducing acrylamide in foods of major importance for dietary exposure (JECFA, 2010). On request from the European Commission EFSA has recently (June 2014) endorsed for public consultation on the scientific opinion on acrylamide in Food.

Concerning the reduction of acrylamide in coffee, modifying the existing roasting technologies has been investigated as possible way to reduce the acrylamide content. Acrylamide is formed exponentially during the beginning of roasting where it reaches a maximum level. Hereafter it decreases rapidly as the rate of degradation exceeds the rate of formation (Taeymans et al., 2004). Light roasted coffee does therefore contain relatively higher amounts of acrylamide than darker roasted coffee. However, storage of coffee for longer time periods is not regarded as an option to reduce acrylamide in coffee, since the quality and organoleptic properties of coffee is affected negatively during storage.

Instead of removing formed acrylamide from the roasted coffee product, reduction of the specific acrylamide precursor, asparagine, in green coffee beans has been investigated as a possible way to limit or reduce acrylamide formation. Studies have e.g. shown a weak positive correlation between the content of asparagine and the formation of acrylamide in coffee (Bagdonaite et al., 2008; Lantz et al., 2006).

A much more powerful way to manipulate asparagine levels in food raw-material is the application of an specific asparaginase during processing (CIAA, 2011; Friedman and Levin, 2008; Hendriksen et al., 2009; Kornbrust et al., 2010; Pedreschi et al., 2008; Taeymans et al., 2004; Zyzak et al., 2003). The specificity of the enzyme allows for a selective removal of asparagine without affecting any other amino acids (Kornbrust et al., 2010).

Asparaginase is an L-asparagine amidohydrolase (E.C. 3.5.1.1) that catalyzes the hydrolysis of asparagine into aspartic acid and ammonia. The asparaginase is commercially available under the product name Acrylaway® from Novozymes A/S. The asparaginase has shown to be an effective tool in reducing acrylamide formation in various food products by removing the specific precursor asparagine. Acrylaway® has e.g. proven to reduce acrylamide levels by up

to 80% in biscuits, 92% in crisp bread, 90% in ginger biscuits, 60% in French fries, and up to 60% in potato chips (Hendriksen et al., 2009).

In the present study experimental evidence is presented for the mechanism by which Acrylaway® CB L (hereafter Acrylaway) from Novozymes causes reduction in asparagine content in green coffee beans and consequently reduction in acrylamide formation in roasted coffee beans. In lab scale, the enzymatic effect of a respond to dosage of Acrylaway is demonstrated on green coffee beans. Subsequent roasting of the Acrylaway treated green coffee beans reveals the effect of Acrylaway on the final acrylamide content.

The steps in the enzymatic treatment of coffee beans (*conf.* Figure 1) can be described as follows: Initial steaming of Robusta beans, followed by enzyme treatment which can be monitored by analysis for asparagine or aspartic acid measurement, drying of the beans prior to roasting, which would be typically followed by acrylamide and sensory analysis.

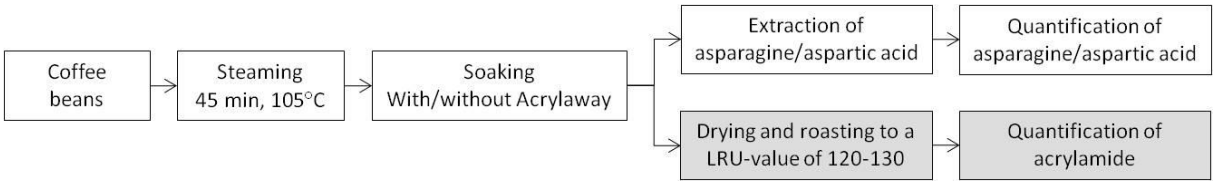

Figure 1. Schematic flow diagram for the enzymatic treatment of coffee beans. The process steps in grey boxes were only applied to the response to dosage trial.

Effect of asparaginase

The results from testing application of Acrylaway at various dosages in green coffee beans and the effect on asparagine content and subsequent acrylamide formation after roasting is shown in Figure 2. In the control sample an asparagine level of 510 mg/kg bean and an acrylamide level of 785 μg/kg bean were measured. This is in good agreement with what has previously been reported in coffee (Alves et al., 2010;Bagdonaite et al., 2008).

When treated with Acrylaway a clear effect of increasing amount of enzyme was observed with a dosage dependent reduction in asparagine from 510 mg/kg bean to 150-90 mg/kg bean and a corresponding reduction in acrylamide from 785 μg/kg bean to 335-220 μg/kg (Figure 2).

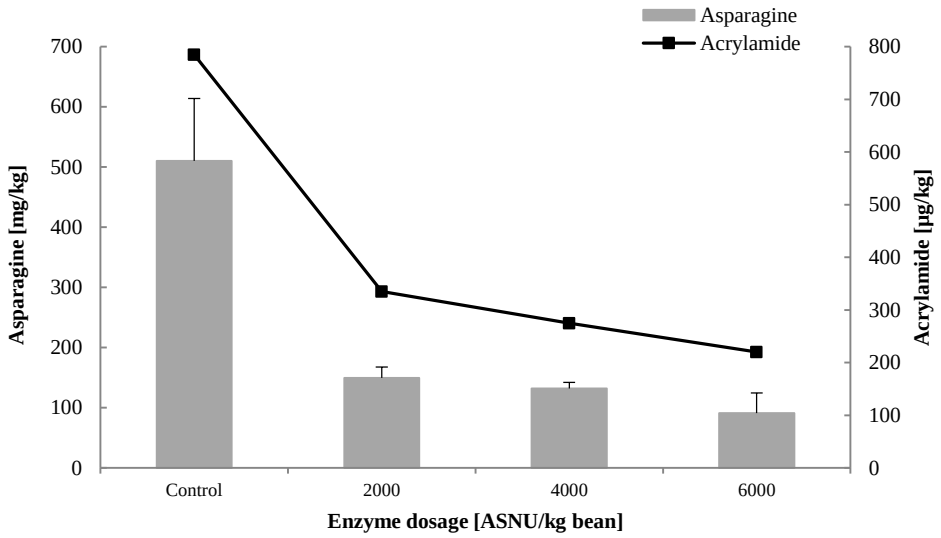

Figure 2. Asparagine content [mg/kg] in green coffee beans after incubation with Acrylaway at various dosages at 60°C for 60 minutes, and the corresponding acrylamide content [µg/kg] in the roasted coffee beans.

The largest reduction in both asparaginase and acrylamide was seen for the sample treated with the highest enzyme dosage of 6000 ASNU/kg bean. At this dosage a reduction in asparagine of 82% and a reduction in acrylamide formation of 72% were obtained.

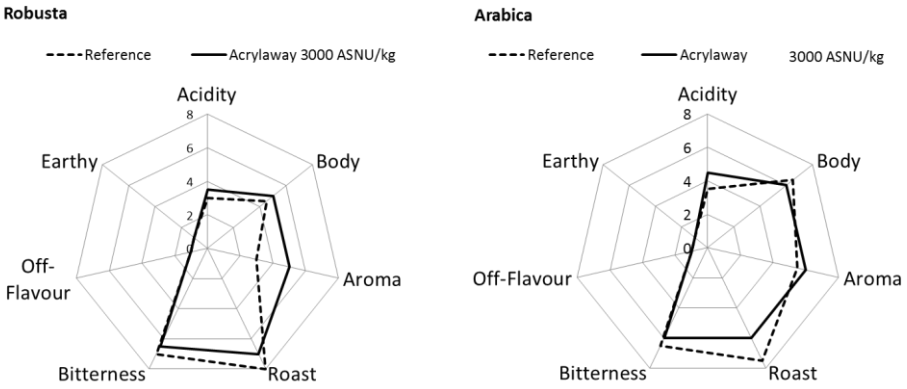

Figure 3. Sensory profile of Robusta (left) and Arabica (right) coffee beans. The green coffee beans were treated with Acrylaway® in a soaking step, the reference beans were processed normally. The asparaginase treatment has only minor impact on the sensory profile of the roasted coffee beans.

Sensory analysis and aroma compounds

In order to evaluate the effect of the application of Acrylaway® a series of sensory studies were conducted. Results of typical sensory evaluation on Vietnam robust beans and unwashed Arabica beans are shown in figure 3. The application of the enzyme requires a soaking step which may affect the quality of the final product. To evaluate this, a series of sensorical evaluations of Acrylaway® treated beans has been carried out at an external partner, results

are shown in Figure 3. In summary the sensory properties for Robusta beans and as well as Arabica beans are very similar with and without treatment with Acrylaway®. In the typical seven sensory dimensions the coffee made from enzyme treated beans does not differ more than one to two evaluation units, which indicates that the application of Acrylaway® and the necessary process changes do not change the cup quality significantly.

The present study clearly illustrates that applying an asparaginase such as Acrylaway to green coffee beans can be used to reduce asparagine content in green coffee beans and thereby also the formation of acrylamide in roasted coffee. However, success in incorporating an enzymatic hydrolysis step in the coffee manufacturing process is dependent on the introduction of a wet process step required for the enzyme to work. Additional experiments are therefore required in order to evaluate if treatment of coffee beans with Acrylaway®. The suggested process has been verified in pilot plant scale and at industrial scale. Further it is important to notice that the use of an asparaginase for Robusta beans has been introduced in the FDE Tool box in the year 2013. (http://www.fooddrinkeurope.eu/)

SUMMARY

The presented results clearly illustrate that significant reduction in the specific acrylamide precursor, asparagine, can be achieved by treating green coffee beans with the asparaginase enzyme product, Acrylaway from Novozymes A/S. The study shows, that coffee beans treated with Acrylaway at a dosage of 6000 ASNU/kg beans exhibited an 82% reduction in asparagine content which resulted in acrylamide reduction of 72% compared to untreated coffee beans. Treating coffee beans with Acrylaway is therefore a very potential strategy to minimize acrylamide formation in coffee.

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Reduction of Acrylamide Contained in Canned Coffee by Heat Treatment with Addition of Amino Acids

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SUMMARY

Acrylamide is a white crystalline solid organic molecule that is odorless with a melting point of 84 °C–85 °C. International Agency for Research on Cancer classified acrylamide into Group 2A as probably carcinogenic to humans. Acrylamide in Food and beverages is formed as a product of Maillard reaction, which involves the reaction of asparagine with reducing sugars such as glucose, galactose, and fructose at high temperatures. Although no country has set any regulations regarding the contents of acrylamide in food and beverages yet, the World Health Organization (WHO) has set a guideline value of 0.5 ppb for acrylamide in tap water. The contents of acrylamide in major food are reported to range from 170 to 3700 ppb for potato chips and crisps, 200 to 12000 ppb for French fries, 64 to 457 ppb for nuts. The contents of acrylamide in coffee brews have been reported to be in the range of 6 ppb to 66.7 ppb. Canned coffee is very popular and approximately 10 billion canned coffees are consumed each year in Japan. In 2012, Yamazaki et al. investigated the contents of acrylamide about 30 types of commercially-available canned coffee and found it ranged from 5 ppb to 14 ppb. Although the canned coffee contains acrylamide, the levels are significantly less than the levels in the processed foods mentioned above.

We investigated the residual contents of acrylamide in canned coffee after heat treatment with addition of each of 20 types amino acid. Heat treatment was carried out at 121 °C for 6 min which is the sterilization conditions during the manufacturing process of canned coffee. The quantification of acrylamide contents was performed using gas chromatography–mass spectrometry. The acrylamide-reducing effects of the addition of L-cysteine were the highest of all, and this was closely followed by the effect of the addition of L-lysine. More than 90% of the acrylamide contents in commercially-available canned coffee were removed using heat treatment with the addition of L-cysteine at 121 °C for 6 min, lowering the acrylamide contents to below the recommended safe value for tap water set by the WHO. Although residual contents of acrylamide in canned coffee after heat treatment with the addition of dithiothreitol, comprising 2 thiol groups, were decreased, the contents of acrylamide in canned coffee after heat treatment with the addition of L-cystine, comprising the structure that two cystein joined by a disulfide bond, were equivalent to levels in canned coffee that only underwent heat treatment at 121 °C for 6 min. From these results, we concluded that thiol groups, found in the amino acid cysteine, have an important role in acrylamide reduction.

INTRODUCTION

Acrylamide (AA) is a white and odorless crystalline organic molecule with a melting point of 84 - 85 °C. International Agency for Research on Cancer classifies AA into Group 2A as a probably carcinogenic compound to humans. AA contained in food and beverages is known to be formed through reactions between asparagine and reactive carbonyls such as glucose,

galactose, and fructose via Maillard reaction at temperatures above 120°C. Although the relevance to human health of dietary exposure to acryl amide is unclear, regulatory agencies such as the World Health Organization (WHO) continue to encourage food manufacturers to take measures to reduce acrylamide levels in processed foods. The contents of AA in major food are reported; 170 - 3,700 ppb for potato chips and crisps, 200 – 12,000 ppb for French fries, and 64 - 457 ppb for nuts. The contents in coffee brews have been reported to be in the range of 6 - 66.7 ppb. Nowadays canned coffee is very popular and approximately 10 billion cans are consumed each year in Japan. In 2012, Yamazaki et al. examined the contents of AA in about 30 types of commercially-available canned coffee and found that they were in the range of 5 - 14 ppb. The AA contents contained in canned coffee are significantly less than those in the processed foods mentioned above.

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) uses the Margin of Exposure (MOE) approach to evaluate the risks of acrylamide to humans. MOE is the quotient of the lowest dose of a substance causing a health issue divided by the estimated intake of that substance by the general human population. According to the JECFA's calculations, MOE values for acrylamide carcinogenicity in humans are 300 for high consumers and 75 for average consumers. These values were estimated by research on mammary tumors in rats. Generally, MOE values less than 10,000 indicate that a substance is "probably carcinogenic to humans." JECFA therefore concluded that the presence of relatively high levels of acrylamide in food is of human health concern.

On the other hand, there is a concept called the "Holistic approach". While brewed coffee does contain trace levels of acrylamide, there is no epidemiological evidence linking consumption of those levels with a risk of cancer in humans. Moreover, the health benefits of coffee (such as anticancer and antidiabetic effects) are often overlooked by public health and regulatory authorities when assessing the overall safety of a food product. We definitely need an assessment of the overall safety and benefits of the whole food product, and not just focus on individual food carcinogens in isolation. However, some evidence has been found of an association between dietary acrylamide and endometrial and ovarian cancers. In 2012, Bongers et al. found that acrylamide may increase the risk of multiple myeloma and follicular lymphoma in men. Little is currently known about adverse effects of very high intakes of acrylamide in humans. Further studies are needed to determine the effects of trace amounts of dietary acrylamide on cancer risk in humans. We are currently carrying out an investigation based on these 2 concepts.

We investigated the residual contents of acrylamide in canned coffee after heat treatment with addition of each of 20 types amino acid. Heat treatment was carried out at 121 °C for 6 min which is the sterilization conditions during the manufacturing process of canned coffee (Fig. 1).

MATERIALS AND METHODS

Materials and reagents

L-Cysteine, L-alanine, glycine, L-glutamic acid, L-glutamine, L-arginine, L-asparatic acid, L-asparagine, L-lysine, L-histidine, L-serine, L-leucine, L-isoleucine, L-valine, L-methionine, L(-)-proline, L-threonine, L-tryptophan, L-tyrosine, L-phenylalanine, dithiothreitol, L-(-)-cystine, *myo*-inositol, L(+)-ascorbic acid, β-cyclodextrin, hexane, acetone, and methanol were purchased from Nacalai Tesque (Kyoto, Japan). Catechin mixture from green tea, casein phosphopeptide, casein sodium, 1 mol/L sodium thiosulfate solution, 0.05 mol/L bromine solution, hydrobromic acid, potassium bromide, AA standard solution (1 mg/mL methanol

solution), and AA (1,2,3-$^{13}C_3$) (1 mg/mL in methanol) were purchased from Wako Pure Chemicals (Osaka, Japan). Glucose and ethyl acetate were from Kanto Chemical (Tokyo, Japan). Canned coffee and roasted barley tea beverages were purchased at local markets and stored in refrigerator at 4°C before use.

Heat treatment

One-hundred-and-ninety grams of the canned coffee and various amounts of the additives were put into a 200-mL flask and mixed at room temperature. The mixture was placed into a new stainless steel can. A stainless steel lid was placed on the can containing the mixture and was sealed using a seamer. Next, the can was treated by a retort sterilization apparatus at 121°C for 6 min. This process is commonly undertaken in manufacturing canned coffee. The heat-treated cans were immediately cooled in an ice water bath and stored in a refrigerator at 4°C until further use.

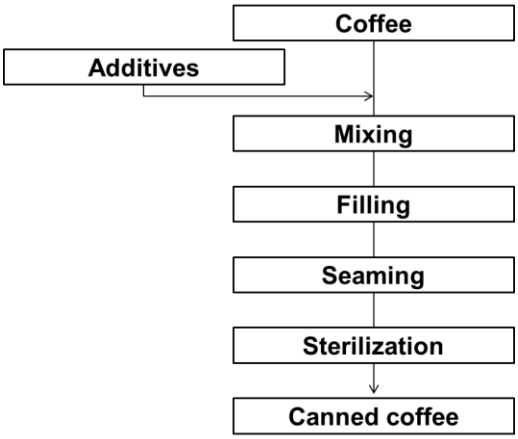

Figure 1. Typical manufacturing process of canned coffee.

Analysis of AA in samples.

The AA content was analyzed according to the procedures previously reported with slight modifications. The quantification of AA in the brominated samples was performed using gas chromatography–mass spectrometry (GCMS-QP2010, Shimadzu, Kyoto, Japan) equipped with a split–splitless injector and a Zebron ZB-1 capillary column (30 m × 0.32 mm i. d., 1.0 μm film thickness) (Phenomenex).

RESULTS AND DISCUSSION

Influence of heat treatment with the addition of additives on acrylamide levels in black canned coffee

AA has been classified by the International Agency for Research on Cancer as a Group 2A carcinogen for humans. It has been detected in a variety of heat-processed foods such as potato crisps, French fries, bread, roasted green tea, black tea, oolong tea, biscuits, cereals, wafers, baby food, and hamburgers. AA that occurs in food and beverages are formed primarily as a product of the Maillard reaction, a process whereby asparagine reacts with reducing sugars such as glucose, galactose, and fructose at high temperatures. The AA content in the commercially-available canned black coffee investigated in this study was 6.2 ± 0.2 ppb (Table 1). Previous studies on approximately 30 types of canned coffee in Japan have shown

results for acrylamide content in the range of 5 to 14 ppb, and our results were within this same range.

When 200 mg of each amino acid, including 18 types of amino acids except Lys and Cys, was added to 190 g of canned black coffee and the mixture was heat treated at 121 °C for 6 min, the residual ratios of acrylamide in the canned coffee was reduced by 97%–104% (Table 1). The AA content in the canned coffee after heat treatment with the addition of 200 mg of Lys at 121 °C for 6 min decreased by approximately 10%, and was 5.6 ± 0.1 ppb (Table 1). In addition, when 200 mg of Cys was used, acrylamide content decreased by approximately 90%, and was 0.7 ± 0.0 ppb (Table 1). The acrylamide-reducing effects using heat treatment with the addition of Cys and Lys at 121 °C for 6 min were similar to the effects found in a previous study on olive oil juice following heat treatment with the addition of the same amino acids at 121 °C for 30 min.

Residual amounts of acrylamide in 190 g of canned black coffee after heat treatment and addition of 200 mg of dithiothreitol consisting of 2 thiol group amino acids at 121 °C for 6 min were 2.8 ± 0.2 ppb, representing 55 ± 3% of the initial amount of acrylamide (Table 2). Residual amounts of acrylamide in 190 g of canned black coffee after heat treatment and addition of 200 mg of L-cysteine comprising the structure of two Cys amino acids combined by a disulfide bond at 121 °C for 6 min were also equivalent to the coffee that only underwent heat treatment. From these results, we can conclude that thiol groups have an important role in the reduction of acrylamide by Cys or dithiothreitol.

Table 1. The AA content in the commercially-available canned black coffee after heat treatment at 121°C for 6 min with the addition of 200 mg of amino acid to 190 g of the canned coffee.

Sample	Acrylamide (ppm)
Canned Black Coffee	6.2 ± 0.2
+ Gly	6.3 ± 0.1
+ Ala	6.1 ± 0.1
+ Ser	6.1 ± 0.1
+ Pro	6.3 ± 0.1
+ Val	6.2 ± 0.1
+ Thr	6.1 ± 0.1
+ Cys	0.7 ± 0.0
+ Leu	6.2 ± 0.1
+ Ile	6.2 ± 0.1
+ Asn	6.4 ± 0.1
+ Asp	6.0 ± 0.1
+ Gln	6.4 ± 0.2
+ Lys	5.6 ± 0.1
+ Glu	6.0 ± 0.1
+ Met	6.2 ± 0.1
+ His	6.0 ± 0.1
+ Phe	6.0 ± 0.2
+ Arg	6.0 ± 0.1
+ Tyr	6.4 ± 0.2
+ Trp	6.4 ± 0.2

Table 2. The AA content in the commercially-available canned black coffee after heat treatment at 121°C for 6 min with the addition of 200 mg of each compound to 190 g of the canned coffee.

Sample	Acrylamide (ppm)
Canned Black Coffee	6.2 ± 0.2
+ Dithiothreitol	2.8 ± 0.2
+ Casein sodium	6.1 ± 0.1
+ Casein phosphopeptide	6.0 ± 0.0
+ Cystine	6.0 ± 0.1
+ Glucose	6.3 ± 0.0
+ *myo*-Inositol	6.1 ± 0.1
+ β-Cyclodextrin	6.0 ± 0.1

Table 3. The AA content in the commercially-available canned black coffee after heat treatment at 121°C for 6 min with the addition of Cys, Lys, and Arg to 190 g of the canned coffee.

Sample	Residual amounts (%)
Canned Black Coffee	100 ± 0
+ Cys 25 mg	89 ± 3
+ Cys 50 mg	59 ± 1
+ Cys 100 mg	35 ± 2
+ Cys 200 mg	11 ± 1
+ Cys 300 mg	4 ± 2
+ Lys 50 mg	100 ± 0
+ Lys 100 mg	98 ± 2
+ Lys 200 mg	90 ± 3
+ Lys300 mg	83 ± 1
+ Arg 50 mg	100 ± 1
+ Arg 100 mg	100 ± 0
+ Arg 200 mg	97 ± 2
+ Arg 300 mg	95 ± 2

Concentration dependence of Cys, Lys, and Arg on acrylamide-reducing effects in canned black coffee

Table 3 shows the acrylamide-reducing effects of heat treatment with the addition of Cys, Lys, and Arg at 121 °C for 6 min in commercially-available canned black coffee. The residual amounts of acrylamide in the treated canned coffee decreased with increases in the concentration of all of these amino acids (Table 3). The additive amounts of Cys required to reduce acrylamide content by 50% in 190 g of canned coffee were calculated to be 69.3 ± 1.9 mg from Table 3. The residual amounts of acrylamide in 190 g of canned coffee after heat treatment with the addition of 200 mg or 300 mg of Cys at 121 °C for 6 min decreased to 0.7 ± 0.0 ppb and 0.3 ± 0.1 ppb, respectively. Although no countries have placed regulations on the content of acrylamide in food and beverages, the maximum safe level of acrylamide in tap water has been set to 0.5 ppb by the WHO. It has been suggested that the acrylamide content in canned black coffee can be reduced to below this value set by the WHO using heat treatment at 121 °C for 6 min with the addition of 200–300 mg of Cys to each 190 g of canned coffee. Similarly, residual amounts of acrylamide following heat treatment and addition of 200 mg or 300 mg of Lys decreased to 5.6 ± 0.2 ppb or 5.2 ± 0.1 ppb, respectively

(Table 3). Results were similar following heat treatment with the addition of 200 mg or 300 mg of Arg, with decreases of 6.0 ± 0.1 ppb and 5.9 ± 0.1 ppb, respectively (Table 3).

Our method can be implemented using normal manufacturing processes, and provides an economical benefit to manufacturers. The only cost associated with our method is the raw material cost of Cys and the heat-treatment time is short. However, there are subjects to be investigated further. It is considered that AA might be decreased in the heat-treatment with addition of Cys by converting to cystein-*S*-β-propionamide and others which Cys and AA combined by the Michael addition reaction. It is necessary to verify about regeneration of AA in the heat-treated canned coffee by our method. In particular, verification of AA regeneration in long-term storage at low temperature (4–25°C) is important, because canned coffee is usually drank as refrigerated goods and stored at refrigerated or room temperature. In addition, it is needed to evaluate the toxicity of these products.

CONCLUSION

Coffee is a popular beverage and is known to contain a small amount of AA. In Japan, canned coffee is popular and approximately 10 billion canned coffees are consumed each year. In this study, we investigated how todecrease the AA content of canned coffee using heat-treatment for sterilization during the manufacturing process. Approximately 95% of AA in both types of canned coffee (black and milk) was removed by the treatment with the addition of Cys at 121°C for 6 min. This method is shown to be superior to previous methods for decreasing AA in canned coffee. However, further verification such as preservation test and toxicity assay is need for practical application of our method to the industrial manufacturing of canned coffee.

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Application of Photo-Ionization Time-of-Flight Mass Spectrometry for the Studying of Flavour Compound Formation in Coffee Roasting of Bulk Quantities and Single Beans

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SUMMARY

This work addresses the online monitoring of organic flavour compounds evolving during coffee roasting of Arabica and Robusta coffee beans, respectively. Both bulk roasting with a laboratory roaster and roasting of single coffee beans were investigated. Detection of the organic species was carried out be photo-ionization time-of-flight mass spectrometry. Different molecular signatures for both coffee types were observed. Arabica contains higher molecular weight lipids in higher concentrations, but less caffeine and phenolic compounds. Statistical analysis of the data revealed influences of roasting conditions on the chemical pattern enabling an online determination of roasting degrees. The observed chemistry in single bean experiments reflects bulk roasting processes well, thus both approaches may be combined to improve the understanding of the chemical mechanisms during coffee roasting.

INTRODUCTION

Coffee roasting is an extremely complex chemical and physical process leading to the formation of a large number of (semi)volatile organic aroma compounds. However, the dynamic and mechanisms of these formation processes are not completely understood. Specific characteristics of the roast process such as the actual roast degree are often determined empirically and based on individual experience. On-line real-time analytical measurements would be best suited to monitor the roasting process, and there are some examples in the literature utilizing chromatographic and spectroscopic methods or even sensor arrays (electronic noses). Direct mass spectrometry with soft ionization methods is another promising approach and has been applied to coffee roasting in the form of proton-transfer-reaction mass spectrometry (PTR-MS) and photo-ionization time-of-flight mass spectrometry (PI-TOFMS). In the first part of this study, the latter technique is used to monitor coffee roasting off-gases generated by a laboratory roaster under different roasting conditions in real time.

In the second part, PI-TOFMS was employed to investigate more fundamental questions to examine chemistry and kinetics of (S)VOC formation in individual single coffee beans. This approach takes into account that the integrity of individual beans plays a decisive role for the

formation of coffee flavour. Both experiments together try to link results on the single bean level with bulk-roasting phenomena and to bridge the processes happening in an individual bean to organic compound formation behaviour in roasting processes of large bean ensembles.

MATERIALS AND METHODS

On-line monitoring of coffee roasting

A small laboratory coffee roaster (Probat PRE 1Z, Emmerich, Germany) allowing batch filling up to 100 g was used for the roasting of coffee beans. The beans were roasted at 200 °C with three different heating rates, representing slow, normal and fast roasting, respectively. The start weight was adjusted to 100 g green coffee beans. Two sorts of beans were investigated, viz. coffea Arabica and coffea canephora (Robusta). For each parameter set, three or four replicates were performed.

Sampling of the evolving (semi)volatile organic compounds was carried out via a quartz tube reaching into the interior of the roaster and a deactivated quartz capillary (200 µm i.d.) running in a heated transfer tube (up to 300 °C) to avoid cold spots. The capillary ended in a heated stainless steel needle, which tip was located inside the ion source of the time-of-flight mass spectrometer, forming an effusive molecular beam. Photo-ionization of the molecules was performed by two different methods. On the one hand, resonance-enhanced multiphoton ionization (REMPI) employing the fourth-harmonic of a Nd:YAG laser (266 nm) served as a selective and sensitive method for the ionization of (poly)aromatic species. Single photon ionization (SPI) utilizing photons of a wavelength of 118 nm generated by frequency tripling of an initial 355 nm laser beam (third harmonic of Nd:YAG) in a xenon gas cell enables a more universal ionization scheme for various organic substance classes dependent on their respective ionization energies, which had to be lower than 10.5 eV. Ions are separated by a time-of-flight mass analyzer (Kaesdorf, Munich, Germany) and signals are processed by Acqiris digital cards and home-written software based on Labview and Matlab. Ten single mass spectra were recorded every second and averaged, yielding a complete mass spectrum every second.

Single bean experiments

Single Arabica and Robusta beans were subjected to a simulated roasting process in a hot air stream of 250 °C. Sampling of the evolving gases was carried out by a µ-probe setup. The µ-probe consists of a conically shaped heated aluminium base body, which is coupled via a heated adapter to the transfer line. Inside the probe, a stainless steel capillary (200 µm i.d.) is connected to the transfer capillary with a capillary union. The stainless steel capillary sticks out of the other side of the conically shaped base body by a length of 4 mm and is heated to 120 °C by conductance. A 5 mm deep hole with a diameter of 1 mm was drilled into the coffee beans and the tip of the steel capillary was inserted. The hole was sealed by inorganic glue based on zirconium oxide. The remaining transfer line up to the mass spectrometer used the same setup as described for the roasting experiments.

RESULTS AND DISCUSSION

Roasting experiments

Figure 1 shows typical SPI-TOFMS spectra of the roast gas composition of Robusta (above) and Arabica (below) coffee, respectively. The spectra are derived from averaging the

consecutive single spectra of a roasting cycle and are presented in both linear and logarithmic scale.

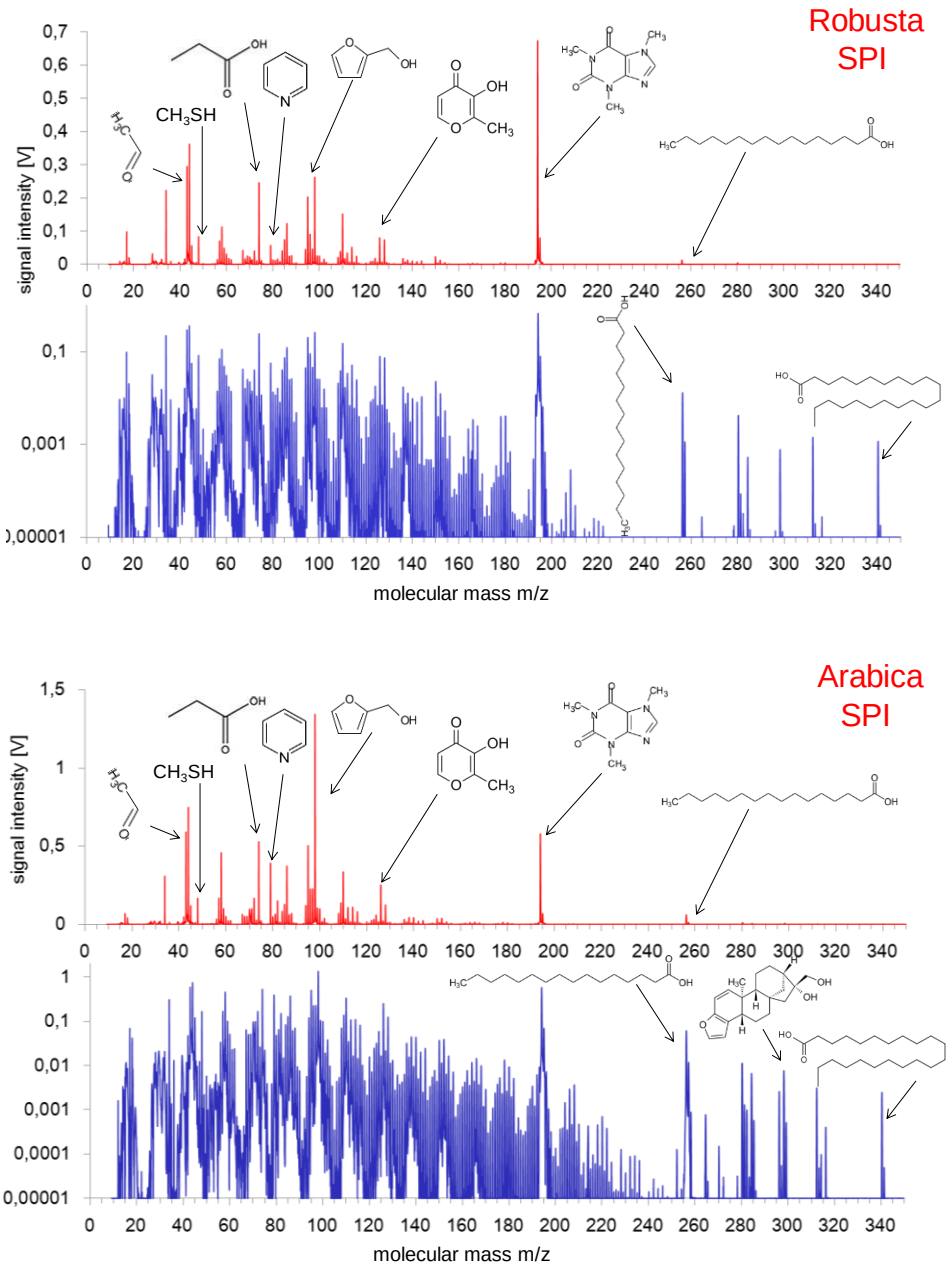

Figure 1. Averaged SPI-TOFMS spectra of roast gas of Robusta coffee beans (above) and Arabica coffee beans (below). The spectra are presented in linear and logarithmic scale.

The basic pattern of the roast gas composition monitored by SPI-TOFMS looks similar in both cases. The main species detected with both coffee sorts are oxygen containing compounds such as acetaldehyde (44 m/z), acetone/propanal 58 (m/z), propionic acid (74 m/z), furfuryl alcohol 98 (m/z), and maltol (126 m/z). Caffeine at 194 m/z is a pre-eminent signal with both bean sorts as well. With methanthiole at 48 m/z a sulfur containing compound is detected. Finally, the occurrence of pyridine (79 m/z) is worth mentioning, since this component is a well-known indicator for over-roasting conditions.

However, if one takes a closer look especially at the logarithmic scaled spectra, certain differences become obvious. First of all, the signal intensity and thus the concentration of caffeine are significantly higher in the roast gas of Robusta coffee. On the other hand, the low concentrated lipid components such as fatty acids show a more complex pattern with Arabica coffee. As a consequence, SPI-TOFMS spectra allow for a clear distinction between the two coffee cultivars.

The main asset of photo-ionization mass spectrometry consists in its capability to monitor the roasting process with a high time resolution. Figure 2 shows typical time traces of selected compounds in the roast gas of Robusta (above) and Arabica (below), both recorded with SPI-TOFMS. During the roast process two cracking events were clearly audible, the points of which are indicated in the graphs by vertical lines. From the various temporal trends of the depicted compounds it becomes obvious that significant changes in concentration are associated with these cracking events. Species such as pyridine and furfuryl alcohol begin to show visible concentration levels only after the second event, other compounds such as H_2S or maltol start to emerge with the first event, and some compounds such as caffeine show distinct changes with the events. This indicated a large influence of the respective coffee bean structures on the emission profiles of the roast gases.

Generally it is interesting to observe that most species show a distinct temporal behaviour during the roasting process. For example caffeine is visible relatively early and its concentration increases throughout the process with the mentioned steeper increases at the time of the cracking events. Other compounds emerge quite late with a strong increase in concentration. Fatty acids pass through a maximum while roasting progresses, which seems to be a unique behaviour. For most of the species, the trends with time show no big differences when both coffee sorts are compared. One striking exception is kahweol, which emerges prominently after the second cracking event with Arabica beans, yet is totally absent with Robusta beans. This provides a further distinguishing feature between the two coffee types.

Multivariate statistics in form of principal component analysis (PCA) using the recorded mass spectrometric data further reveals connectivity and differences especially when one aims at a comparison of the varying roasting speeds. Figure 3 shows a score plot derived from the chemical profile SPI-TOFMS data of five different experiments. Valuable information derived from the plot is the reasonable repeatability of the experiments, since the replicate measurements cluster together accordingly. Principal component 1, which accounts for 51 % of the variance of the data set, separates the experiments with Robusta and Arabica, respectively. Principal component 2, which also represents a considerable part of variance with 38 %, roughly reflects the separation due to the roasting speed. This indicates that SPI-TOFMS monitoring of coffee roasting is capable of distinguishing between different coffee types and variations in roasting parameters at the same time. This concept can be easily expanded to the variation of other parameters such as pressure, moisture, chemical additives, and bean pre-processing.

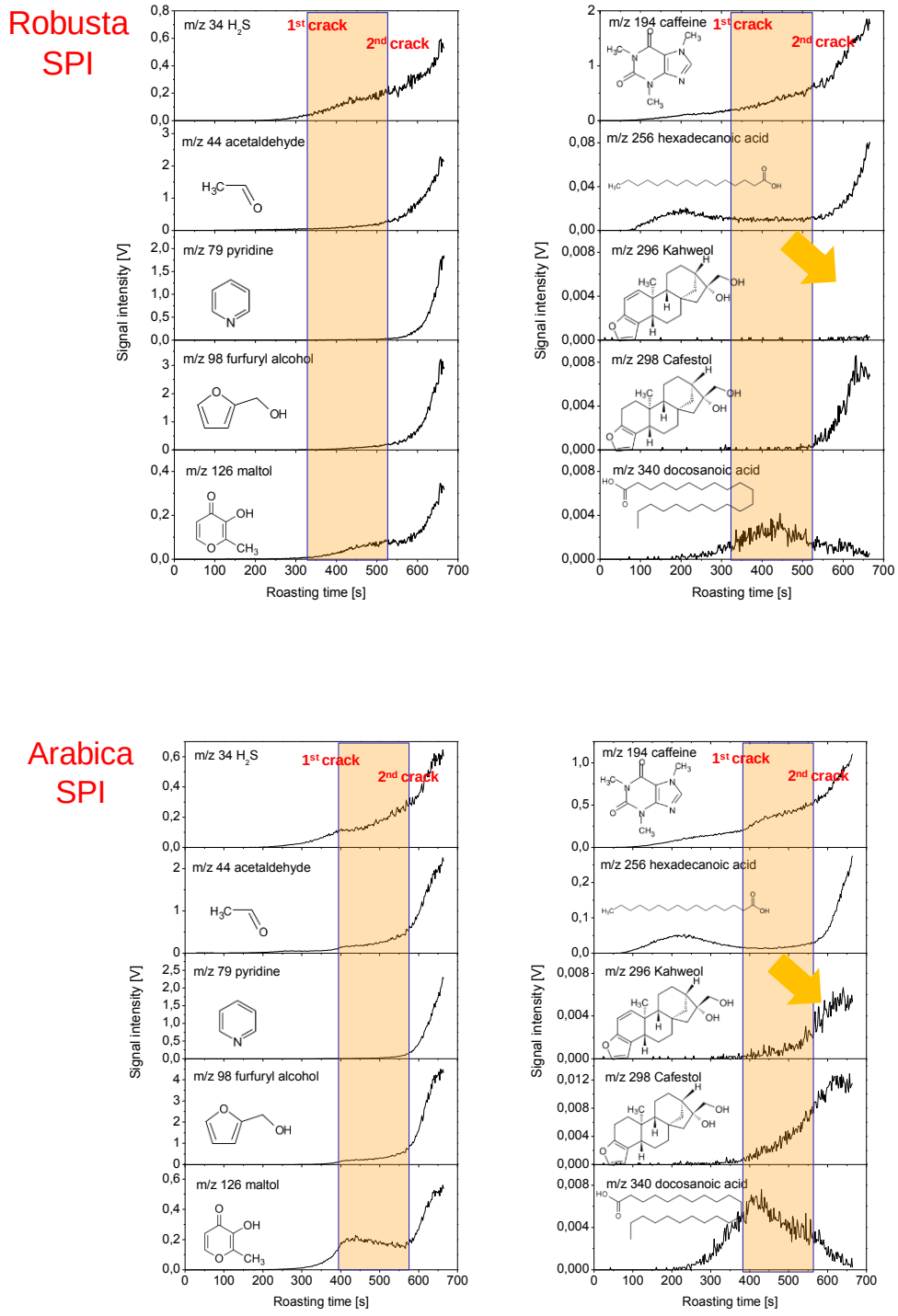

Figure 2. SPI-TOFMS monitoring of selected species contained in the roast gas of Robusta coffee beans (above) and Arabica coffee beans (below) showing the changes in concentration with the progress of the roasting process. The vertical lines depict the points of audible cracking events.

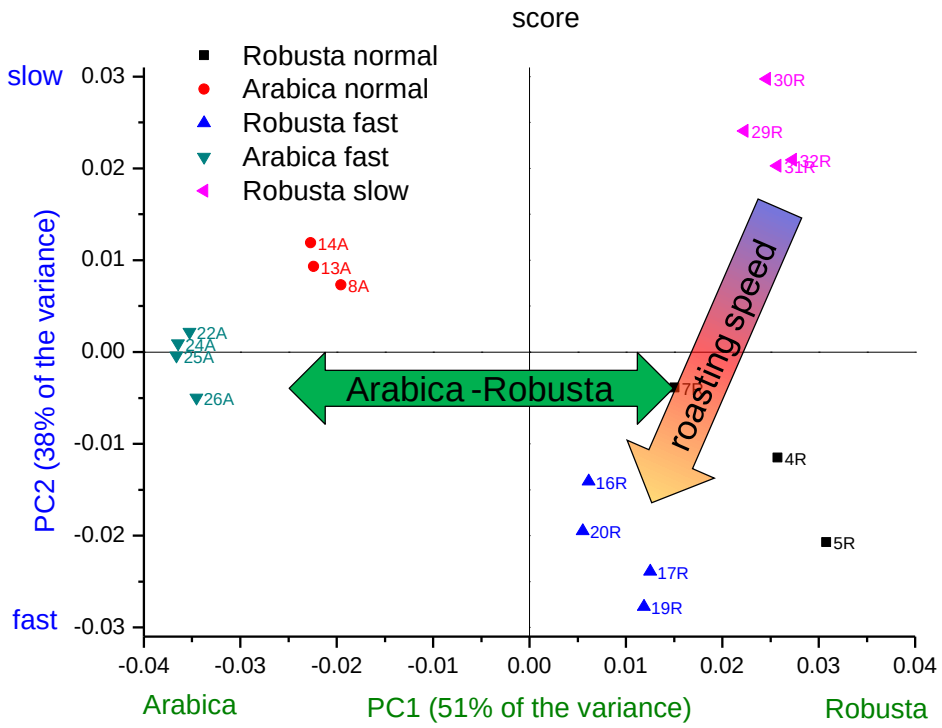

Figure 3. Score plot of the principal component analysis (PCA) of SPI-TOFMS chemical profile data of five different roasting experiments, each conducted with several replicates. A and R indicates Arabica and Robusta beans, respectively. Each number represents a single experiment.

Single bean experiments

Figure 4 depicts results from the µ-probe sampling within single coffee beans of Arabica and Robusta, respectively, during simulated roasting in a hot air stream. The mass spectra were recorded using REMPI-TOFMS. The three-dimensional plots on the left hand side of the figure give a general overview of the whole experiment, depicting signal intensities of the measured species' concentration as a function of time. On the right hand side mass spectra averaged over the whole duration of the experiment are shown in both linear and logarithmic scales. The selectivity of REMPI emphasizes the phenolic species within the evolved gases, which are mainly derived from aromatic coffee-oil components such as chlorogenic acid. Dominating signals belong to 4-vinylguiacol (150 m/z) and caffeine. The most striking difference between Arabica and Robusta is the occurrence of signals above 200 m/z with Arabica beans, which are almost totally absent with Robusta beans. This is especially visible in the logarithmic plot. Since Robusta coffee contains more chlorogenic acid than Arabica, the concentrations of phenolic compounds are higher in the off-gases released by Robusta beans. Indole (117 m/z), formed by degradation of phenylalanine, shows stronger signals with Robusta beans as well. The higher caffeine content of Robusta observed in the roasting experiments is reflected here as well. On the other hand, Arabica beans show higher concentrations of C3-alkylamines (59 m/z), which are formed from pyrolysis of amino acids, contained in higher amounts in Arabica beans.

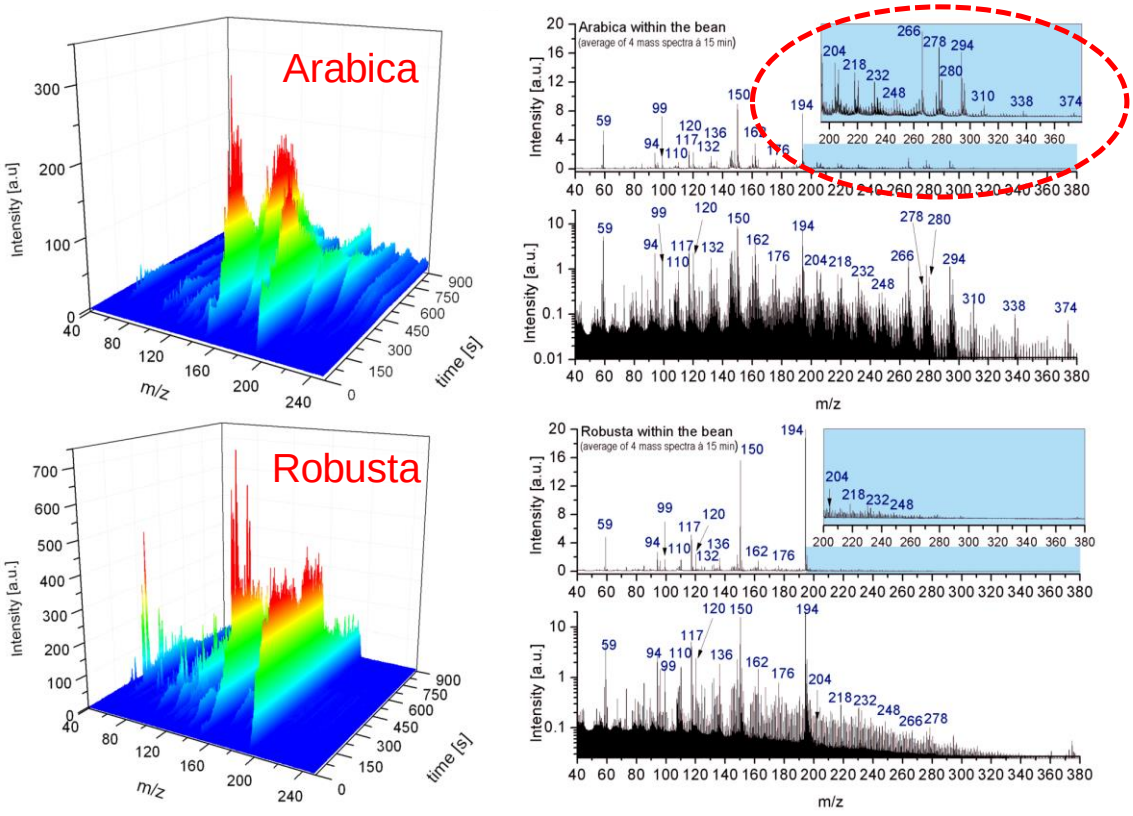

Figure 4. Score plot of the principal component analysis (PCA) of SPI-TOFMS chemical profile data of five different roasting experiment, each conducted with several replicates. A and R indicates Arabica and Robusta beans, respectively, each number represents a single experiment (revised from [6]).

Figure 5 depicts time-intensity graphs of selected phenolic species monitored in the off-gas of a single Robusta bean subjected to simulated roasting. These compounds are well known intermediate products of the thermal decomposition of chlorogenic and ferulic acid. The first step of this reaction scheme is decarboxylation leading to vinylcatechol and vinylguaicol, respectively. Vinylcatechol is then subsequently reacting to ethylcatechol and catechol, while vinlyguiacol is further decomposed to vanillin, guiacol, and phenol. This sequence of reactions is well reflected in the temporal trends of the monitored species. Vinylcatechol and vinylguiacol emerge first in the off-gas, showing a relatively steep increase and reaching their maximum concentration early (in the case of vinylcatechol after 105 s). The other compounds are rising delayed and reach their maximum significantly deferred at about 220 s or in the case of guiacol even later. Concentration of phenol increases steadily throughout the experiment, seemingly reaching its maximum at the end of the process.

The monitoring of this behaviour in single bean experiments, which is known to occur in real roasting processes as well, suggests the feasibility of single bean experiments to reflect bulk roasting processes. Hence, results from both experiments may be combined for a better understanding of the mechanisms leading to the formation of flavour compounds in coffee roasting and the influence of varying parameters on these mechanisms. The conclusion from such experiments can then be translated to industrial roasting devices.

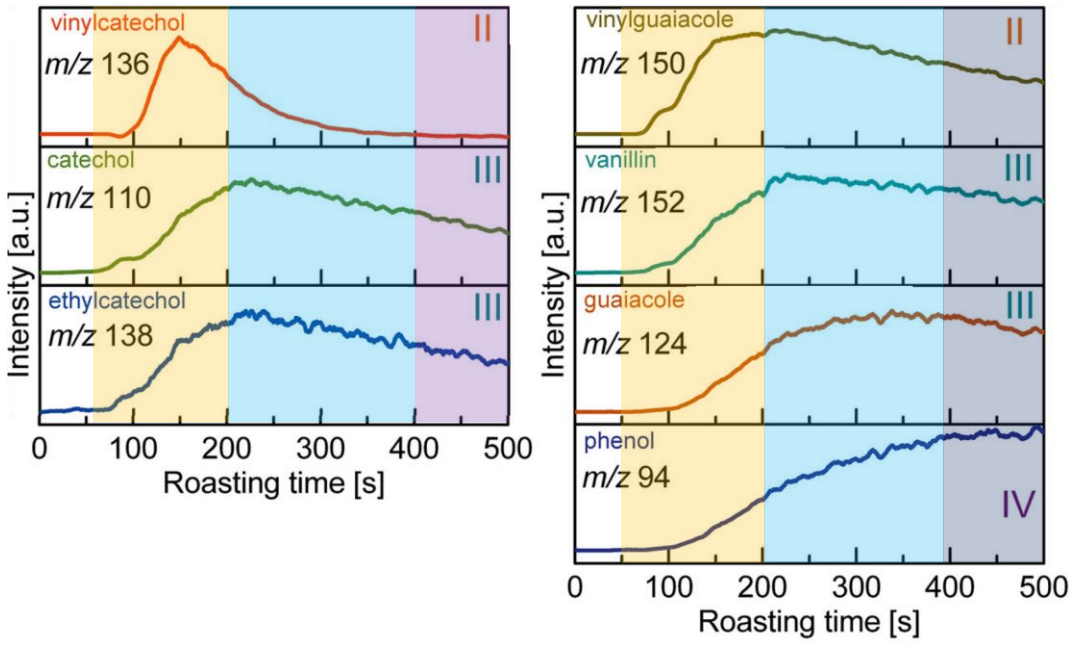

Figure 5. Concentration profiles of selected compounds as a function of roasting time from a single Robusta bean experiment monitored by REMPI-TOFMS. The roman numerals indicate products of subsequent reaction steps (revised from [6]).

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Extraction of Espresso Coffee: Time-Resolved Analysis of the Extraction Kinetics of VOCs by PTR-ToF-MS

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SUMMARY

The extraction dynamic of 95 ion traces in real time (time resolution: 1 second) was investigated during espresso coffee preparation, using proton-transfer-reaction time-of-flight mass-spectrometry (PTR-ToF-MS). Fifty-two of these ions were tentatively identified. This was achieved by on-line sampling of the volatile organic compounds (VOCs) in close vicinity to the coffee flow, at the exit of the extraction hose of the espresso machine (single serve capsules). Ten replicates of six different single serve coffee types were analyzed. The results revealed considerable differences in the extraction kinetics between compounds, which led to a fast evolution of the volatile profiles in the extract flow and consequently to an evolution of the final aroma balance in the cup. Besides exploring the time-resolved extraction dynamics of VOCs, the dynamic data also allowed the coffees types (capsules) to be distinguished from one another. Both Hierarchical Cluster Analysis (HCA) and Principal Component Analysis (PCA) showed full separation between the coffees types. The methodology developed provides a fast and simple means of studying the extraction dynamics of VOCs and differentiating between different coffee capsules.

INTRODUCTION

Progress in the on-line analysis of VOCs has progressively transformed aroma analysis and process monitoring in coffee research. Nearly 20 years ago Chahan Yeretzian and coworkers started introducing PTR-MS (in collaboration with Werner Lindinger) and REMPI-ToF-MS[4] (in collaboration with Ralf Zimmermann) into coffee research. Today, PTR-MS is an established technology in this field, and REMPI-ToF-MS will most probably soon be established as well. While the more traditional GC/MS is highly suitable for identifying and quantifying flavor-active compounds, it performs less well when it comes to monitoring the temporal evolution of fast dynamic processes and needs to be complemented with other analytical techniques when processes such as flavor generation, in vivo release or process monitoring are explored.

Here, we focus on the analysis of the dynamic extraction of an espresso coffee using PTR-ToF-MS. The extraction technique and conditions used for coffee preparation strongly influence the flavor profile in the cup and is often the only parameter that can be influenced by the consumer at home. Several studies have investigated how the extraction of flavor compounds is affected by the brewing technique, temperature, pressure, water composition and cup length. In all of these studies, measurements were carried out on the final extract, but there is a lack of information on how the above mentioned parameters affect the kinetics of

extraction. Few quantitative studies have been published to date on the time-resolved extraction of volatile coffee compounds. By using different volumes of water in the extraction process or taking fractions over the whole extraction time/volume, some authors have published findings on the extraction dynamics of acrylamide, caffeine and antioxidants. To the best of our knowledge, only the recently published work by Mestdagh et al. has reported data on the kinetics of extraction for selected aroma compounds, using solid phase micro extraction (SPME)-GC/MS.

The approach taken here examines whether it is possible to measure VOC release from the coffee flow at the exit of the extraction hose using PTR-ToF-MS. We make the assumption that each compound in the liquid extract is partitioned in the gas phase, so that the gas phase concentration of VOCs at the exit of the hose is proportional to the liquid concentration, with the Henry's Law Constant (HLC) being the proportionality constant. Hence the time-evolution in the gas-phase mimics the extract concentration. An analytical approach that is based on on-line sampling of the volatiles released from the coffee flow was developed and tested for real-time monitoring of the extraction of volatile aroma compounds from single serve coffee capsules.

MATERIALS AND METHODS

Coffee

Six commercial Delizio coffee capsule types (Delica, Birsfelden, Switzerland) were selected: Ristretto Forte (RF), Espresso Intenso (EI), Espresso Alba (EA), Espresso Classico (EC), Lungo Fortissimo (LF), and Lungo Crema (LC). All capsules of a given type were from the same production batch and were characterized according to (i) total weight in the capsule and (ii) roasting degree, measured with a Colorette 3B instrument (Probat, Emmerich am Rhein, Germany), as summarized in Table 1.

Coffee preparation

Ten different capsules of each coffee type were extracted using a Delizio Compact Automatic coffee machine (Delica, Birsfelden, Switzerland). These were operated according to the factory settings to pump three different volumes of water in an unrestricted mode (no capsule in the brewing unit): 40 mL for Ristretto, 72 mL for Espresso and 131 mL for Lungo. Depending on the coffee inside each capsule type, the actual weight of the extract in the cup (final column in Table 1) showed significant variations, but was very stable for repetitions of the same type. The total time for extraction of the cup and its final weight were measured (Table1). Note that the expression "espresso" can have two meanings. Either it describes the general fact that coffee was prepared using a pressurized brewing/extraction process and may refer to different extracted volumes (Ristretto, Espresso and Lungo), or it designates the volume of the extract (here: ~ 50 mL) - the context clarifies the meaning. Just before extraction of each capsule, 110 mL of water was passed through the circuit to remove possible residues from the previous extraction and to preheat the circuit. Both for cleaning and extraction, tap water was mixed with filtered water (PURITY 600 Quell ST, BRITA Professional, Taunusstein, Germany) to adjust the extraction water to 6° ± 1° dH (German water hardness).

Table 1. Characterization of the coffee capsules.

Capsule type	Powder weight /g	Roast degree /Pt	Extraction time /s	Extracted weight /g
RF	**6.01 ± 0.17**	**69**	**14.2 ± 0.6**	**20.06 ± 1.37**
EA	**5.95 ± 0.11**	**77**	**28.9 ± 0.8**	**47.21 ± 1.57**
EC	**6.24 ± 0.09**	**88**	**22.0 ± 0.0**	**62.10 ± 1.05**
EI	**6.00 ± 0.15**	**67**	**23.5 ± 0.8**	**49.81 ± 1.40**
LC	**6.30 ± 0.02**	**98**	**41.1 ± 0.5**	**111.64 ± 2.81**
LF	**6.04 ± 0.07**	**73**	**42.0 ± 0.4**	**117.80 ± 2.27**

Sampling set up

VOCs released from the coffee flow were measured with the set up shown in figure 1. Coffee was extracted over an ice-cold water bath to ensure that interference of volatiles from the collected extract was eliminated. The sampling lance was positioned 0.5 cm from the coffee flow and coupled to the inlet of the PTR-ToF-MS. Using a custom built gas dilution system, adapted from Wellinger et al., the sampled VOCs were diluted 7.5 fold to avoid condensation of VOCs on the tubing and to adjust their concentration to within the dynamic working range of the mass spectrometer. The dilution gas was dry compressed air containing 2-isobutyl-3-methylpyrazine as a standard for mass range calibration. All the sampling and dilution lines were heated to 90 °C and all flows were controlled by mass flow controllers (Bronkhorst, Ruurlo, The Netherlands) and verified using a bubble meter.

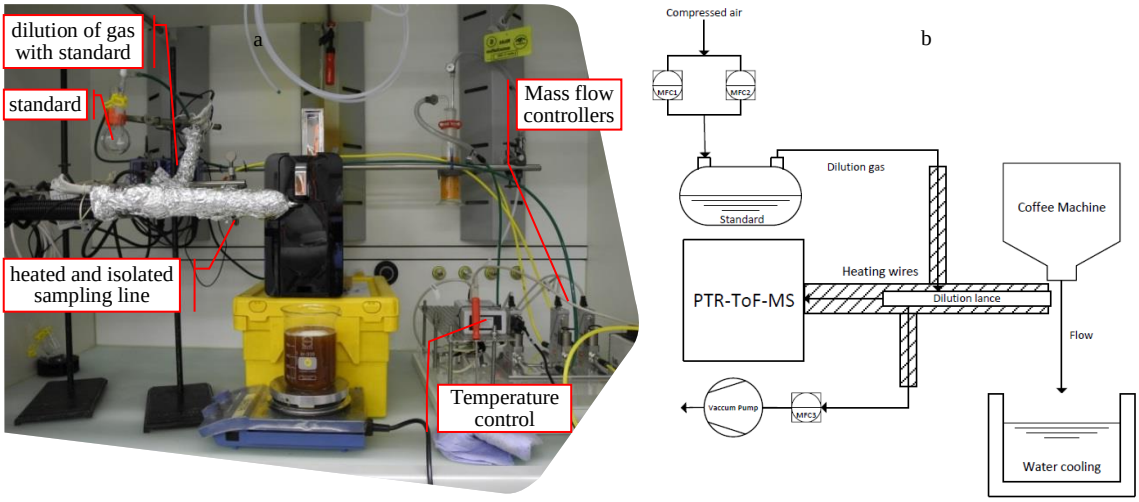

Figure 1. Set up for sampling VOCs from the coffee flow. Volatiles were introduced into the dilution lancet by a flow created with a vacuum pump and were then diluted 7.5 fold using dried compressed air containing a standard for mass calibration.

PTR-ToF-MS

A commercial PTR-ToF-MS 8000 instrument (Ionicon Analytik GmbH, Innsbruck, Austria) was used. The diluted sample was introduced with a flow of 200 sccm into the drift tube, which was operated at 2.2 mbar, 70 °C and 600 V drift voltage. PTR-ToF-MS data were recorded by TOFDAQ v.183 data acquisition software (Tofwerk AG, Thun, Switzerland). Mass spectra were recorded in the mass-to-charge (*m/z*) range of 0-205 with one mass-spectrum recorded per second. Mass axis calibration was performed on $[H_3{}^{18}O]^+$, $[C_3H_7O]^+$ and $[C_8{}^{13}CH_{15}N_2]^+$.

Data Processing

A PTR-TOF DATA Analyzer software v4.1736 was used for data analysis. Duty cycle corrected signals were normalized to 10^6 H_3O^+ primary ions. During extraction, fluctuations in the flow (ml/sec) and the foam (different bubble sizes) were observed. To correct for small differences in the absolute intensity and allow for a better comparison between capsules, the intensity of the VOCs was normalized to the maximum intensity of the m/z 69.035 ion trace, before averaging for replicates.

Mass peaks selection

Ten replicates for each of the six coffee capsule types (RI, EA, EC, EI, LC and LF) were analyzed with the set up. Around 300 mass peaks were found in the *m/z* range recorded, although the exact number was dependent on the capsule type. Only peaks that changed over time and that were present in all samples were included in the subsequent data analysis, yielding a list of 95 ion traces. Out of these, 52 were tentatively identified, based on the literature and were reduced to 47 after removing fragments and isotopologues[37-39].

Statistical analysis

The area under the time-intensity profiles, from t = 0 s to the end of the extraction (depends on capsule type) were calculated/integrated, and normalized/divided by the amount (in grams) of the extracted coffee. The set of 60 samples (10 replicates of 6 different capsule types) with 95 different variables (one for each VOC) were subsequently subjected to statistical analysis. Hierarchical Cluster Analysis (HCA) was performed by Ward's minimum variance method using half-squared Euclidean distances. Principal Component Analysis (PCA) was performed on mean-centered scaled data. All analysis and graphs were performed with packages and scripts in R (R foundation for statistical computing, Vienna, Austria).

RESULTS AND DISCUSSION

The time-intensity profiles show different extraction dynamics for the VOCs analyzed (Fig.2A). The time at which the maximum intensity was reached ranged from 2 to 24 seconds, although for 95 % of the compounds it was reached in less than 10 seconds. Once the maximum had been achieved, the intensity fell at different rates, depending on the compound. This decrease of intensity provides information on how the compounds are extracted. A fast decrease implies that the compound is extracted over a relatively short time period while a slow decrease implies that the compound is extracted over a longer period. Using $t_{1/2}$ as a measure of the intensity decrease, we observe a large variability between the different VOCs, encompassing a range of 3 to 25 seconds for $t_{1/2}$. A few compounds did not fall below 50 % of the maximum intensity by the end of the extraction and hence their $t_{1/2}$ could not be determined. Although the extraction of some compounds was relatively slow, 70 % of them reached $t_{1/2}$ in less than 10 seconds and showed intensities lower than 20 % of the maximum by the time that the coffee had been prepared (~24 seconds). Plotting the integrated intensity of the time-intensity curves for each VOC, we obtained the cumulative concentration of the VOC released from the flow at each time-point (Fig. 2B). The slope of these curves reflects the extraction rate. Normalizing this data to the total amount of compound extracted (intensity at the end of the extraction time was set to 100 %), it is possible to compare the extraction behavior of the different compounds within a coffee capsule. For each VOC, and as a function of time, these curves represent the extracted fraction with respect to the total amount in the final cup. We can observe that, as a consequence of the different extraction behavior of the

different compounds over time, the VOC profiles and ratios of aroma compounds in the samples differ at each time point.

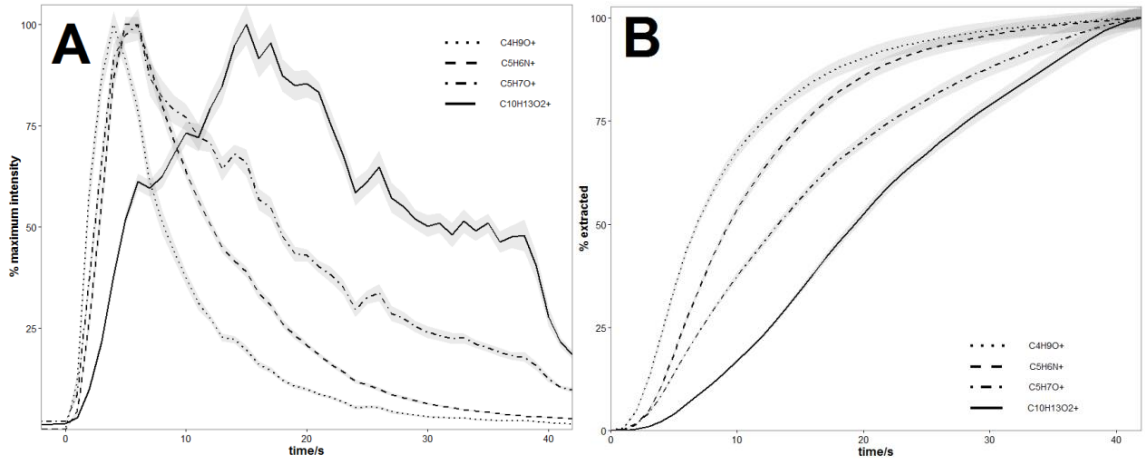

Figure 2. Time intensity profiles in the LC capsule showing differences in extraction. (A) Data normalized to the maximum intensity of four *m/z*. (B) Integration of the area under the curve at each time point as a percentage of the total area at the end of the extraction for the four selected *m/z* and Shaded ribbons show the 95 % confidence interval.

Extraction of single serve capsules is similar to espresso extraction, where hot water at high pressure passes through the ground coffee bed and results in an extract containing dissolved compounds, suspended solid particles and emulsified oil and foam. The high pressure at which the water is pumped through the coffee makes espresso extraction much faster than other coffee brew techniques (e.g. compared to filter coffee extraction by gravitational force). A simple visual inspection of the coffee flow out of an espresso machine shows that the color of the extract becomes progressively lighter with extraction time. This indicates that most of the colored compounds are extracted at the beginning of the extraction, in the first few seconds (first few mL). The same happens with the VOCs, although it is expected that VOC extraction is even faster and occurs more quickly than the colored, higher molecular weight compounds. Extraction of VOCs mostly occurs at the very beginning of the espresso extraction, resulting in an intense signal at the start of the time intensity profile, which is expressed as a steep slope on the integrated curve. Our results agree with those of Mestdagh et al[28], who extracted *Nespresso* coffee capsules stepwise with increasing volumes of extracts, from 10 mL up to 150 mL, and quantified 20 flavor active VOCs using GC-MS and isotopically labelled standards. Despite the variance associated with the use of different capsules for each volume point and the low time resolution (six points for 150 mL), they were able to describe the kinetics of extraction for 20 compounds and found some correlation between the polarity of the compound and extraction efficiency – more polar compounds were extracted faster. The same behavior was observed by Ludwig et al. for non-volatile compounds such as caffeine, 3-, 4- and 5-caffeoylquinic acids. They found that 70 % of these compounds were extracted in the first 8 seconds while only 50 % of the total 3,4-,3,5- and 4,5-dicaffeoylquinic acids were extracted in the same 8 second time window, showing slower rates during the whole process of making an espresso coffee. Diccaffeoylquinic acids are less polar than monocaffeolyquinic acids and have stronger chemical interactions with melanoidins, due to potential esterification. This suggests that not only polarity but also possible interactions with other polymers present in the coffee powder modulate the rate of extraction of the different compounds.

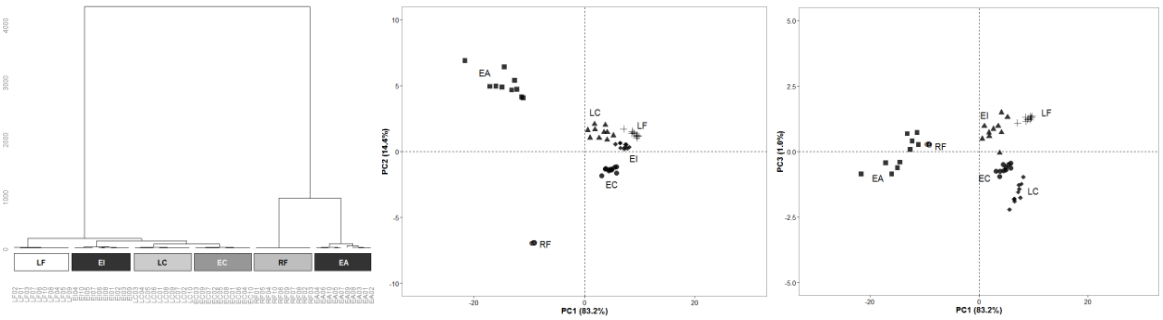

Figure 3. Hierarchical clustering and score plots for the first three dimensions of PCA of the six capsule varieties using the integrated area at full time corrected/normalized by the weight of the extract. A full separation of all ten repetitions for each capsule type is achieved, as shows in the HCA as well as on the PCA.

Differentiation of Capsules

The dynamic time-intensity data discussed above provide insights into the extraction rates of the different compounds in the coffee capsules. However, besides exploring the extraction dynamics, the data were also used to distinguish between coffee types, by means of statistical analysis - Hierarchical Cluster Analysis (HCA) and Principle Cluster Analysis (PCA).

Integrating the 95 time-intensity profiles are over the full extraction time (which varies between the different coffee types), and normalizing/dividing by the amount of coffee extracted transforms the dynamic data of each extraction process into a set of 95 intensities, which reflect the volatile profiles above the cup (the headspace). Indeed, one of the advantages of PTR-MS is that the signal is proportional to the measured concentration. Therefore integrating over the full time-intensity curve provides a measure of the VOC extracted over the extraction time. Dividing subsequently by the weight of the final cup corrects for dilution and gives a measure for the concentration in then cup and can hence be linked to the headspace concentration via the Henry Law Constant (HLC). Both HCA and PCA showed good clustering for all the capsules. Therefore, we conclude that the aroma profile of the extracts and consequently the HS profiles are different for the six capsule types investigated here.

On-line analysis by PTR-ToF-MS not only provides valuable insight into the extraction dynamics of individual flavor active VOCs. It also allows separating between different capsule types.

CONCLUSION

We have presented a novel, high time-resolution methodology for monitoring the extraction dynamics of espresso coffee and applied it to six different capsules types. The results presented in this work show the suitability of PTR-ToF-MS for monitoring changes in the volatile composition of a liquid flow in an open atmosphere. Online analysis of coffee extraction revealed the kinetics of extraction for different VOCs and highlighted the differences between commercial coffee capsules over the whole extraction time. The presented method overcomes the problems of previous GC-based approaches: (i) it increases temporal information, from a few data points over the whole extraction time to a one second resolution, and (ii) it reduces sources of variability, as the time-evolution of each VOC is monitored on-line in a single extraction process and is not a combination of multiple different extracts. The simplicity, high sensitivity and time resolution of the method makes it a perfect

approach for investigating the impact of different parameters that affect extraction dynamics of flavor compounds. Based on such data, the process can be fine-tuned in order to achieve the desired aroma balance in the final cup.

The methodology also allows the user to differentiate between coffee types, by applying HCA and PCA on the cumulated intensities of VOCs over specific time windows.

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Coffee Diterpenes: from Green Beans to Espresso Coffee

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SUMMARY

The most important coffee diterpenes are kahweol, cafestol and 16-O-methylcafestol (16-OMC). These compounds are mostly esterified with various fatty acids and only a small amount is present in the free form. In addition to both minor diterpenes (e.g., 16-O-methylkahweol) and degradation products (e.g., dehydrocafestol, dehydrokahweol, kahweal and cafestal) they are important constituents of the unsaponifiable fraction of coffee oil. Both desirable and undesirable -biological activities have been ascribed to these compounds. In addition to anticarcinogenic, antioxidant, anti-inflammatory properties and hepatoprotective effects, a hypercolesterolemic activity (particularly for cafestol) has been reported. The interest towards coffee diterpenes is also triggered by their potential use as chemical markers for roasting intensity and for authentication purposes. *Coffea arabica* and *Coffea canephora* var. Robusta, the commercially exploited coffee species, differ not only in their agronomical performances, morphological characteristic, and organoleptic properties, but also in chemical composition, including diterpenes. Specifically, whereas cafestol is present in both species, kahweol is present in higher amount in *C. arabica* than Robusta and 16-OMC is present exclusively in Robusta, according to available literature. The quantitative determination of 16-OMC is routinely performed by the German standard method DIN 10779. The methodology is rather tedious, time- and solvent-consuming, but the separation of 16-OMC is quite good.
The present work is aimed at characterizing diterpenes content in several coffee samples of both coffee species, from green beans to *espresso* coffee. Special focus has been placed on the determination of 16-OMC in the attempt to explore analytical methods alternative to the DIN standard that could guarantee the authenticity of 100% *C. arabica* blends by detecting possible commercial frauds in a rapid and reliable way.

INTRODUCTION

In spite of the important role played by coffee diterpenes in bean biology, human physiology, and industrial applications, studies focused on these coffee components are still rather scarce. One drawback in getting qualitative and quantitative data could be the necessity to resort to different experimental approaches based on coffee oil extraction, saponification and classical analytical instrumentation (eg. CG or HPLC), which can be relatively time-consuming, laborious and tedious depending on the starting sample to be examined (green coffee, roasted coffee or coffee brews).

In the framework of coffee authenticity (roasted coffee beans), the German standard method DIN no. 10779 uses the determination of 16-OMC to detect *C. canephora* in blends. This method is rather tedious but the separation of 16-OMC is quite good as well as that of the other diterpenes. For this reason, the German standard has been used not only to characterize roasted coffee beans, as originally intended, but also green coffee beans and coffee brews. In

the present work, diterpenes in several coffee samples of both Arabica and Robusta coffee species, from green beans to *espresso* coffee have been determined by using the DIN standard in order to assess its performance. In view of promising results obtained on green coffee oil, attention has been paid to the determination of coffee diterpenes by NMR techniques in order to explore possible analytical alternatives to the DIN standard method.

MATERIALS AND METHODS

C. arabica (Brazil and Colombia) and *C. canephora* (Ivory Coast gr. 2 and India parchment AB) hereafter named A1, A2, R1 and R2, respectively were used as green coffee beans and they were roasted to a medium roasting degree in a Probat lab roaster. The roasted samples were properly ground and prepared as *espresso* brews according to the standard traditional method (Espresso Machine "La Marzocco", 7 g coffee dose and 25 ± 1 mL cup volume). Several commercial roasted coffee samples (in whole beans) were purchased in local markets. These samples were named TS0, …, TSn.

Major and minor diterpenes were quantitatively determined in duplicate according to the DIN method n. 10779. In the case of beverage samples, the saponification step was performed directly on the liquid sample. Two different wavelengths were used for detection: 220 and 290 nm. Standard diterpenes (cafestol, kahweol, 16-O-methylcafestol and dehydrocafestol) were used for quantification and when the standard was not available, the quantification was carried out using the standard with more chemical similarity with the analyte.

A Bruker (Bruker, Rheinstetten, Germany) Avance DMX600 spectrometer was used for one-dimensional ^{1}H spectra operating at 599.90 MHz and equipped with a 5 mm TXI xyz- triple gradient probe. The spectra were acquired in deuterated chloroform, (CDCl3,99.96% purchased from Sigma Aldrich) by using a common one pulse sequence with a spectral width of 6000 Hz, 32768 data points, the receiver gain ranged from 64 to 128, depending on signal intensity, and the number of scans varied from 64 to 128 depending upon the 16-OMC concentration:

The ACD software (ACD labs 12.0) was used to process the spectra. Fourier transformation was performed after exponential line-broadenings of 0.2-0.4 Hz. Integrations were manually obtained after careful manual phase and baseline correction-

RESULTS AND DISCUSSION

In Table 1, the major diterpenes determined in green coffee samples are reported. As far as Arabica is concerned, cafestol and kahweol contents are within the range of reported data (cafestol approx. 2.0 – 10.2 mg/g and kahweol approx. 1 – 10 mg/g. In Robusta samples, cafestol and 16-OMC are within the range of reported data (cafestol approx. 1.5 – 6.8 mg/g and 16-OMC range approx. 0.4 – 2.5 mg/g), even though at the lower limit of the range. Kahweol was found in traces. No 16-O-methylkahweol was detected (in spite of the use of 220 and 290 nm wavelengths for detection) nor diterpenes degradation products.

The same samples analyzed after roasting led to the experimental data reported in Table 2. As expected, all samples are characterized by the presence of diterpenes deriving from the thermal treatment (dehydrocafestol, dehydrokahweol, cafestal and kahweal). In Arabicas, the content of the major diterpenes decreased remarkably whereas in Robustas the figure is paradoxically almost opposite. Specifically cafestol seems rather unaffected by roasting whereas 16-OMC remarkably increases even if its content is in agreement with literature data (mean total 16-OMC content of 1.7 mg/g (free + esterified).

In our opinion, this incongruence could be ascribed to the different behavior of the two Coffea species in green beans form, when subjected to grinding. The different particle size distribution obtained after grinding which may reflect the different cell structure of the two commercially exploited species as well as the different amount of defective beans in the present Robusta samples, could greatly influence the extraction of lipids and subsequent quantitative data. This view is corroborated by the coffee oil yield measured before and after roasting on the same ground material used for diterpenes determination. As a matter of fact, the yield from roasted samples is in very good agreement with the typical oil content expected for Arabicas and Robustas (mean 16.4 % and 11.8 % respectively in the examined samples). On the other hand, the increase of coffee oil yield expected in passing from green to roasted beans, is compatible with the obtained roasting degree only in the case of Arabicas being that of Robustas (particularly R1 sample) remarkably higher. This finding is compatible with a not exhaustive oil extraction from green Robustas. In Table 3, experimental data of traditional espresso beverages are reported.

Table 1. Major diterpenes in green coffee samples

Diterpene	A1 (mg/g)	A2 (mg/g)	R1 (mg/g)	R2 (mg/g)
Cafestol	5.1 ± 0.1	1.9 ± 0.1	1.1 ± 0.2	1.6 ± 0.2
Kahweol	6.1 ± 0.5	2.9 ± 0.3	traces	traces
16-OMC	nd	nd	0.4 ± 0.1	1.1 ± 0.1

nd: not detected

Table 2. Major and minor diterpens in roasted coffee samples

Diterpene	A1 (mg/g)	A2 (mg/g)	R1 (mg/g)	R2 (mg/g)
Cafestol	2.8 ± 0.5	0.8 ± 0.1	1.2 ± 0.2	1.3 ± 0.1
Kahweol	5.1 ± 0.6	1.1 ± 0.2	traces	traces
16-OMC	nd	nd	2.0 ± 0.1	1.60 ± 0.2
DehydroC	0.30 ± 0.04	0.03 ± 0.003	0.65 ± 0.04	0.27 ± 0.02
DehydroK	0.25 ± 0.04	0.02 ± 0.002	traces	nd
Cafestal	0.02 ± 0.004	nd	0.04 ± 0.01	0.05 ± 0.004
Kahweal	0.02 ± 0.004	nd	traces	traces

nd: not detected

Cafestol and kahweol content is in agreement with literature data (cafestol up to 2.48 mg/cup; kahweol up to 3.12 mg/cup). Degradation products were detected and quantified but no data on espresso beverage (25 mL cup) have been reported in literature, so no comparison may be performed. In all cases, however, the content of roasting-induced diterpenes, when detected, appears to be very low.

As far as authenticity of roasted coffee is concerned, often the high-quality Arabicas, described as ‘‘100% Arabica’’ or ‘‘Highland coffee’’, are mixed with the less expensive

Robustas. The quantification of 16-O-methylcafestol is particularly useful to control the authenticity of the products as well as Robusta content in blends.

However, in view of the wide range of 16-OMC content found in Robustas, the determination of this molecular marker is more appropriate for authentication of 100% Arabica blends rather than to control Robusta content in blends.

Table 3. Major and minor diterpenes in *espresso* coffee samples

Diterpene	A1 (mg/cup)	A2 (mg/cup)	R1 (mg/cup)	R2 (mg/cup)
Cafestol	1.64 ± 0.01	0. 0.80 ± 0.1	0.85 ± 0.1	1.65 ± 0.1
Kahweol	3.01 ± 0.01	1.3 ± 0.1	nd	nd
16-OMC	Nd	nd	0.7 ± 0.1	1.45 ± 0.2
DehydroC	0.12± 0.01	0.03 ± 0.007	0.15 ± 0.01	0.1 ± 0.03
DehydroK	0.10± 0.001	0.01 ± 0.001	nd	nd
Cafestal	0.03 ± 0.003	nd	0.03 ± 0.01	0.03 ± 0.005
Kahweal	0.01± 0.001	nd	nd	nd

nd: not detected

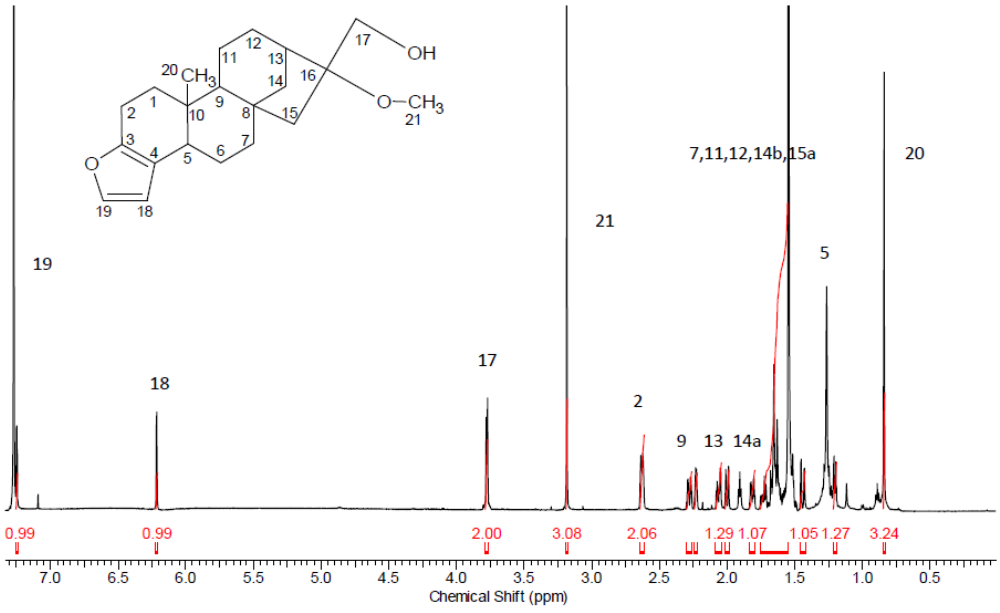

Figure 1. ^{1}H-NMR spectrum of standard 16-OMC in $CDCl_3$. 16-OMC structure and atom numbering is shown.

In the present work we explored the possibility to quantify 16-OMC by mean of high-resolution ^{1}H-NMR spectroscopy. The ^{1}H-NMR spectrum of standard 16-OMC in $CDCl_3$ is shown in Figure 1. Protons 17 and 21 are diagnostic of the presence of 16-OMC in a roasted coffee chloroform extract. Different from protons 21, which originate a singlet signal very slightly shifted in esterified 16-OMC (3.17 ppm), protons 17, going from free to esterified 16-OMC, changed from an apparent singlet signal to a doublet of doublets, with only one of which is resolved at 4.45 ppm. A plot of the integrals of these signals as a function of % of

Arabica in appropriately produced blends, shows a very good linearity (R^2 is higher when the singlet at 3.17 ppm is used). Moreover, protons 19 and 18 for both cafestol and kahweol, as well as protons 1 and 2 for kahweol (Fig. 2) may reveal additional information on possible presence of Robusta in blends.

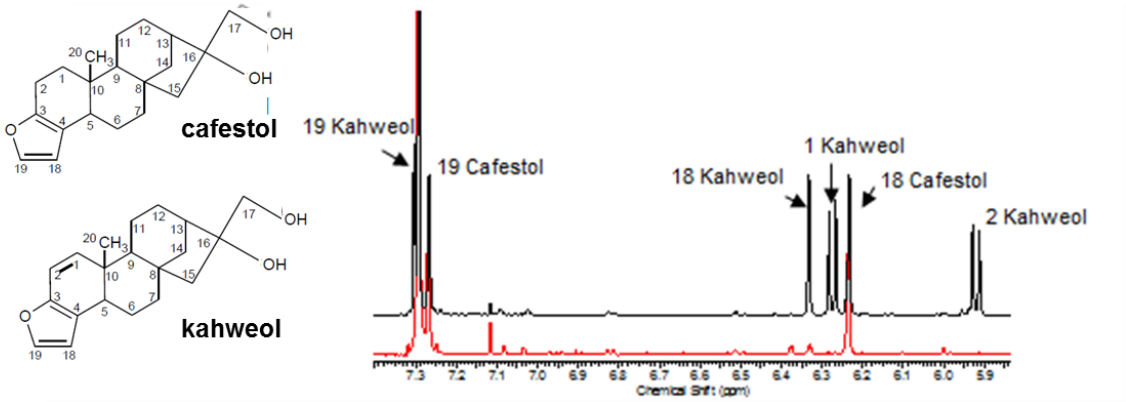

Figure 2. ^{1}H-NMR spectra of $CDCl_3$ roasted coffee extracts in the 5.8 – 7.4 ppm region: black: pure Arabica, red: pure Robusta. Cafestol and kahweol structures and atom numbering is shown.

By selecting proper NMR experimental conditions it is possible to quantify 16-OMC in roasted coffee chloroform extracts.

In Table 4, a comparison between 16-OMC content determined by both DIN and NMR methods is reported.

The agreement between the two different determinations is very promising and in view of the several advantages offered by the NMR method, it opens a new scenario into coffee diterpenes characterization and analysis.

In fact high-resolution NMR spectroscopy has the considerable advantage that it can yield information simultaneously in a rapid and non-destructive way on small amount of sample.

In many cases, the 16-OMC content determined by DIN is lower than that determined by NMR suggesting a possible underestimation by DIN method, possibly related to the complex manipulation necessary to perform the analysis. In this regards previous studies suggested that the extraction step according to DIN method seems to be crucial in possible underestimation.

However this point, as well as the validation of the NMR method and the NMR characterization of other coffee diterpenes will constitute the grounds for further studies.

Table 4. 16-OMC content in commercial roasted coffee samples.

Sample	Blend	DIN 10779 (mg/kg) [st. dev]	1H-NMR (mg/kg) [st. dev.]
R1	100% R	1976 [100]	1693 [1]
R2	100% R	1630 [240]	1735 [7]
TS0	100% R	1428	1418 [11]
TS1	100% R	1450 [18]	1723
TS4	A/R	1193 [51]	1155 [16]
TS8	nd	434 [10]	624 [9]
TS10	nd	945	703 [16]
TS12	100% R	1008	1491 [40]
TS13	100% R	1279 [129]	1759 [20]
TS15	A/R	1135 [18]	1545 [25]
TS16	nd	1305	1315 [14]

nd: not declared

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Single-Dose Espresso Coffee Capsules: a Complete Data Set Characterization of Body, Color and Aroma

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SUMMARY

This study concerns the characterization of a wide range of espresso coffee blends from the same manufacturer (Delta Q) using a single-dose coffee capsules system. Total solids content ranged from 0.99 to 1.67 g *per* cup, ashes content from 139.5-176.0 mg *per* cup and the protein content ranged from 8.58 to 12.56 % (w/w). Total lipids content greatly varied from 7.40 to 35.1 mg, but the fatty acids profiles determined was shown to be quite similar among all blends. Coffee variety affects caffeine content that ranged from 56.2 to 79.6 mg *per* cup in regular blends, while the one supplemented with natural extracts blend was higher. The degree of roasting affects coffee brown color and volatile composition analysis showed that the furan derivatives are the predominant family of compounds present in espresso coffee brew.

INTRODUCTION

Espresso coffee is a worldwide consumed beverage with more than 50 million cups consumed every day. Aroma, distinctive flavour, intense colour, bitter/acid balanced taste, full-bodied appearance and persistent hazelnut foam are some of the distinguishable characteristics of a perfect espresso coffee. An enormous diversity of compounds are extracted during espresso coffee preparation. Coffee capsules systems were welcomed by the consumers, enabling that the espresso coffee with regular quality of aroma and body reaches every home. Furthermore, these systems brought to consumer the opportunity to choose between several blends commercially available and experience different espresso coffee aromas, body, color and intensity. However, the knowledge about the chemical composition of the espresso coffee from single-dose capsules is quite scarce and an analysis of a range of blends could give useful information about the relationship between composition and sensorial properties of the espresso coffee. *Coffea arabica* (70-75% of total coffee production) and *Coffea canephora* var. *robusta*, commonly referred as Arabica and Robusta, respectively, are the two species of *Coffea* genus (*Rubiaceae* family) cultivated for commercial production. The different variety as well as distinct geographical origin affects chemical properties of coffee beans and consequently may be reflected in the espresso brew characteristicts. The chemical composition of the coffee beans are also affected by the degree of roasting and the polysaccharides structures present influences the espresso coffee stability. The sensations that will be perceived by the consumer are closely related to these chemical factors. Thus, seting a detailed chemical characterization of each blend becomes greatly important for coffee industry as well as try to relate the knowledge obtained with consumer preferences.

MATERIALS AND METHODS

Sample Preparation

All coffee samples (40 ± 2 mL) were prepared on a Delta Q Qosmo machine by extraction with tap water. The single-dose coffee capsules of 10 blends Delta Q were kindly provided by Novadelta, SA. Blend 1 corresponded to a decaffeinated coffee, Blends 2-7 to regular ones and Blend 8 had ginseng and guarana extracts in its constitution. According to data provided by the manufacturer, Blends 2, 3 and 4 may be related by the degree of roasting (Blend 4 > Blend 3 > Blend 2), while Blends 5, 6 and 7 may be related by their *Coffea arabica* content (Blend 5 < Blend 6 < Blend 7). Blends 9-10 are coffee blends with exactly the same constitution that only differ in its degree of roasting (Blend 9 < Blend 10).

Density, Total Solids, Ashes and pH

The determination of density, total solids and ashes were performed cumulatively since one coffee extraction allowed the determination of the three parameters (6 replicates). Total solids content was determined by gravimetry (105°C, 24 h). Ashes were determined by incineration (700°C, 6 h). The density was determined at 20°C before the aforementioned measurements. For pH determination, the coffee samples were previously cooled (20ºC) and homogenized (3 replicates).

Total Lipids and Fatty Acids Profile

Soxhlet extraction with petroleum ether was used for determination of the total lipids content using freeze-dried extracts (3 replicates). Total lipids content were determined gravimetrically. The lipidic fractions were used to determine the fatty acid profile by transesterification to the corresponding fatty acids methyl esters and GC-FID analysis.

Diterpenes Content

For diterpenes determination, the total lipids fractions were used (3 replicates). The coffee samples were saponified with a 2.5 M KOH solution and extracted with diethyl ether. Analysis by HPLC (UV-vis - 220 nm) was used for diterpenes determination.[6]

Dialysis

The defatted coffee samples were dialyzed against distilled water (MW cut-off 12-14 kDa) at 4°C. The dialysis process proceeded until conductivity was lower than 5 μS cm^{-1} (3-4 water renewals).

Protein Content and Amino Acids Profile

The protein content was estimated multiplying the nitrogen content determined by elemental analysis by a factor of 5.5.[7] The high molecular weight material soluble in cold water (the principal polymeric fraction) obtained after dialysis was used for this analysis (3 replicates). GC-FID analysis was performed to obtain the amino acid profile after acid hydrolysis and derivatization to heptafluorobutyl isobutyl derivatives.[8]

Carbohydrate Analysis

Polysaccharides (sugar composition) was determined after acid hydrolysis and derivatization to alditol acetates and analysis by GC-FID.

Melanoidins content, $K_{mix,\ 405\ nm}$ and *MBI*

Dilutions of the 1 mg mL^{-1} freeze-dried coffee sample were used to spectrophotometrically determine $K_{mix,405nm}$ measuring the absorption at 405 nm. The difference between the total content of polymeric material and the content of carbohydrates and proteins gave an estimate of melanoidins content. *MBI* was calculated by the ratio of $K_{mix,405nm}$ values and the relative content of polymeric material present not identified as polysaccharides or proteins.

Caffeine and Chlorogenic Acids Content

Aliquots of 10 mg mL^{-1} of freeze-dried coffee samples (2 replicates) were used to determine caffeine and chlorogenic acids (as 5-caffeoylquinic acid equivalents) by HPLC at 280 nm and at 325 nm, respectively.

Volatile Composition

Espresso coffee volatile compounds present in the vapour phase of the beverage were extracted by solid-phase microextraction (SPME) and analysed by comprehensive two-dimensional gas chromatography and detected by mass spectrometry with time-of-flight analyzer (HS-SPME/GC x GC ToFMS). The sample vial was maintained in a water bath at 60ºC. The sample of espresso coffee (40 ± 2 mL) was extracted directly to the thermostated flask, which was sealed and placed at 60ºC under constant stirring. The fiber (DVB/CAR/PDMS) was exposed for 3 min prior to GC x GC-ToFMS Pegasus 4D injection. Each sample was analyzed in triplicate.

RESULTS AND DISCUSSION

The results obtained from the analysis of pH, density, total solids, ashes and protein content are represented in Fig. 1. Each graph represents the mean values for each parameter, as well as the minimum and maximum values obtained among all blends.

1. *pH*: The pH values ranged from 5.07 and 5.32, which is within the limits of acceptance (4.8-6.0) and in accordance with literature data for coffee espresso. The highest value (5.32) was obtained with the decaffeinated coffee blend (Blend 1), although the difference to some other regular blends were not significant.
2. *Density*: Some variability among the blends tested were observed considering density analysis (1.0040-1.0109 g cm^{-3}), although these results were in accordance with the mean value reported in literature 1.010 g cm^{-3}.
3. *Total solids*: Fig. 1c) seems to indicate a wide variability of total solids content among the blends tested. However, a detailed view (Fig. 1f) highlights two blends from the remaining ones. Thus, Blend 1 (decaffeinated coffee) showed a substantial lower content of total solids (0.99 g *per* cup) which seems to indicate that the decaffeination process affects the extraction of coffee compounds. Moreover, the supplemented blend that had the addition of plant extracts (Blend 8) exhibited clearly the highest total solids content (1.67 g *per* cup). Therefore, this indicates that the content of natural extracts should be easily extracted to the beverage during espresso preparation. Finally, the remaining regular blends (Blends 2-7) showed an intermediate total solids

content (1.21 g *per* cup in average) similar to each other and in accordance with the results obtained with other espresso coffee capsules systems.

4. *Ashes*: The ashes content ranged from 139.5 mg to 176.0 mg *per* cup. Fig. 1g) shows that their content tends to decrease with increasing of roasting degree, since Blend 2 exhibited higher content of ashes than Blend 3, and the latter showed higher amount of ashes comparing with the darker Blend 4. The analysis of the blends 9 and 10, allowed confirming this conclusion since these blends have exactly the same composition and only differ in the degree of roasting. In fact, the darker Blend 10 exhibited a lower ashes content comparing with the milder roasted Blend 9 (214.35 mg > 172.4 mg *per* cup).

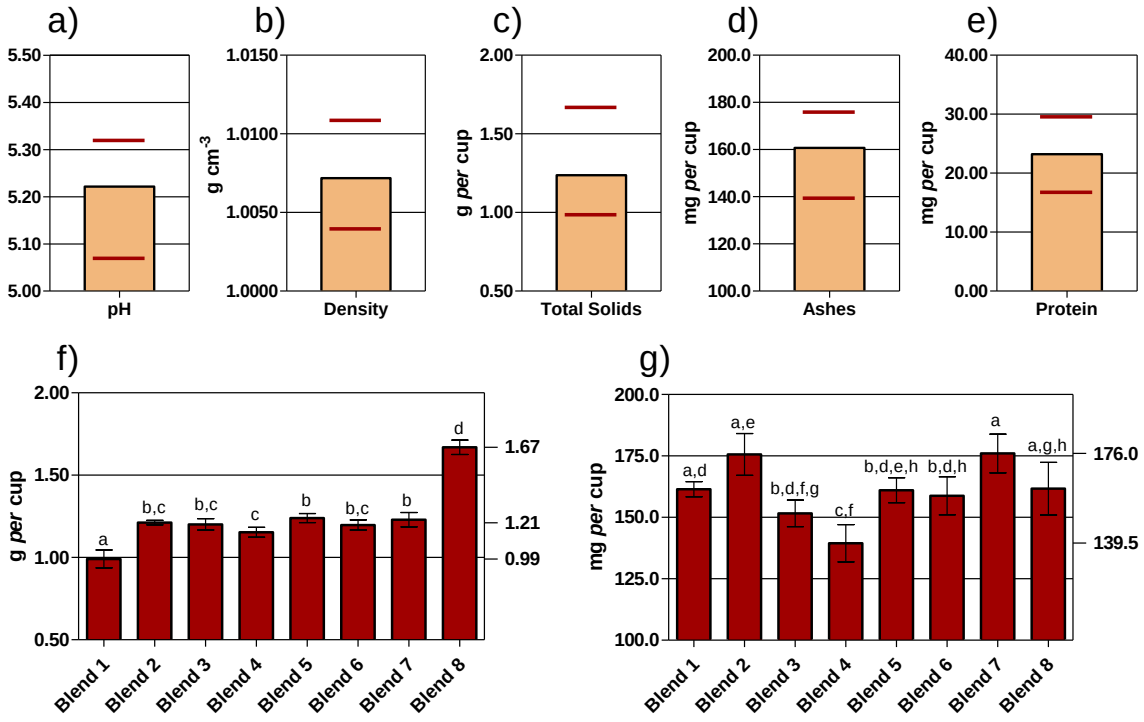

Figure 1. pH (a), density (b), total solids (c), ashes (d) and protein (e) content of the blends analyzed. The result show the mean value and the stripes in each graph represent the maximum and minimum values obtained. The total solids and ashes content of each blend are highlighted (f and g). Data represent the mean ± standard deviation and bars with the same letter represent values that are not significantly different ($p<0.05$).

5. *Protein*: The protein content *per* cup of coffee ranged from 16.93 mg to 29.70 mg (Fig. 1e). The minimum and maximum values were obtained for the decaffeinated blend (Blend 1) and for the supplemented blend (Blend 8), respectively, which may be related to their different values of total solids. However, considering the weight percentage of protein, these relations are reversed. In fact, Blend 8 exhibited the lowest value (8.58 %w/w), as the extracts added should contain low protein content and Blend 1 exhibited the highest percentage value (12.56 %w/w) despite the smaller amount of protein present.
6. *Amino Acids*: Glutamic acid was the most abundant amino acid in all blends (22.0-36.7 %mol), followed by leucine, glycine, aspartic acid and proline at intermediate values, which is in accordance with literature data.
7. *Total lipids*: The analysis of total lipids showed a wide range of values between the blends (7.40 mg to 35.10 mg *per* cup). The amount of total lipids in the brew (Fig. 2b) decreases with the increase of degree of roasting (Blend 2 < Blend 3 < Blend 4), as

already reported. Furthermore, it was observed that the blend with highest *Coffea arabica* content (Blend 7) exhibited the highest amount of total lipids showing also that coffee variety should affect lipids content.

8. *Fatty acids profile*: Although the amount of total lipids widely varies, Fig. 2c shows that the fatty acids profiles determined were quite similar among all blends tested. The main fatty acids present were linoleic (C18:2, 44.4%) and palmitic (C16:0, 35.0%) acids. The overall profile was in agreement with those reported concerning single-dose capsules.
9. *Diterpenes*: Moreover, kahweol was the main diterpene found, followed by cafestol and 16-*O*-methylcafestol and it was observed that their content decreased with increasing degree of roasting. In fact, diterpenes content was even below the detection limit for the darker blends (Blend 3 and 4) with the exception of Blend 2. The blend with the highest *Coffea arabica* content (Blend 7) exhibited the highest diterpenes content, regardless the kind of diterpene, whereby the coffee variety should also affect its content.

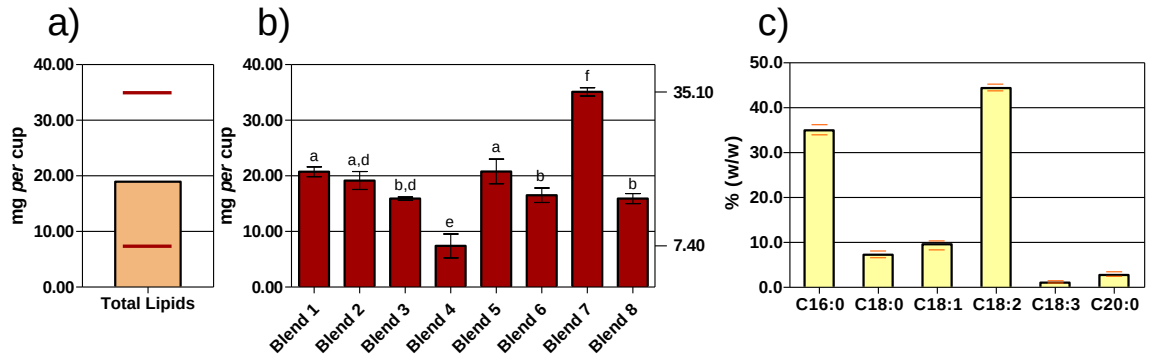

Figure 2. (a) Amount of total lipids present in espresso brews. The result show the mean value and the stripes in each graph represent the maximum and minimum values obtained. (b) Total lipids of each coffee blend are highlighted. Data represent the mean ± standard deviation and bars with the same letter represent values that are not significantly different ($p<0.05$). (c) Fatty acids profile of the espresso coffee analyzed.

10. *Caffeine*: The caffeine content present in the decaffeinated blend (Blend 1) was quite low (2.4 mg *per* cup) (Fig. 5). On the other hand, Blend 8 exhibited an amount of caffeine *per* cup of coffee (113.5 mg *per* cup) quite higher than the caffeine content of the remaining regular coffees analyzed. Thus, the addition of ginseng and guarana extracts affords a coffee brew with higher caffeine content than usual which is indeed the aim of the addition of these extracts by the manufacturer. Concerning the remaining regular Blends (Blends 2-7), caffeine content ranged from 56.2 to 79.6 mg *per* cup of coffee. The caffeine content seems to be directly related with coffee variety since an increase in its amount was observed with increasing Robusta variety content in the blend (Blend 5 > Blend 6 > Blend 7). The degree of roast seems not to affect significantly the caffeine content among the blends.
11. *Chlorogenic acids*: Regarding chlorogenic acids content, the analysis performed showed a range of 57.24 mg to 72.90 mg *per* cup of coffee, with 5-CQA as the main chlorogenic acid present.

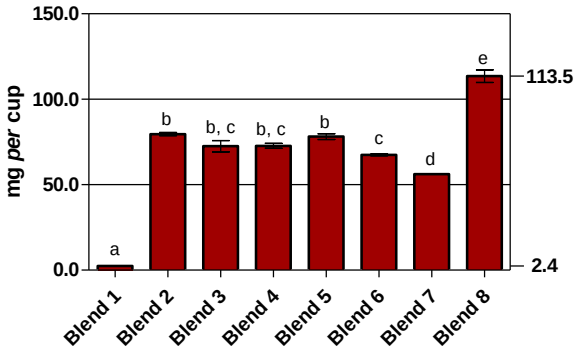

Figure 3. Caffeine content in the blends analyzed. Data represent the mean ± standard deviation and bars with the same letter shows results that are not significantly different ($p<0.05$).

12. *Polymeric material*: The dialysis process allows the separation of the material according to its molecular weight. The polymeric material (>12 kDa) that remains in the membrane was separated from the dialyzed material (low molecular weight material). The high molecular weight material (>12 kDa) represents 16.4 to 22.7 % (w/w) of coffee dry matter. Blend 8, having the addition of plant extracts, exhibited a significant higher content of polymeric material. Contrarily, deccafeinated blend (Blend 1) showed the lower content in all fractions, in agreement with its lower total solids amount. Sugars analysis of polymeric fractions of espresso coffee blends highlighted two blends from the remaining ones for having a lower (Blend 1) and a higher (Blend 8) polysaccharide content (Fig. 4) which is correlated with total differences of high molecular weight material content. Figure 4 also shows that the major sugar present in almost all blends was mannose followed by galactose, except for Blends 1 and 8. Rhamnose, arabinose and glucose were present in substantial lower quantity. The results obtained are in accordance with the presence of galactomannans and arabinogalactans which are the main polysaccharides reported for coffee. The first is mainly composed by mannose and some galactose, while for the latter, galactose and arabinose are the principal sugars present. Decaffeinated blend (Blend 1) showed galactose as the major sugar and this particularity may be related to the decaffeination process. In supplemented Blend 8, the glucose appears clearly as the principal sugar, resultant from ginseng and guarana glucose-rich polysaccharides.

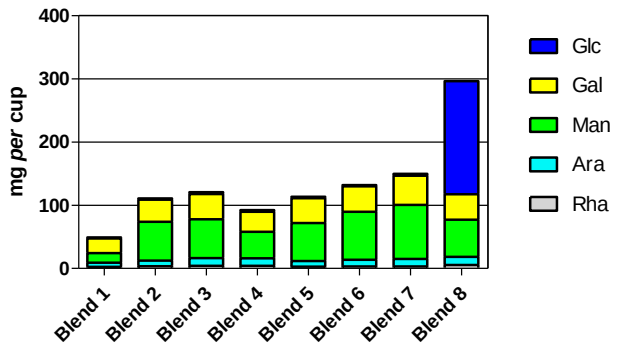

Figure 4. Composition of polysaccharides present in the blends analyzed.

13. *Melanoidins*: The content of melanoidins, the fraction of polymeric material with a somewhat complex and unknown structure, ranged from 69.8 to 145.6 mg *per* cup. $K_{mix,405nm}$, a specific extinction coefficient, gives information about the brown color and its determination showed that the highest value was obtained with the blend with

the highest degree of roasting (Blend 4). This result suggests a higher intensity of the brown coffee color for this blend when compared with others. The melanoidin browning index (*MBI*) parameter integrates the brown color estimated by $K_{mix,405nm}$ and the high molecular weight unknown material estimated by the difference between high molecular weight material and the content of polysaccharides and protein. This parameter evidences that the increase in the degree of roast increases the contribution of these unknown compounds to brown color intensity (Blend 4 > Blend 5 > Blend 6). It was also observed that the fraction of melanoidins had a great contribution for the brown color of Blend 8 espresso coffee.

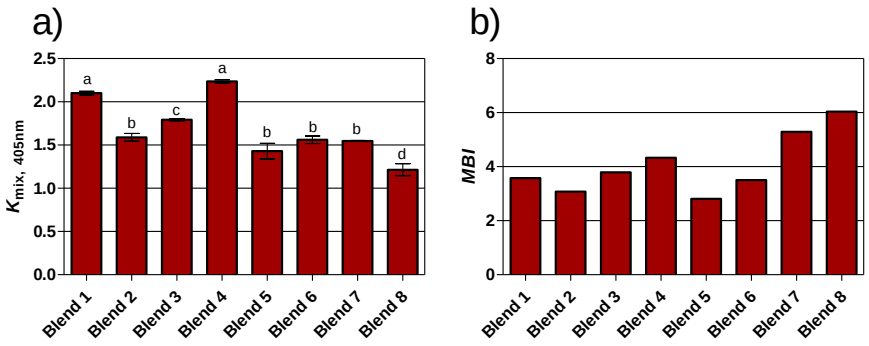

Figure 5. (a) $K_{mix,405nm}$ values for the blends analyzed. Data represent the mean ± standard deviation and bars with the same letter shows results that are not significantly different ($p<0.05$). (b) *MBI* values found for the blends analyzed.

14. *Volatile compounds*: The analysis of espresso coffee brews volatile composition was performed by solid-phase microextraction (SPME) with an extraction time of 3 minutes. This period was used in order to simulate the consumer perception of the aroma. Figure 6 shows an example of bidimensional (2D) and tridimensional (3D) chromatograms obtained during volatile composition analysis performed by comprehensive two-dimensional gas chromatography GCxGC-ToFMS. The compounds are separated by volatility and by polarity in the first and second dimension, respectively.

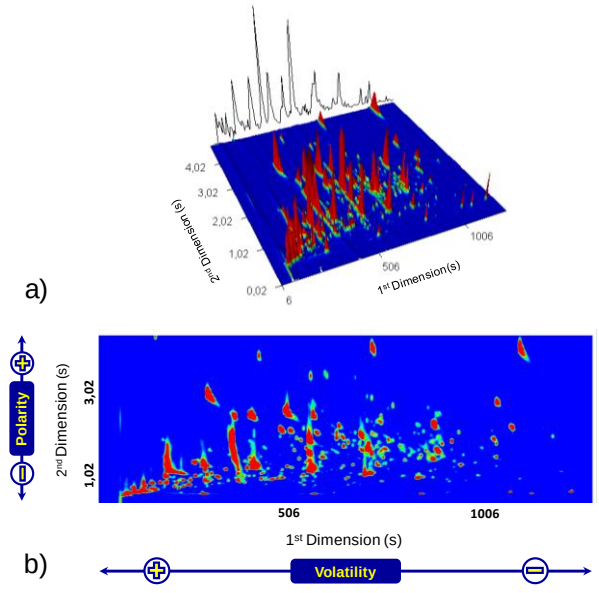

Figure 6. Example of a 3D (a) and 2D (b) chromatograms obtained during volatile composition analysis.

More than 600 compounds were identified in this study. This high quantity of compounds was organized by their chemical families. Figure 7 allows comparing the chromatographic areas of each family of volatile compounds present in espresso coffee obtained for Delta Q capsules.

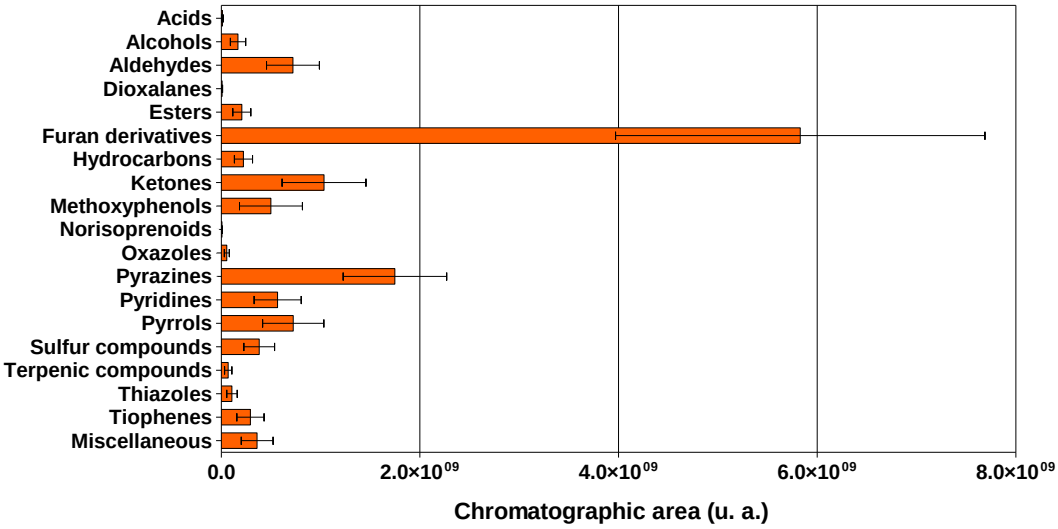

Figure 7. Total chromatographic areas of the different families of volatile compounds found in the espresso brew. Data represent the mean ± standard deviation considering all blends analyzed.

Furan derivatives, a family of compounds associated with roasting process, were the main family present in the espresso coffee brew accounting their chromatographic areas. Pyrazines, pyrrols and ketones had intermediate chromatographic areas, while alcohols, dioxalanes and norisoprenoids were families of compounds with lower chromatographic areas. Volatile compounds analysis showed some differences in the chromatographic areas of the families among all blends analyzed. It was observed that the supplemented blend showed the lowest volatile compounds content and a decrease in the total content of volatile compounds with increasing degree of roasting.

Final Remarks: The results achieved so far are the first step to know more about the characteristics of Delta Q capsules. Their relation with the sensorial characteristics are the next step in order to prepare single-dose espresso coffee capsules with body, color and aroma according to the consumer preferences.

ACKNOWLEDGEMENTS

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Prediction Models for Twelve Chemical Compounds Linked to Coffee Quality Obtained by Near Infrared Spectroscopy - NIRS (C13)

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SUMMARY

A database was constructed with 32000 NIRS spectra, in order to develop calibration equations (CEs) for twelve chemical compounds associated with coffee quality.. Sample selection for Ces development was made taking into account a priori differences in coffee crops system, origin within Colombia, and chemical values for contrasting varieties of Cenicafé's germplasm bank. The CEs were made for caffeine, trigonelline, sucrose, total chlorogenic acids (CQA Total), total lipids and the following fatty acids: palmitic, stearic, oleic, linoleic, linolenic, arachidic and behenic. CEs were developed by Partial Least Squares regression (PLS) with cross validation, compliance and acceptance parameters as defined by Shenk and Westerhaus (1996). Excellent prediction models were obtained for caffeine, trigonelline , lipids and the stearic fatty acid; Good prediction models resulted for sucrose, oleic, linoleic, linolenic, arachidic and behenic fatty acids, as well as for Total CQA. Finally, a Low prediction model was obtained for palmitic fatty acid. These CEs are implemented for research purposes in germoplasm characterization, assisted selection by biochemical compounds, improved new varieties,Origin characterization, trazability and authenticity of commercial programs by the National Federation of Coffee Growers of Colombia (FNC).

INTRODUCTION

Coffee is considered one of the most important beverages in the world, Coffee quality can be assessed globally by sensory evaluation and physical analysis, these are the only parameters used to determine the price and the final product quality (Clarke & Macrae, 1988).

Coffee consists in over 1000 different chemicals compounds including amino acids and other nitrogenous compounds, polysaccharides, sugars, triglycerides, linoleic acid, phenolic compounds (chlorogenic acid), caffeine, volatile substances (about 800 identified of which 60-80 contribute to the aroma of coffee). There are significant variations in the concentration of these components depending on the coffee variety and the roasting process. (Spiller, 1984).

Near infrared spectroscopy (NIRS) was developed for the estimation of chemicals in foods (Osborne, Fearn, & Hindle, 1993). NIRS is a rapid, non-destructive or contaminating and highly accurate technique provided the proper procedures are followed to create the calibration equations (Alomar, Fuchslocher, & De Pablo, 2003; Gous et al., 2012) and allows to quantify and classify various agricultural products, including coffee (Downey & Boussion, 1996).

The optical properties of foods, and in particular the application of NIRS technique has been used since the 70s in the petrochemical food, pharmaceutical, and as an alternative to

traditional chemical methods (Norris, Barnes, Moore, & Shenk, 1976, Cozzolino, Cynkar, Shah, & Smith, 2011).

In the developing of a NIRS calibration, spectral information is related by an algorithm with information from the physicochemical (reference method) composition through the application of statistical models such as multiple regression, principal components analysis and partial Least Squares.

MATERIALS AND METHODS

Coffee samples

The initial database (32.000 spectrum) was built with samples obtained from different experiments of Cenicafé's breeding program, from regional trails, multiple crosses, which included the two most important species in the coffee market; gene bank accessions, samples from farmers' fields to represent all the possible variability in field conditions were included. In addition, samples from different coffee producing countries, which include countries in Central America, South America, Asia and Africa.

Green coffee samples were ground in a cryogenic mill RETSCH at 12,000 rpm to obtain a fine powder of particle size of less than 1 mm, 3 g per sample was packed in circular cups with a diameter of 3.75 cm.

Reference analysis

After extraction and purification, the content of caffeine, trigonelline, sucrose and fatty acids were determined by high performance liquid chromatography (HPLC) Chlorogenic acids were determined by ultraviolet visible spectrophotometry (UV-VIS) and lipid and fatty acids by gravimetric for gas chromatography detector SID. All analytical methods were validated in terms of precision, accuracy and repeatability.

NIRS analysis and calibration equation (CE)

The samples were scanned in reflectance mode (400–2500 nm) using a NIRSystemsTM 6500 monochromator (NIRSystems, Silver Spring, MD, USA) in a small circular cup (50mm diameter) (Part number IH-0325, NIRSystems, USA). Reflectance data were stored as the logarithm of reciprocal of reflectance ($1/R$) at every 2 nm interval to give a total of 1050 data points.

The selection of samples for the development of prediction equations was performed taking into account a priori differences in origin, harvest, and values of chemicals quantification in contrasting genotypes had an amplitude in the range to quantifying an additionally database of 32000 espectra files, samples were selected by discriminant analysis through SELECT WINISI algorithm program, which is based on Mahalanobis distance with a limit H> 3 as control.

Calibration equations were developed for samples selected from the population set, using modified partial least squares regression (MPLS) with cross-validation. Spectra were corrected for scattering by using standard normal variate (SNV) and detrend. Different derivative treatments of the spectra were used. Calibration statistics included the standard error of calibration (SEC), the coefficient of determination in calibration (*RSQ*), the standard error of cross-validation (SECV), and the variance residual (*1 - VR*) (Shenk & Westerhaus,

1996). Optimum calibrations were selected based on the highest R^2 CAL and lowest SECV. Thereby, the coefficient of simple correlation (RSQ), the standard error of prediction, the slope and bias were calculated.

RESULTS AND DISCUSSION

The parameters values used in the development of calibration equations is given in Table 1. The first aspect is, that the minimum number of samples with reference data used was 101 samples for total chlorogenic acids, the second group of compounds with a number between 231 and 237 for fatty acids and the third group is comprised of sucrose (290), lipids (235), caffeine (329) and trigonelline (328) with the highest numbers of reference analysis. The calibration models was an iterative process beginning with 50 discriminant samples selected from a first database of 10000 spectra files.

The parameters SEC, RSQ, SECV and 1-VR were classified as excellent in the next compounds: caffeine, trigonelline, lipids and stearic acid; classified as Good prediction models for sucrose, and the fatty acids: oleic, linoleic, linolenic, arachidic and behenic, and Total CQA, finally, a low prediction model was obtained for the palmitic fatty acid.

Table 1. Statistical parameters of the prediction equations of chemical compounds in coffee (expressed in % of dry matter).

Compound		N	μ	STDEV	MIN	MAX	SEC	RSQ	SECV	1-VR	Quality equation
Caffeine		329	1.32	0.29	0.45	2.19	0.04	0.98	0.06	0.97	Excellent
Trigonelline		328	0.93	0.15	0.48	1.39	0.05	0.90	0.07	0.80	Excellent
Sucrose		290	5.79	1.29	1.91	9.67	0.45	0.88	0.62	0.78	Good
Lipids		235	14.25	1.09	10.97	17.52	0.24	0.95	0.29	0.93	Excellent
Fatty Acid	Palmitic	235	35.95	2.61	28.13	43.77	1.23	0.78	1.53	0.65	Low
	Stearic	233	7.69	1.26	3.91	11.47	0.38	0.91	0.50	0.84	Excellent
	Oleic	234	8.93	0.94	6.11	11.76	0.35	0.86	0.45	0.77	Good
	Linoleic	239	40.22	3.62	29.36	51.08	1.50	0.83	1.86	0.73	Good
	Linolenic	237	1.45	0.16	0.97	1.93	0.06	0.85	0.08	0.76	Good
	Arachidic	233	3.00	0.46	1.61	4.39	0.18	0.85	0.23	0.75	Good
	Behenic	231	0.71	0.13	0.31	1.11	0.06	0.82	0.07	0.70	Good
Total CQA		101	5.20	0.51	3.68	6.73	0.24	0.83	0.46	0.82	Good

N: Number of samples; μ: mean, STDEV: standard deviation of the concentration values, MIN: minimum, MAX: maximum, SEC: Standard error of calibration; RSQ: coefficient of determination; SECV: standard error of cross validation; 1-VR: coefficient of determination after cross-validation.

In this case the National Coffee Growers Federation of Colombia and the National Coffee Research Center have implemented the use of the NIRS prediction models for the following routine analysis:

1/ Genebank Characterization by chemical compounds, and the monitoring of lines developed in the breeding program; 2/ Response to chemical compounds in advanced lines of the breeding program under regional trials; 3/ Implementation in studies of Designation of Origin (DO) and Geographical Indication (GI) of Café de Colombia; 4/ Implement the strategy of the origin 100% Café de Colombia identifying adulteration with other origins that produce Arabica and canephora coffee. These model applications are displayed next:

Genbank evaluation

Figure 1 shows a dendrogram constructed with the prediction of the compounds: caffeine, trigonelline and chlorogenic acids in samples from the Ethiopian collection and some materials developed in the breeding program. This figure shows the formation of three groups, the first correspond to materials developed in the breeding program of Cenicafé, the second correspond in the Ethiopian materials and the third is a mixture between Ethiopians and improved progenies. The variability allows searching parentals for the development of new varieties with contrast contents of chemical compounds associated with quality in *Coffea arabica*.

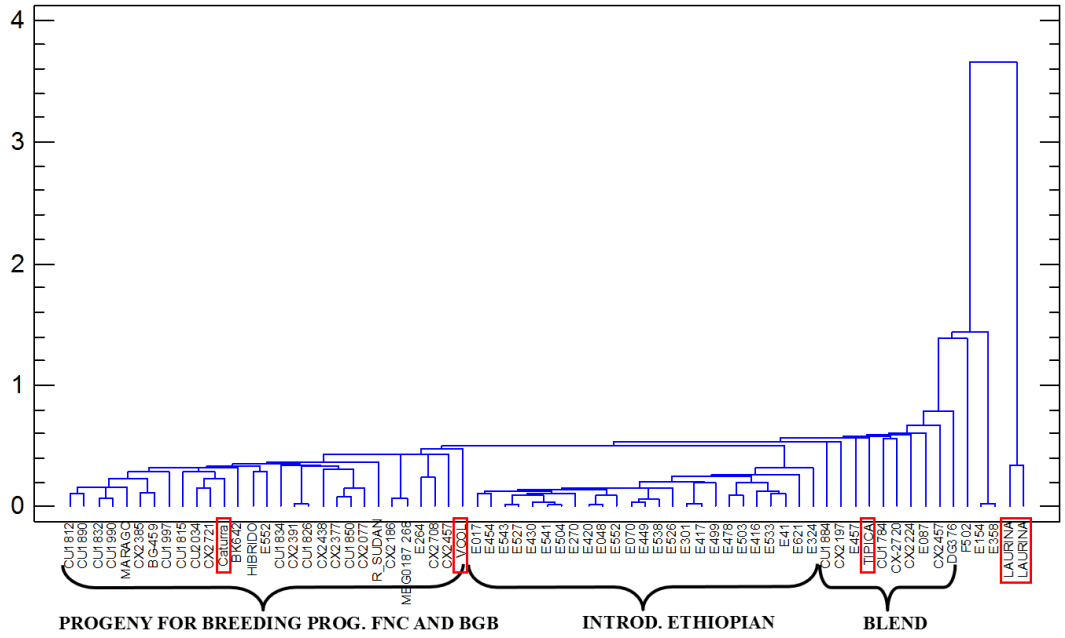


Figure 1. Diversity samples of the Colombian Coffee Collection by chemical compounds (Caffeine, trigonelline and chlorogenic acids).

Regional trials: advanced lines (Breeding program of Cenicafé)

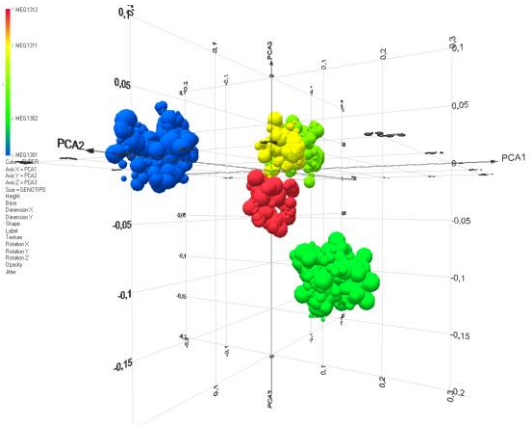
The breeding program of Cenicafé has been focused on the development of varieties that assemble characteristics of agronomic importance, especially in productivity, resistance to pests and diseases and quality aspects (bean sizes, sensory attributes and recently chemical compounds) that characterized the Café de Colombia.

A necessary evaluation of new materials in the regional trails, which involves the evaluation of any outstanding materials that are in an advanced generation, with the aim of establishing their productive potential, and adaptation to particular conditions and knowledge of the genotype by environment interaction of each coffee area in Colombia.

Table 2, shows the Mahalanobis distance that were statistically different in 5 locations of the Colombian coffee zones; these distances were determined for green and roasted coffee for the same genotypes at each site. It is observed that the spectral signature is different at each site and is independent of the materials evaluated.

Table 2. Mahalanobis distance between 5 regional trail sites of Colombia for green and roasted coffee.

COFFEE SAMPLE	REGION	2	3	4	5
Green	1	31.8	56.8	70.6	36.5
	2		51.4	34.0	38.4
	3			40.4	36.0
	4				39.7
Roasted	1	14.4	12.0	46.3	21.4
	2		19.2	63.1	32.2
	3			38.0	12.7
	4				39.6



1: Caldas. 2: Quindío. 3: Antioquia. 4: Cauca. 5: Cesar.

The table 3, shows the prediction for 6 compounds using the NIRS equations on 7 locations in Colombia coffee regions. After performing the analysis for three consecutive years, significant differences for concentration of all compounds determined by NIRS were found. Gradient in the concentration of caffeine, trigonelline between the north and south of Colombia was found. The higher lipid content were found in areas with production systems of shade trees (Santander and Cesar), sucrose content for the region with the highest concentration was Quindío and lower was found in Cesar.

Table 3. Chemical compounds for 7 regional trail in Colombia.

SITE	Caffeine		Trigonelline		Lipids		Arachidic acid	Total CQA	Sucrose
RISARALDA	1.24	b	0.85	cd	13.80	bc	3.29a	5.82de	5.97c
CALDAS	1.14	e	0.88	b	13.81	bc	3.28a	5.93c	6.02bc
CESAR	**1.32**	**a**	**0.81**	**e**	**12.69**	**d**	3.28a	6.21b	**5.22e**
QUINDIO	1.17	d	**0.92**	**a**	13.97	b	3.25a	**5.76e**	**6.58a**
ANTIOQUIA	1.21	c	0.84	d	13.89	b	3.24a	5.91cd	5.66d
SANTANDER	**1.11**	**f**	0.85	c	**14.25**	**a**	**3.02b**	**6.39a**	5.76d
CAUCA	1.21	c	**0.90**	**a**	13.60	c	3.24a	5.74e	6.14b
AVERAGE	**1.20**		**0.86**		**13.72**		**3.23**	**5.96**	**5.91**

Protected designation of origin

The information obtained from the experiments of regional trails, were used in value-added strategy of the National Coffee Growers Federation of Colombia to regionalize the quality of Café de Colombia within the concept of Protected Designation of Origin and Protected Geographical Indication.

Using geographic information systems and intensive sampling scheme for 5 years in field conditions has spatialized the chemical compound calibrated by NIRS, allowing a better understanding of the interaction between chemicals and product quality of Café de Colombia.

Figure 2 shows the spatial distribution of two chemicals linked to quality (caffeine and lipids) in two coffee grower departments (Tolima and Huila), a gradient between north and south for

the content of Caffeine was found, being lower in Huila. For the lipid content the south of Tolima and northern of Huila has the highest content of 14 to 17%.

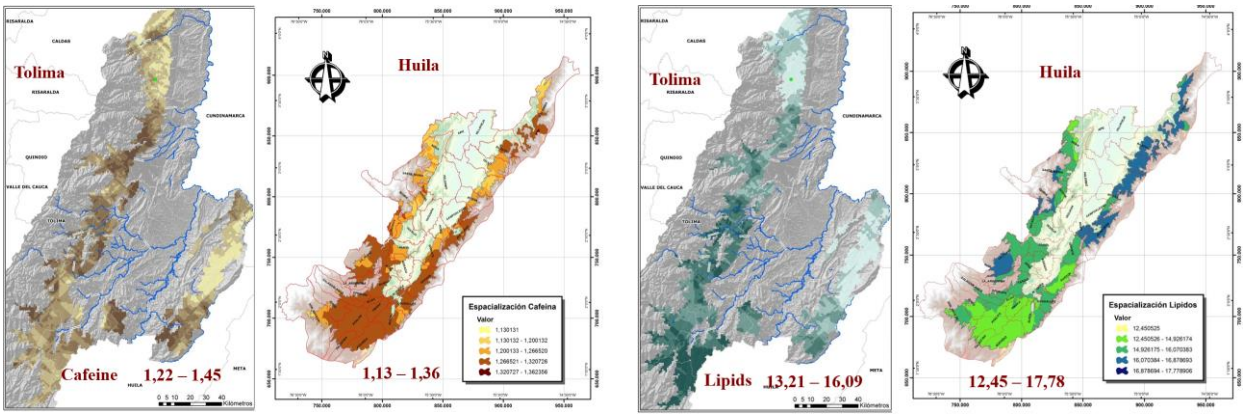


Figure 2. Spatial distribution of two compounds associated with quality in *Coffea arabica* for two Colombian coffee regions (Tolima and Huila).

NIRS databases have provided the spectral fingerprint (Figure 3) of 5 coffee regions of the country (Nariño, Huila, Cauca, Santander and Tolima) and this information has been used to protect coffee areas which are recognized as the source of high quality under the concept of DO recognized by the Superintendence of Industry and Commerce (SIC) in Colombia, under the following resolutions: Café de Nariño: (Resolution 06093 of February 11th, 2011), Café de Cauca: (Resolution 41788 of August 10th, 2011), Café de Huila: (Resolution 17989 of April 16th, 2013) and Café de Santander: (Resolution 50042 of August 25th, 2014.

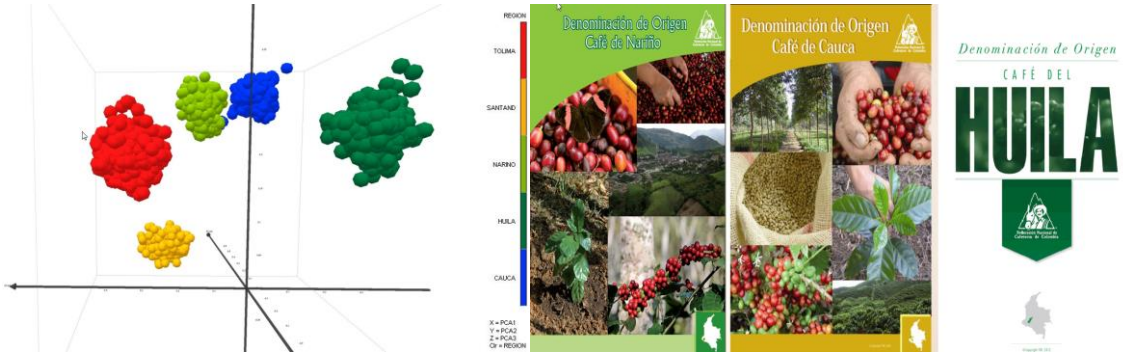


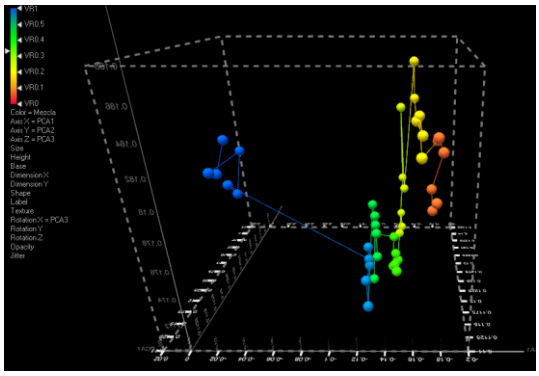
Figure 2. Spectral Signature for 5 regions in Colombia and three regions protected with PDO.

Defense of origin 100% Colombian Coffee

To identify blends of Café de Colombia with robusta coffee from other origins the chemical composition and behavior of the spectrum were quantified by NIRS. The results show an additive behavior for compounds calibrated by NIRS, especially caffeine, lipids, linoleic acid, arachidic acid and sucrose associated with blend ratio of 100% between the Café de Colombia and robusta (table 3).

Table 3. Chemical composition from eleven mixtures between 100% Café de Colombia and robust coffee.

Chemical compound	ARABICA (%)	ROBUSTA (%)					
	100	**100**	**10**	**20**	**30**	**40**	**50**
Caffeine	**1.3**	**2.0**	1.4	1.5	1.5	1.6	1.7
Trigonelline	**1.0**	**0.8**	1.0	1.0	1.0	0.9	0.9
Lipids	**16.1**	**12.2**	15.7	15.5	15.2	14.9	14.8
Palmitic	**35.2**	**46.6**	33.4	35.5	34.8	35.3	35.5
Stearic	**7.5**	**1.5**	7.0	6.5	5.4	4.6	4.7
Oleic	**9.8**	**17.1**	11.5	11.5	13.4	14.0	15.9
Linoleic	**38.6**	**32.7**	38.5	37.9	38.8	38.6	35.7
linolenic	**1.4**	**2.4**	1.4	1.5	1.7	1.8	1.8
Arachidic	**2.9**	**2.0**	2.9	2.8	2.5	2.4	2.4
Behenic	**0.8**	**0.4**	0.8	0.7	0.6	0.5	0.6
Sucrose	**5.3**	**2.6**	5.1	5.0	4.4	4.2	3.8



CONCLUSION

NIRS technique allows the analysis of chemical compounds in a fast, reliable and accurate analysis. We have developed 12 prediction models for chemical compounds with good parameters of acceptance (R2, SEP, SED, SECV).

The use of prediction equations allow the evaluation of the genebank and the advanced lines in which the chemical compounds can be associated with the quality.

This models and spectral database was used to support the technical studies of the Protected Designation Of Origin and used to defend the origin 100% Colombian Coffee.

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Impact of Various Extraction Parameters on the Sensory Qualities of Coffee

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SUMMARY

The way coffee is consumed has changed over recent years. With the arrival of pods and capsules, coffee preparation has been simplified, though "filter" coffee is still consumed. This raises a question: On what parameters of extraction is it necessary to act to obtain the best coffee whatever is the used machine?

To answer that question, we turned to sensory analysis as a tool for measuring how the sensory characteristics of these coffees have evolved. The sensory assessment was carried out by five experts from CIRAD, trained in accordance with standards ISO 8586-1 and 8586-2, 2008 version.

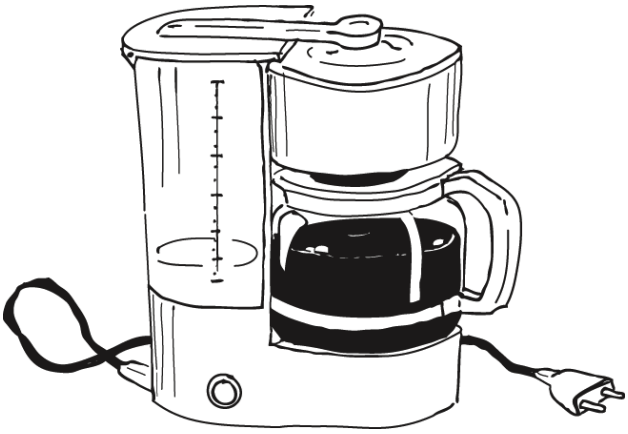
The tests were carried out on the same washed Arabica coffee from Honduras, taken from the same batch, roasted in the same roaster, at the same temperature, but at different times to obtain varying degrees of roasting. Different degrees of grinding were tested. In order to preserve the freshness of the coffee, all the tests were carried out over a very short time span and the coffee was packed in waterproof, opaque packs fitted with a one-way valve for degassing. The packs were then stored at minus 80°C pending tasting, so that the organoleptic qualities were not altered. Each sample was prepared as an espresso and in a filter coffee maker. In order to not influence the panellists, the coffees were blind tasted and the crema of the espressos was removed.

The results showed that the sensory characteristics of the coffees varied depending on different parameters, such as water hardness, degree of grinding, roasting time and type of extraction.

INTRODUCTION

The way coffee is consumed has changed over recent years. With the arrival of pods and capsules, coffee preparation has been simplified, though "filter" coffee is still consumed. This raises a question: On what parameters of extraction is it necessary to act to obtain the best coffee whatever is the used machine?

This study is showing how adjust technical parameters of extraction to correct the coffee cup quality of one origin on two ways of extraction: Filter & expresso.



MATERIALS AND METHODS

The tests were carried out on the same washed Arabica coffee from Honduras, taken from the same batch, roasted in the same roaster, at the same temperature, but at different times to obtain varying degrees of roasting. Different degrees of grinding were tested. In order to preserve the freshness of the coffee, all the tests were carried out over a very short time span and the coffee was packed in waterproof, opaque packs fitted with a one-way valve for degassing. The packs were then stored at minus 80°C pending tasting, so that the organoleptic qualities were not altered. All the parameters are recapitulated in the board below.

Parameters	**Espresso**	**Filter**
Roasting	Low (11') & rapid (9') velocity	Low (11') & rapid (9') velocity
Grinding	300µm, with 2 different grinder (2 or 3 cylinders)	600µm, with 2 different grinder (2 or 3 cylinders)
Water Hardness	100µS & 300µS	100µS & 300µS
Volume of water	80ml & 160ml	1l & 700 ml

Each sample is prepared as an espresso and in a filter coffee maker.

In order to not influence the panellists, the coffees were blind tasted and the crema of the espressos was removed.

We turned to sensory analysis as a tool for measuring how the sensory characteristics of these coffees have evolved. The sensory assessment was carried out by five experts from CIRAD, trained in accordance with standards ISO 8586-1 and 8586-2, 2008 version.

As coffees have different dry extract, we have diluted the more concentrated before cupping in order to allow comparison of coffee sensory attributes.

Sensory analysis was chosen as a tool for measuring how the sensory characteristics of these coffees have evolved.

The sensory attributes choose was: body, acidity, bitterness, astringency, sourness, fruity, harshness, green, earthy, metallic, phenol, burned.

RESULTS

Data was analyzed with a correlation matrix in order to highlight difference between each coffee. As the board shows (table 1), there is not much difference in spite of the used techniques of extraction. The variation of those parameters: grind size, type of extraction, impact the beverage quality on bitterness, acidity, body, astringent and harshness. Quality of water has an effect on fruity, green & burned taste.

The average of each attribute is used to build the spider graph below. As we can see the profile of the entire sample is very similar, and it's difficult to discriminate a coffee from another.

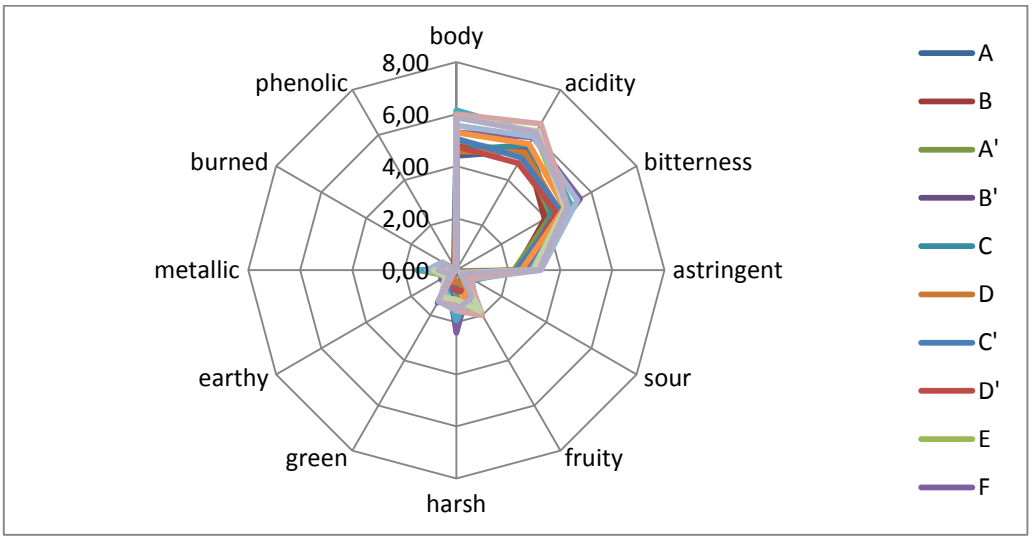


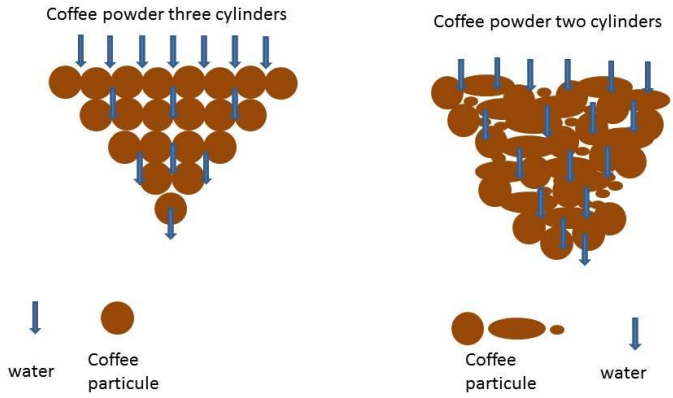
Figure 1.

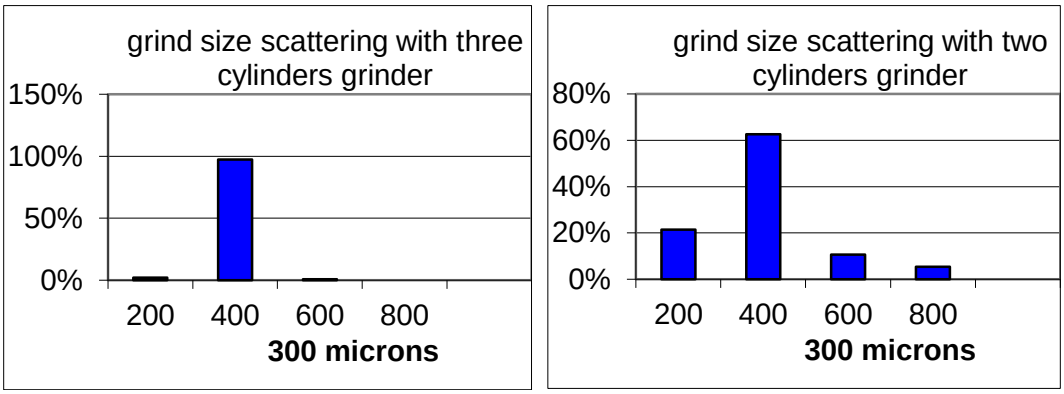
Table 1.

Variables	Body	Acidity	Bitterness	Astringent	Sour	Fruity	Harsh	Green	Earthy	Metallic	Phenol	Burned
Body	**1,000**	0,689	0,671	0,641	0,238	0,532	0,733	0,308	0,330	0,235	-0,289	0,179
Acidity	0,689	**1,000**	0,453	0,540	0,257	0,592	0,467	0,146	0,148	0,144	-0,276	-0,100
Bitterness	0,671	0,453	**1,000**	0,603	0,203	0,352	0,767	0,191	0,471	-0,081	-0,083	0,304
Astringent	0,641	0,540	0,603	**1,000**	0,178	0,448	0,533	0,131	0,383	0,153	-0,429	0,391
Sour	0,238	0,257	0,203	0,178	**1,000**	-0,107	0,479	-0,223	0,159	0,129	-0,164	0,091
Fruity	0,532	0,592	0,352	0,448	-0,107	**1,000**	0,154	0,262	0,033	-0,051	-0,134	0,114
Harsh	0,733	0,467	0,767	0,533	0,479	0,154	**1,000**	0,014	0,530	-0,009	-0,155	0,215
Green	0,308	0,146	0,191	0,131	-0,223	0,262	0,014	**1,000**	0,000	0,046	0,003	0,176
Earthy	0,330	0,148	0,471	0,383	0,159	0,033	0,530	0,000	**1,000**	-0,129	-0,005	0,363
Metallic	0,235	0,144	-0,081	0,153	0,129	-0,051	-0,009	0,046	-0,129	**1,000**	-0,330	-0,129
Phenol	-0,289	-0,276	-0,083	-0,429	-0,164	-0,134	-0,155	0,003	-0,005	-0,330	**1,000**	-0,109
Burned	0,179	-0,100	0,304	0,391	0,091	0,114	0,215	0,176	0,363	-0,129	-0,109	**1,000**
Roast time-9	0,172	0,058	-0,035	0,088	0,284	0,191	-0,044	0,126	0,065	0,358	-0,052	-0,005
Roast time-11	-0,172	-0,058	0,035	-0,088	-0,284	-0,191	0,044	-0,126	-0,065	-0,358	0,052	0,005
Grind size-300	0,841	0,795	0,740	0,655	0,215	0,505	0,764	0,254	0,338	0,129	-0,305	0,129
Grind size-600	-0,841	-0,795	-0,740	-0,655	-0,215	-0,505	-0,764	-0,254	-0,338	-0,129	0,305	-0,129
cylindre-2	-0,111	0,201	0,018	0,147	0,204	0,220	-0,013	-0,290	0,053	-0,021	-0,265	-0,090
cylindre-3	0,111	-0,201	-0,018	-0,147	-0,204	-0,220	0,013	0,290	-0,053	0,021	0,265	0,090
Espresso extraction	0,841	0,795	0,740	0,655	0,215	0,505	0,764	0,254	0,338	0,129	-0,305	0,129
Filter extraction	-0,841	-0,795	-0,740	-0,655	-0,215	-0,505	-0,764	-0,254	-0,338	-0,129	0,305	-0,129
Water quality-100	-0,073	-0,067	-0,148	-0,345	0,330	-0,586	0,246	-0,402	-0,012	0,307	-0,033	-0,406
Water quality-300	0,073	0,067	0,148	0,345	-0,330	0,586	-0,246	0,402	0,012	-0,307	0,033	0,406

DISCUSSION

In fact all the results of the espresso extraction are not completely correct. In lot of studies we can find that to have a « good » espresso, time of extraction must be between 20 &25 seconds. When we have realized the coffees we have set time of expresso extraction to 25sec. It is not a problem with a grinding of 300µm done by a grinder in two cylinders. But when we use the grinder in three cylinders the extraction becomes irregular and time of extraction passes below 20 seconds.





This problem is due to the homogeneity of the grind. In fact the regularity of coffee particles from the three cylinder grinder does not retain enough the water in the porta filter during extraction. To solve this problem we have reduce the number of hole in the porta-filter by adding a piece of metal (picture on the right).

CONCLUSION

It's really important to know & control all parameters of extraction to obtain a good coffee. Technology could help us to adjust the extraction whatever the initial quality of your coffee. The results showed that the sensory characteristics of the coffees varied depending on different parameters, such as water hardness, degree of grinding, roasting time and type of extraction.

Control of Volatile Aroma Compounds Release during the Rehydation of Soluble Coffee

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SUMMARY

The aim of this study was to review the current state of knowledge surrounding the key drivers that regulate the bio-accessibility of volatile aroma compounds released during instant coffee preparation and propose and ultimately evaluate practical mechanisms to manage aroma bio-accessibility during instant coffee preparation from a mechanistic perspective.

Dry instant coffee powders were prepared with modified lipidic, proteinaceous and carbohydrate fractions to represent the widest range of raw materials that may be realised through traditional coffee processing and production. Dry instant coffee powders were also prepared using a technology developed in the beverage industry through which high pressure pockets of inert gas are included within spray dried coffee powder, the pockets are released on hydration.

The impact of co-ingredients and the inclusion of high pressure pockets of inert gas was evaluated on the release of key aroma compounds during coffee preparation. It was shown that aroma release kinetics could be singificantly modifed through both approaches and that ultimately an inteligent approach to product design taking into account the presented results would offer product developers new tools to control consumer perception and liking.

INTRODUCTION

Coffee is a brewed product derived from the roasted seeds of *Coffea arabica* or *Coffea canephora.* This brewing process can be carried out in a number of ways, these may include high pressure extraction in the form of expresso preparations, boiling, steeping or filtration or coffee may be sold pre-extracted in the form of soluble or instant coffee, instant coffee will the topic of this work.

High quality coffee beans and an effective extraction confers a deep aroma, roasted taste with a good balance of acidity or bitterness. Incorrect choice of green beans, roasting process or extraction protocols will result in an imbalanced product with poor quality aroma. A balanced coffee aroma drives consumer liking and controls both product choice and repeat purchase frequency, it is therefore important to understand the mechanisms controlling aroma release and to develop tools to efficiently manage the abundance of volatile aroma compounds during beverage preparation

If aroma is considered, there are several stages during instant coffee preparation when the aroma of the coffee can be perceived by the consumer: the opening of the jar, mixing during

preparation of the dried powder in the cup with water, orthonasal perception of the brew after preparation and retronasal perception of the beverage on consumption. The rehydration phase of instant coffee preparation is an underexploited point of consumer interaction, as there is little or no flavour perception during this process.

This study will therefore evaluate two mechanistic approached to understand the impact of ingredient chemistry on aroma release and to evaluate the inclusion of pressurised gas within soluble coffee as a new technology for the enhancement of aroma release during hydration.

MATERIALS AND METHODS

Instant coffee powder was hydrated and the relative abundance of volatile organic compounds measured in the headspace during the hydration phase. Methods are described in detail within prior publications.

In brief, coffee samples were hydrated in a reaction cell and the headspace analysed using MS-NOSE technology as demonstrated previously in [1].

Samples of instant coffee were prepared by blending casein, fructose or sunflower oil to generate samples with equal coffee concentrations but varying levels of co-ingredients (lipid, sugar, protein).

Spray dried instant coffee was treated such that high pressure gas was included within the internal pores of the coffee matrix. To acheive this coffee (3 g) was placed within a 10 mL stainless steel pressure vessel; the vessel was pressurised to 40 bar (nitrogen). The pressure vessel was heated (60 ^{o}C, 90 ^{o}C, 100 ^{o}C, 110 ^{o}C or 120 ^{o}C) and held for 10 min. The pressure vessel was then cooled to 30 ^{o}C in ambient air, depressurised slowly and the coffee containing internalised gas was isolated [8].

Aroma release was measured using a bespoke powder dissolution cell connected APcI-MS headspace sampling interface [9-11] via a heated transfer line (60^{o}C) to prevent condensation. The dissolution cell consisted of a 450 mL vessel with sample introduction and analysis ports fitted within the lid. The vessel had a 70 mm elevation, and contained the dissolution water and a magnetic stirrer (200 rpm). Samples (1.5 g coffee equivalent) were introduced through a cylindrical entry port and physically pushed under the surface of the water (40 ^{o}C) with a syringe plunger, to minimise contact with the headspace gas.

SEM images were generated using a FEG SEM XL30 Philips SEM (USA). Samples were crushed and viewed using tungsten filament, secondary electron mode, spot voltage of 20.0 KV and spot size of 6.0.

RESULTS AND DISCUSSION

Spray dried instant coffee was prepared either through blending with co-ingredients (fructose, sunflower oil or casein) or through pre-treatment to included pressurised inert gas (nitrogen) within the internal voids of the structure. The structure of fragmented soluble coffee is shown in Figure 1, the internal voids can be clearly seen across the fractured planes of the coffee granules. The internal pores are heterogeneous but distributed ubiquitously across the soluble coffee matrix.

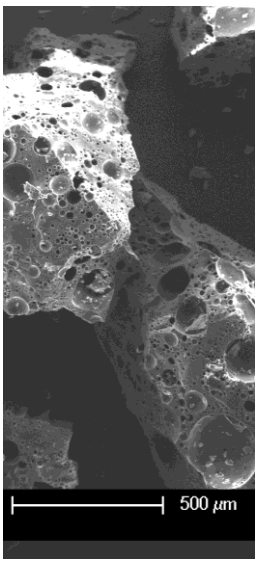

Figure 1. SEM micrographs of fractured soluble coffee showing the internal pore structure. Imaged were generated using a FEG SEM XL30 Philips SEM, (tungsten filament, secondary electron mode, spot voltage of 20.0 KV, spot size of 6.0).

Spray dried instant coffee was then hydrated within a bespoke reaction cell, on hydration volatile aroma compounds were released into the headspace, and an equilibrium was formed. Maximum intensity (Imax) was calculated from the different between the initial equilibrium point and the highest achieved headspace intensity, and the time to achieve this maximum intensity was denoted as Tmax. Standard soluble coffee hydrated with a smooth hydration curve which is typical of any dissolution reaction. The inclusion of high pressure inert gas within the voids of the soluble coffee resulted in a release of aroma that exceeded the equilibrium headspace concentration.

It is presumed that during the initial phase of hydrations, regions of highly concentrated solubilised aroma will be formed and released gas will deliver this to the headspace such that it exceeds the natural equilibrium or partitioning state. Over time the coffee will be fully dissolved and the elevated concentration will be lost and equilibrium will return, this can be clearly seen in Fig. 2.

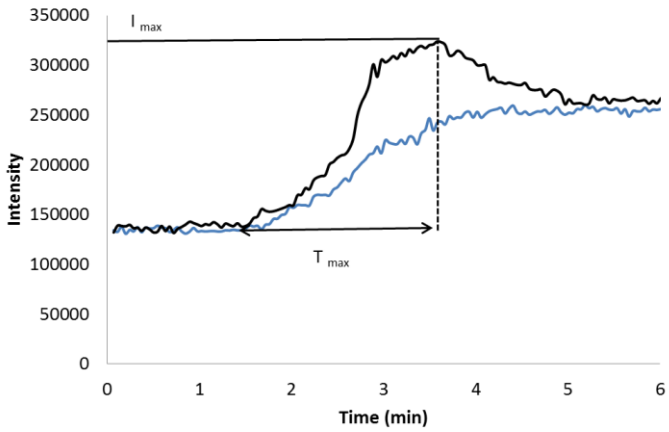

Figure 2. MS-NOSE release profiles illustrating the relative abundance of key aroma compounds in the headspace above hydrating instant coffee (lower), and the headspace above hydrating instant coffee containing high pressure internalised gas (higher).

The calculated Tg of soluble coffee under study was 45±1.0 °C. It can therefore be assumed that at process temperatures above 45 °C some gasification will occur, samples were prepared at 6 temperatures ranging from room temperature (25 ^{o}C), coffee samples prepared at temperatures above 60 ^{o}C showed an increase in Imax with a maximum observed at 120 ^{o}C. (Table 1). The high temperatures were observed to damage the structure, but temperatures below 110 ^{o}C produced an instant coffee with enhanced aroma delivery.

The time at which the maximum intensity was achieved varied across all samples and it was shown that a significant reduction in Tmax was achieved at 90 ^{o}C and 100 ^{o}C, the acceleration in delivery of aroma was not achieved at higher process temperatures (Table 1), this is presumed to be due to loss of structural integrity and reduced solubility at higher temperatures.

Table 1. Extracted parameters from MS-NOSE release profiles illustrating the maximum intensity (Imax) of key aroma compounds in the headspace above hydrating instant coffee (A), and the time to achieve maximum headspace intensity (Tmax) above hydrating instant coffee containing high pressure internalised gas (B).

A			
Temperature (^{o}C)	Imax		stdev
25	256667	±	15144
60	266333	±	21733
90	312800	±	18399
100	356333	±	20232
110	382667	±	22723
120	411000	±	38743
B			
Temperature (^{o}C)	Tmax		stdev
25	1.6	±	0.12
60	0.8	±	0.16
90	0.4	±	0.03
100	0.5	±	0.09
110	1.4	±	0.30
120	1.9	±	0.21

Spray dried instant coffee was then blended with co-ingredients to replicate the addition of dried milk powders, creamer or sugars. The impact of co-ingredient addition on aroma release during hydration was evaluated and is show in Table 2. The inclusion of either lipid or carbohydrate significantly suppressed the maximum headspace concentration and at higher concentrations the Tmax was reduced. The addition of protein had not significant impact on the release kinetics of aroma during the hydration process.

Table 1. Extracted parameters from MS-NOSE release profiles illustrating the maximum intensity (Imax) of key aroma compounds in the headspace above instant coffee (A), and the time to achieve maximum headspace intensity (Tmax) above instant coffee (B) containing varying concentrations of lipid, carbohydrate and protein.

[Lipid]	Imax	SD
0%	139743.3	51795.6
6.6%	115128.0	17380.8
11.8%	73649.3	27293.1
50%	31091.0	9894.8

[Lipid]	Tmax	SD
0%	1.097	0.175
6.6%	1.314	0.066
11.8%	1.084	0.131
50%	0.867	0.043

[Carbohydate]	Imax	SD
0%	102597.7	41692.7
8.5%	62298.3	20664.4
15.6%	39368.0	12741.6
50%	24480.7	6397.5

[Carbohydate]	Tmax	SD
0%	1.285	0.100
8.5%	1.170	0.130
15.6%	1.069	0.050
50%	0.997	0.044

[Protein]	Imax	SD
0%	67869.0	29184.3
6.6%	62298.3	20664.4
12.4%	41015.3	15288.7
50%	32922.0	9013.5

[Protein]	Tmax	SD
0%	1.141	0.132
6.6%	1.170	0.130
12.4%	1.271	0.090
50%	1.083	0.075

Results have been presented that will showed that increased concentrations of carbohydrate and lipidic material accelerated the rate of release but suppressed total aroma release; protein had no impact of aroma release kinetics. Naturally entrapped inert gas enhanced both the rate of release of volatile aroma compounds and the maximum gas phase concentration. There was a compound specific impact of both technologies.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Acrylamide Content of Soluble Coffee: Effects of Extraction Conditions, Extraction Scheme and Concentration and Spray Drying Processes

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SUMMARY

Coffee is consumed for its hedonic, stimulant and, in recent times, its antioxidants properties. On the other hand, it is also a source of acrylamide, a neurotoxic and carcinogenic compound which is formed during the roasting process. Acrylamide extraction from R&G coffee with commonly used brewing methods has been intensely studied. In this work we addressed the acrylamide extraction in a typical soluble coffee production process. Specifically, the effects of water temperature and drawn off extract:R&G coffee ratio on the acrylamide content of the obtained soluble coffee and its extraction yield were investigated. In addition, the effect of a fractionated extraction scheme on the acrylamide content of the obtained soluble coffee in each fraction was studied. Finally, the effect of the vacuum-evaporation and spray drying process on the acrylamide content of the soluble coffee was evaluated. Obtained results indicated that acrylamide content of extracted soluble coffee is affected by both feed water temperature and drawn off extract:R&G coffee ratio. Acrylamide extraction yields was high in all studied extraction conditions but it is slightly reduced with low drawn off extract:R&G coffee ratio. Higher overall extractions yields reduced acrylamide content of the obtained soluble coffee, but acrylamide content of the roasted coffee has to be taken into account. A fractionated extraction scheme produced 2 fractions of soluble coffee with the same concentration of acrylamide. Finally, it was observed that acrylamide content of extracted coffee is not affected by the vacuum-evaporation process but slightly reduced in the spray drying process.

INTRODUCTION

Coffee is a known source of polyphenols and their health benefits. However, it also contains acrylamide (AA) a neurotoxic, genotoxic and probable carcinogenic substance. Other heat treated products as french fries, breakfast cereals, biscuits are also sources of AA in human diet. Direct evidence of carcinogenic effects in humans as a result of AA intake is not available. Nevertheless, reduction of AA content in food is a major topic of interest.

The coffee roasting process and its influence in AA formation has been studied in detail. It has been established that the variables that do mainly influence AA formation are roasting time and temperature and species of coffee beans. Indeed, higher AA content is obtained in roasted Robusta than Arabica beans. Similarly, lighter roasted coffee contains higher amounts of AA than darker roasted coffee.

AA is highly water soluble (2155 g/l) thus it is easily extracted during the coffee beverage preparation. With most commonly coffee brewing methods, AA extraction yield is nearly 100% and only diminishes in low-volume beverages as espresso. Extraction of AA in an

industrial soluble coffee production process has not been reported. However, it is generally observed that AA content in instant coffee is higher than in roasted coffee.

In the present work it was studied the effects of extraction conditions on the AA content of obtained soluble coffee and the AA extraction yield. In addition a fractionated extraction scheme (aroma and hydrolysis) was studied to assess whether one of these fractions produced a soluble coffee with a low AA content. Finally, after the extraction process the AA content of the soluble coffee along the production line (evaporation, and spray drying) was studied.

MATERIALS AND METHODS

Roasted coffee: Dark roasted 100% Arabica coffee was degassed for 8-16 hours prior the extraction process.

Extraction process: COLCAFE pilot and industrial extraction plants were used in the present work. Both plants are geometrical similar with a size ratio of 1:10. Initially, water temperature and drawn off extract:R&G coffee ratio were modified to obtain different overall extraction yields. After stabilizing plant conditions 3 none-consecutive extractions were drawn off. Afterwards, a fractioned extraction process was performed in which the fresh percolation column was fed with the coffee extract drawn off from the previous percolation column. The initial obtained extract (Aroma) was separated from the later drawn off extract (hydrolysis) in a 1:9 ratio (w/w).

Evaporation and spray-drying process: Coffee extract from 6 different extraction trials were independently evaporated in an industrial-size evaporator. Operation conditions were 45°C and -0.78 bar, with the inlet and outlet concentrations ranging from10-15°Brix and 45-50°Brix, respectively. Each of the extracts coming out of the evaporator were independently dried in a pilot plant rotary disk spray dryer (Disk rotating speed, 3600 rpm and air inlet temperature, 108°C)

AA quantification: Both roasted coffee and liquid coffee extracts were analyzed for AA content by HPLC/MS/MS at Covance Laboratories (Greenfield, USA). The method for extraction and quantification of AA is based on the one reported by the United States Food and Drug Administration.

Yields calculation: Overall extraction yield was calculated as the soluble coffee obtained from the R&G coffee loaded in the fresh percolation column. The AA extraction yield was calculated as AA in the soluble coffee obtained from the AA present in the coffee beans loaded in the fresh percolation column.

Experimental design and data analysis: Initially a 2^2 factorial design with central point, with water temperature and drawn off extract:R&G coffee ratio as independent variables, was used to determine their effect on the AA concentration in the obtained soluble coffee and the AA extraction yield. Later on the differences of the AA content of the soluble coffee coming from a fractionated extraction were compared by an ANOVA analysis. Finally, coffee extract from 6 different extraction trials were subjected to evaporation and spray drying. The AA content of the soluble coffee coming in and out of these operation units was compared by an ANOVA analysis. Statistical analysis was performed with a statistical software MINITAB.

RESULTS AND DISCUSSION

After stabilizing plant conditions drawn off extracts were collected and the AA content of the obtained soluble coffee and AA extraction yield were calculated. It was observed that AA content of obtained soluble coffee was significantly affected by water temperature and drawn off extract:R&G coffee ratio ($p<0.05$), with no interactions among them (Fig. 1). Minimum AA concentration (dry base) in the soluble coffee was nearly 3 times higher than the roasted coffee fed on the percolation column (data not shown).

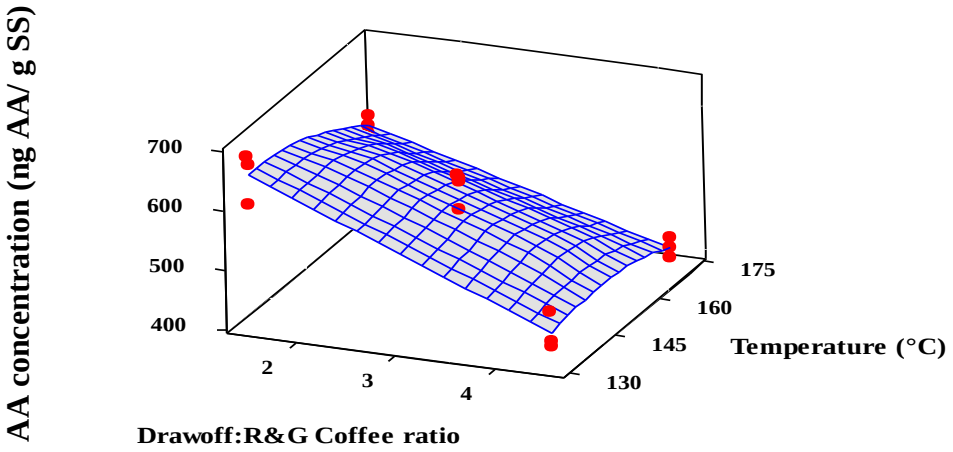

Figure 1. Effects of water temperature and drawn off extract:R&G coffee ratio on the acrylamide concentration of obtained soluble coffee.

At all tested conditions, AA extraction yield was high, with minimum yields around 80% (Fig 2). Interestingly, AA extraction yield was affected only by the drawn off extract:R&G coffee ratio ($p<0.05$) and not by the water extraction temperature (Fig. 2). A Tukey's multiple comparison test confirmed differences of AA extraction yield among all tested drawn off extract:R&G coffee ratios (Fig. 2). This result is in accordance to what has been observed with AA extraction in most commonly brewing methods, in which only low-volume extractions, as espresso, do not extract the full AA content of the R&G coffee. High AA extraction yields are also in accordance to how other small molecules behave in an industrial percolation process. Indeed it has been proposed that small molecules such as caffeine, are readily extracted by water and therefore be thoroughly extracted from the coffee particles.

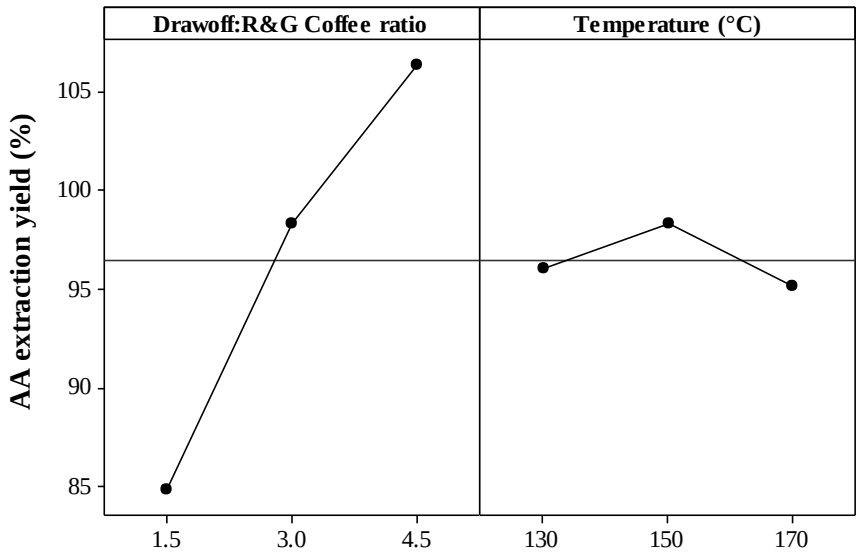

Figure 2. Effects of drawn off extract:R&G coffee ratio and water temperature on the acrylamide extraction yield.

A slight correlation (r^2=0.62) was observed between the overall extraction yield and the final AA concentration in the obtained soluble coffee (Fig 3). This correlation improves when the AA concentration of the loaded R&G coffee is taken into account (r^2= 0.79) (data not shown). Both correlations indicate that as AA is nearly fully extracted at all tested conditions (Fig 1), its concentration in the obtained soluble coffee decreases as a result of a dilution effect of the additionally extracted coffee solubles.

A fractionated extraction scheme (aroma and hydrolysis) was studied to determine whether soluble coffees with different AA content could be obtained in each fraction. For this, the initial extract coming out of the percolation column was separated from the extract coming out afterwards. In accordance to what has been reported soluble coffee concentration of the aroma extract was higher than the hydrolysis extract (Fig 3). However, AA content of the soluble coffee drawn off in both fractions were not significantly different (Fig 4). Caffeine content of the instant coffee obtained in both fractions was also not significantly different (Data not shown).

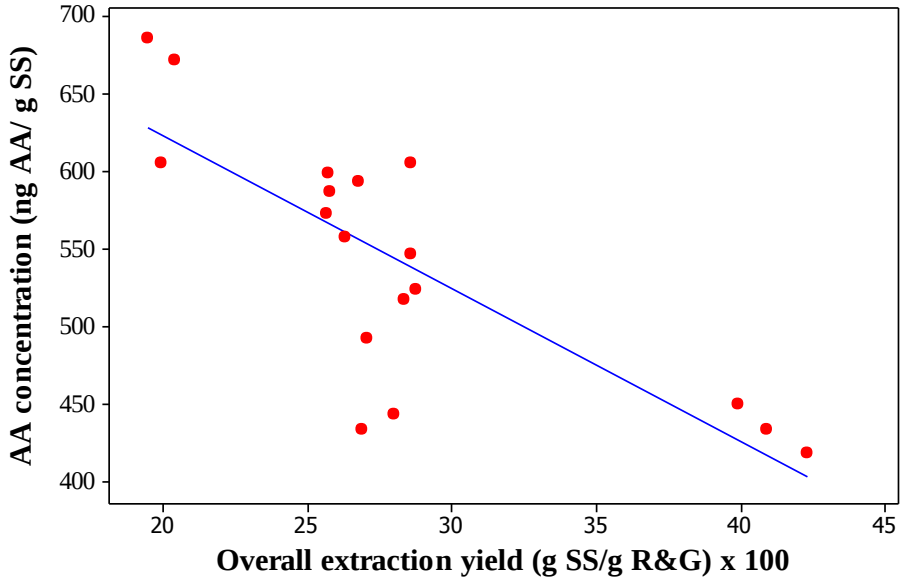

Figure 3. Influence of the overall extraction yield on the acrylamide concentration of obtained soluble coffee. AA (ng/ g SS) = 821 - 9.88 Yield (r^2 = 0.62)

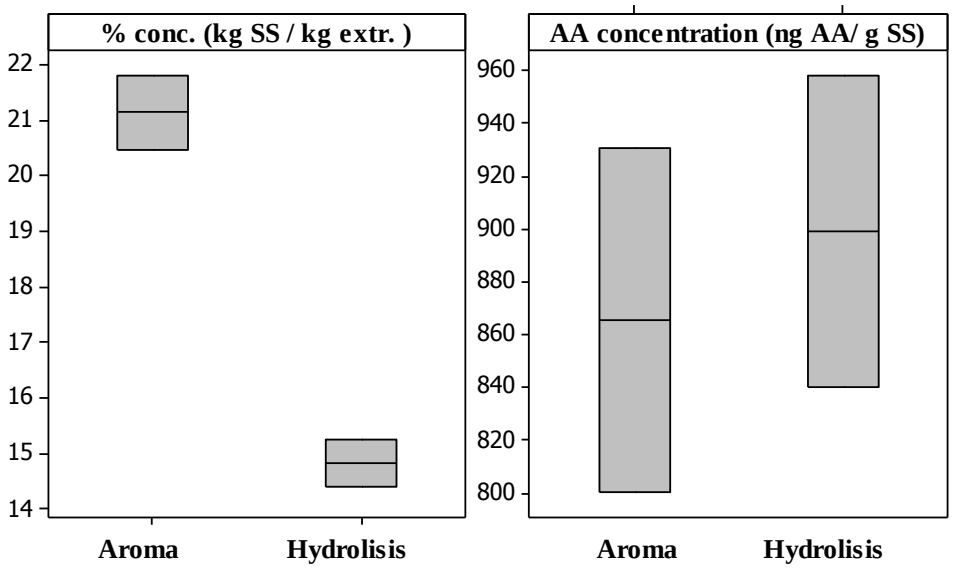

Figure 4. Effect of a fractionated extraction scheme on A) Concentration of coffee solubles in the obtained extract and B) Acrylamide concentration in the obtained soluble coffee.

Finally, it was observed that AA content of extracted coffee was affected along the production process ($p<0.05$). It was not affected by the evaporation process but slightly reduced in the spray drying process (Fig. 5). Indeed, a Tukey's multiple comparison test confirmed that AA concentration of the dried product was different to previous steps. During potato frying, at 150-180°C, AA evaporation alongside water has been observed, although in insignificant values. The surface area of the coffee drying droplet is enormous, compared to the potato chips. However, during coffee drying the coffee droplet does not reach so high temperatures as the ones reached by the potato. In fact, in used spray dryer this temperature should not have

been over 93°C, which should not result in AA evaporation. Therefore, the most probable cause of this reduction could be reactions of AA with melanoidins which are very highly temperature dependent.

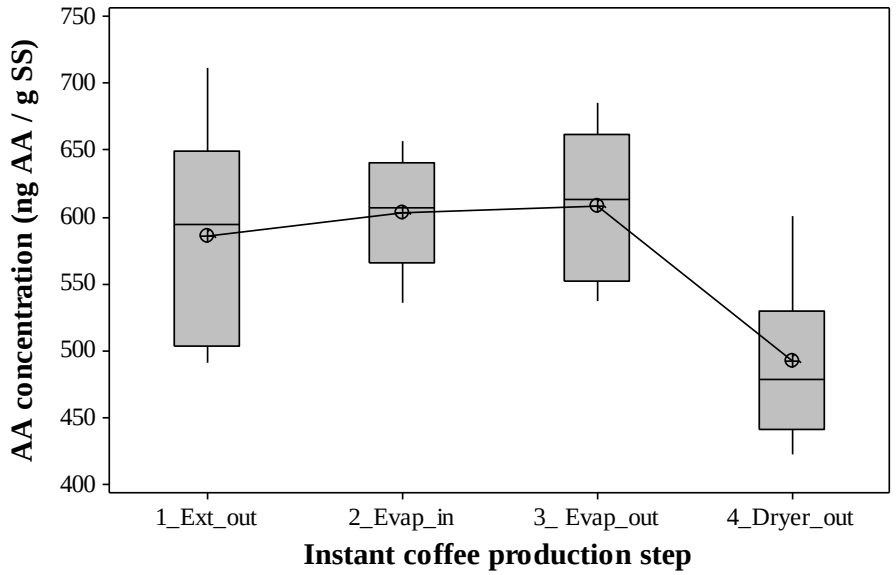

Figure 5. Acrylamide content of soluble coffee at different stages of the production process. 1) Extraction outlet, 2) Evaporator inlet, 3) Evaporator outlet, 4) Spray-dryer outlet.

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Descriptive Cupping – a Rapid Coffee Flavour Profiling Method Using the Specialty Coffee Association of America (SCAA) Cupping Protocol

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SUMMARY

The aim of this study was to assess the use of a cupping panel to obtain descriptive coffee flavour profiles. Specifically, this study focused on characterising the sensory profile of natural coffee using descriptive data generated using the SCAA Cupping Protocol. In this protocol, quality scores are used to negotiate coffee price and the descriptive data is disregarded. However, the descriptive data collected by the SCAA Cupping Protocol may provide a means to obtain a coffee flavour profile in origin countries that lack advanced sensory facilities and descriptive panels. Natural (dry method), green coffee samples (22) from 7 countries were prepared according to SCAA protocol. Samples were presented to a panel of 7 cuppers. Cuppers assessed the samples according to the SCAA protocol and provided descriptive information to support their quality scores. Descriptors from the cuppers were grouped into categories. A contingency table was constructed with samples as columns and categories as rows. The descriptive space was visualized using non-symmetric correspondence analysis (NSCA). Factor 1 (F1 – 30% total variation) described the samples based on origin. Nicaraguan coffees were negatively loaded on factor 1 and described as 'red-fruity', 'tropical-fruity' and 'sweet-acid'. Brazilian samples were positively loaded on factor 1 and described as 'toasty', 'caramelly' and 'phenolic'. Flavour differences may be linked to the drying rates used. This shows Descriptive Cupping has application as a rapid method to study flavour profile variation.

INTRODUCTION

In the case of coffee, flavour is the most important consumer parameter. Understanding the effect of local conditions and technology on regional coffee flavour would help coffee producers better extract value from their coffee. Conventional descriptive sensory methods, as *"the most sophisticated tools in the arsenal of the sensory scientist"*, would normally be the method of choice. However, training and maintenance of a descriptive panel requires substantial financial and administrative support. A cost-efficient methodology for profiling coffee flavour is needed for many origin countries. To this extent, coffee has an important resource in its certified coffee cuppers. Cuppers are expert coffee graders focusing on quality [4], but it should be possible to extract flavour profiles from the cupping data.

During the last 13 years the specialty coffee sector, namely the Specialty Coffee Association of America (SCAA), has adopted sensory evaluation procedures. The SCAA and the Coffee Quality Institute (CQI), have worked to overcome the cupping method shortcomings identified in 2002 by Feria-Morales through use of trained panels, blind testing, identification of important characters for the different coffee styles, assessment of a broader range of attributes, and emphasis on the importance of sensory quality. The term 'specialty coffee' and

the specifications of the ‘Q Coffee’ Certification Mark were defined through a standard and a grading protocol was issued for determining compliance with the standard. Only cuppers licensed as ‘Q Graders’ through a certification process by the CQI can be used to assess the compliance of a sample with the Q standard. Currently, there are 4,000 Q-Graders licensed in more than 60 countries, and more than 7,000 people have been trained. Licensed Q Graders need to pass 20 different tests, covering subjects such as general coffee knowledge, tasting acuity, smelling acuity, organic acids profile in coffee flavour, roasting standards for cupping, green coffee standards, roasted coffee standards, cupping skills and olfactory vocabulary. This ensures they have the knowledge and the sensory skills required for grading coffee. They are also trained and tested in a standard, structured vocabulary for the coffee bouquet, supported by the ‘Le Nez du Café®’ set of olfactory standards (Table 1).

Table 1 Descriptor structure from 'Le Nez du Café®' used in Q Grader training.

Enzymatic	Sugar Browning	Dry Distillation	Taints and Faults
Vegetable • Potato • Sweet peas • Cucumber	Nutty • Roasted almonds • Roasted hazelnuts • Walnuts	Spicy • Cloves • Black pepper • Coriander seed	Earthy • Earth • Straw • Leather
Floral • Tea rose • Coffee blossom • Honey	Caramelly • Fresh butter • Caramel • Roasted peanuts	Resinous • Cedar • Blackcurrant • Maple syrup	Fermented • Coffee pulp • Basmati rice • Medicinal
Fruity • Lemon • Apricot • Apple	Chocolaty • Vanilla • Toasted bread • Dark chocolate	Pyrolytic • Malt • Pipe tobacco • Roasted coffee	Phenolic • Boiled beef • Smoke • Rubber

The SCAA Cupping Protocol details the sample preparation protocol, the cupping procedure and the scoring criteria for grading coffees. The cupping procedure describes the different assessment stages: fragrance assessment, aroma assessment and liquoring at hot, warm and tepid brew temperatures. Ten different attributes are scored: ‘Fragrance/Aroma’, ‘Flavour’, ‘Aftertaste’, ‘Acidity’, ‘Body’, ‘Uniformity’, ‘Balance’, ‘Clean Cup’, ‘Sweetness’ and ‘Overall’. Cuppers are expected to rate the intensity and to describe the qualities (i.e. using the descriptors from Table 1) of some of the attributes, as a justification for their quality scores.

SCAA cupping has been used to assess the effects of yeast strain, method of storage, and type of processing conditions on coffee quality. SCAA cupping has also been used to compare quality attributes of regional coffees. Studies requiring coffee flavour profiling have used other sensory techniques. Temporal Dominance of Sensations (TDS) was used to study the effect of yeast strains on natural coffee flavour, though these results were not compared to the cupping data. Traditional descriptive sensory analysis methods were used to investigate effect of coffee storage study on the brew flavour. Time Scanning Descriptive Analysis (TSDA) was used to evaluate the flavour of coffee brew as it cools down. To the best of our knowledge, SCAA cupping has not been used as a means to acquire information about coffee flavour. However, Q-Graders and other SCAA Protocol-trained cuppers have many advantages as trained sensory assessors. Cuppers have been screened for sensory skills, are trained in the use of the SCAA Cupping Protocol and standard descriptive terms, use a common descriptor language worldwide, justify their cupping scores with descriptive information, are widely

available in origin countries and are enthusiast volunteers. The aim of this study is to assess the use of trained cuppers for coffee flavour profiling and hence to define this approach to coffee flavour profiling as 'Descriptive Cupping'.

In this study, Descriptive Cupping was investigated as a means to characterise the sensory profile of 'natural coffee'. 'Natural coffee' is produced using the dry method, whereby the whole coffee fruit is dried after harvest. Conflicting information about the quality of Natural Coffees and their flavour exists in the industry. However, Natural Coffees are becoming an increasingly valuable part of the specialty coffee industry. Recently the CQI introduced a natural coffee cupping session in the Q-Grader exam. Despite increased interested in natural coffee, no studies have characterised the flavour of natural coffee.

MATERIALS AND METHODS

Samples

Natural coffee samples (22) were provided by exporters and farmers from 7 natural coffee producing countries (Table 2). Coffees were received as green beans, harvested in the 2011-2012 crop year. All samples were processed using the dry method, with drying times to achieve a final moisture content of 12% ranging from 4-21 days. Coffee samples were selected to enable the flavour spectrum of natural coffee to be covered.

Table 2. Origin and variety information for natural coffees evaluated by descriptive cupping.

Sample code	Origin	Variety
BNI	Brazil	Catuaí & Catucaí
BNO	Brazil	Mondo Novo
BNX	Brazil	Unknown
CNX1	Colombia	Unknown
DNT	Dominican Republic	Typica
ENX	Ethiopia	Unknown
PNG	Panama	Gesha

Sample	Origin	Variety
HNX	Mexico	Un-known
MNM	Mexico	Marago-gype
MNT	Mexico	Typica
VNG	Mexico	Gesha
NNB1	Nicaragua	Bourbon
NNB2	Nicaragua	Bourbon
NNB3	Nicaragua	Bourbon
NNB4	Nicaragua	Bourbon

Sample	Origin	Variety
NNB5	Nicaragua	Bourbon
NNC	Nicaragua	Caturra
NNH	Nicaragua	H2 Hybrid
NNP1	Nicaragua	Pacamara
NNP2	Nicaragua	Pacamara
NNR	Nicaragua	Mara-caturra
NNY	Nicaragua	Yellow Catuaí

Experimental

A cupping panel (5 males, 2 females) was recruited and trained over 9 sessions in Dunedin, New Zealand. Assessors were trained to recognize and describe the standard coffee aroma descriptors used by Q Grader training (Table 1) and also in four additional descriptor categories specific to natural coffees. These included sweet spices (cinnamon and cardamom), dried fruit (prunes, raisins, dates and figs), red fruit (açaí flavouring, redcurrant jelly) and tropical fruit (passion-fruit flavouring).

In session one, assessors were trained on the SCAA Cupping Protocol. During sessions 2-8, assessors were trained on six cupping sections. These included 'Fragrance/Aroma', 'Flavour', 'Aftertaste', 'Acidity', 'Body', 'Balance' and 'Overall' . The last session focused on the use of the Catador® iPad® App to record the cupping data. In each training session, two coffees were cupped. Coffee pairs were selected for their contrasting attributes.

Samples, roasted following the SCAA Protocol, were cupped in duplicate over 6 sessions and evaluation recorded using Catador® App. During each session, 6 samples were cupped by the panel following the SCAA protocol. This produced three types of data: attribute intensities for fragrance, aroma, acidity and body, attribute quality scores for all the cupping sections, and freely-elicited terms.

Data Analysis

Flavour profiles for the coffees were prepared from freely-elicited terms using a sequential protocol: (a) preparation of contingency table, (b) tests of significance, (c) visualisation of flavour profiles.

The contingency table was prepared by incorporating the freely-elicited terms into separate lists based on bouquet, taste, aftertaste duration, acidity, and mouthfeel. Bouquet consisted of fragrance, aroma, and the olfactory components of flavour and aftertaste terms. Within each list, terms with the same meaning were merged into one descriptor. For example, 'chocolates', 'dark chocolate' and 'chocolaty' were merged into the descriptor 'chocolate'. Next, the descriptors were merged by category. In addition to the categories in Table 1 and the four specific to naturals, supplementary bouquet categories were added based on cuppers' comments. Descriptors for taste, aftertaste duration, acidity, and mouthfeel were also listed and categorised. Finally, the contingency table was constructed with the categories as columns and the samples as rows. Each cell contained the sum of mentions for a given category for each sample.

Significance tests were run on the contingency table. The dependence of descriptor category on the sample was tested using the Monte Carlo method (5000 simulations, $\alpha = 0.05$). A global χ^2 was performed to identify the most and least discriminant descriptor categories. χ^2 per-cell was carried out to determine significant descriptor categories for individual samples ($\alpha = 0.05$). Duplicate samples were aggregated for Monte Carlo and χ^2 methods.

The visualisation of the flavour profile was obtained by applying non-symmetrical correspondence analysis (NSCA) to the contingency table. To further understand the links between the flavour profile and quality scores, the correlations between the descriptors and the other data sets (attribute intensities and scores) were explored using multifactorial analysis (MFA). All statistical analyses were carried out using XLstat (Addinsoft SARL).

RESULTS AND DISCUSSION

Flavour profiles for the cupped samples were generated from the freely-elicited terms using a sequential protocol:

(a) For the preparation of the contingency table, the terms were classified in a total of 33 categories. Bouquet descriptors included from the 'Le Nez du Café®' vocabulary were *caramelly, chocolaty, citrus-like, earthy, fermented, floral, fruity, nutty, phenolic, pyrolytic, resinous, spicy, stone-fruity, toasty, woody* and *vegetable;* from the supplementary natural coffee vocabulary, *dried-fruity*, *red-fruity tropical-fruity*, and from the freely-elicited categories, *acid-smell*, *fungal*, *complex past-croppish* and *pungent.* As stated previously, additional bouquet descriptors to those of the standard and natural categories were included to account for supplementary cupper comments. Taste descriptors included *bitter* and *sweet.* Aftertaste duration consisted of the term *long-aftertaste.* The acidity category contained the

descriptors *dry-acidity*, *medium-acidity* and *sweet-acidity*. Mouthfeel contained *rough-body*, *smooth-body* and *astringent* descriptors.

(b) Significance was tested on the contingency table. The dependence between descriptors and samples was significant ($p = 0.003$), as shown by the 'Monte Carlo' method applied on the frequency table (aggregated duplicates, 5000 simulations). The global χ^2 identified *Past-croppish* ($\chi^2 = 52.7$), *Phenolic* ($\chi^2 = 48.1$) and *Fungal* ($\chi^2 = 46.2$) as the most discriminant categories. The least discriminant categories were *Chocolaty* ($\chi^2 = 10.0$), *Complex* ($\chi^2 = 10.4$) and *Medium-acidity* ($\chi^2 = 11.5$). The least discriminant categories would be typical of natural coffee profile findings, as least discriminant would suggest that they are common in naturals regardless of origin. Application of χ^2 per-cell found that, for eight samples, one or two descriptor categories were significantly above the theoretical average. These categories were *earthy*, *fermented*, *fungal*, *past-croppish*, *phenolic*, *pyrolytic*, *woody* and *vegetable* ($\chi 2$ test per-cell, $\alpha = 0.05$). This would indicate that these eight samples had distinct character.

(c) The flavour profiles were plotted. NSCA on the frequency table (non-aggregated duplicates) shows that 75.3% of the total information was represented in the first 8 factors. Inertia took values of 31.3% and 9.0% on Factor 1 (F1) and Factor 2 (F2), respectively. Samples positively loaded on F1 were described as *red-fruity* (26.4% of contribution) and *tropical-fruity* (8.5%) (Figg. 1 and 2). Those negatively loaded on F1 were described as *Phenolic* (9.9%). This corresponds to two commonly recognised natural coffee profiles. The first is the so-called 'Mocha' profile, which is characterised by its fruitiness. The second is a profile closer to that of washed coffee with little or no fruitiness. In this study, these two profiles appear to be due to a process factor having a geographical concentration. The Nicaraguan samples are negatively loaded on F1, and described by the Mocha-like descriptor categories. The Brazilian samples are positively loaded and described as more phenolic and caramelly. The Mexican samples sit between Nicaraguan and Mexican samples, near the origin.

Factor 2 (F2) is positively loaded with *Fungal* (10.4%), opposing *Chocolaty* (21.6%) and *Pyrolytic* (10.0%) on the negative side. The level of experimental error along this factor begins to be important, as can be seen by the placement sample duplicates. However, it is still clear that *Chocolaty*, with such a large contribution, is a character that does not depend on the origin, but is found in many types of coffee. Natural coffees combining fruity and chocolaty characters, for example, are not uncommon.

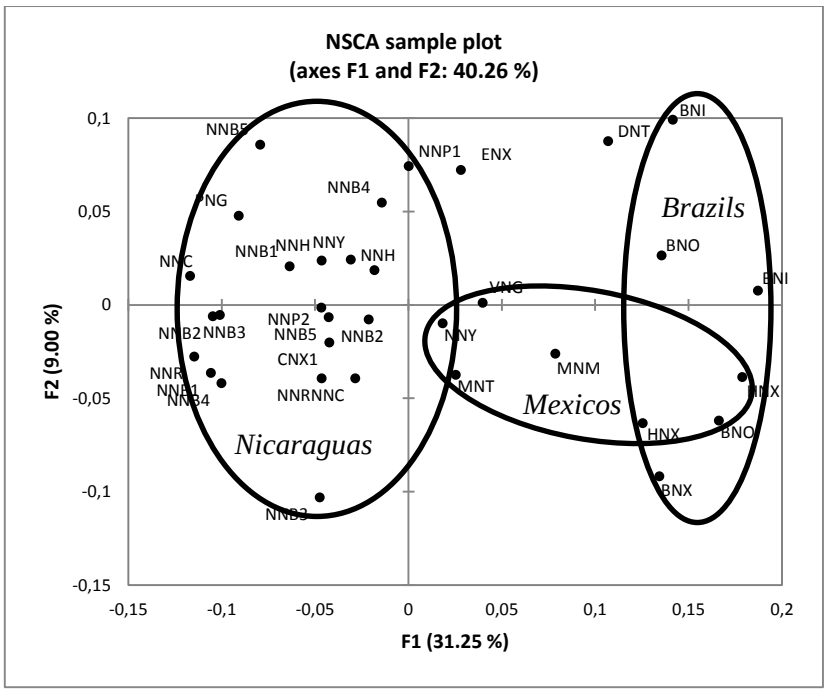

Figure 1. Non-symmetrical correspondence analysis (NSCA) map representing the projection on F1 and F2 of natural coffees for 22 samples, evaluated using the descriptive cupping method. Samples with the same name are duplicates.

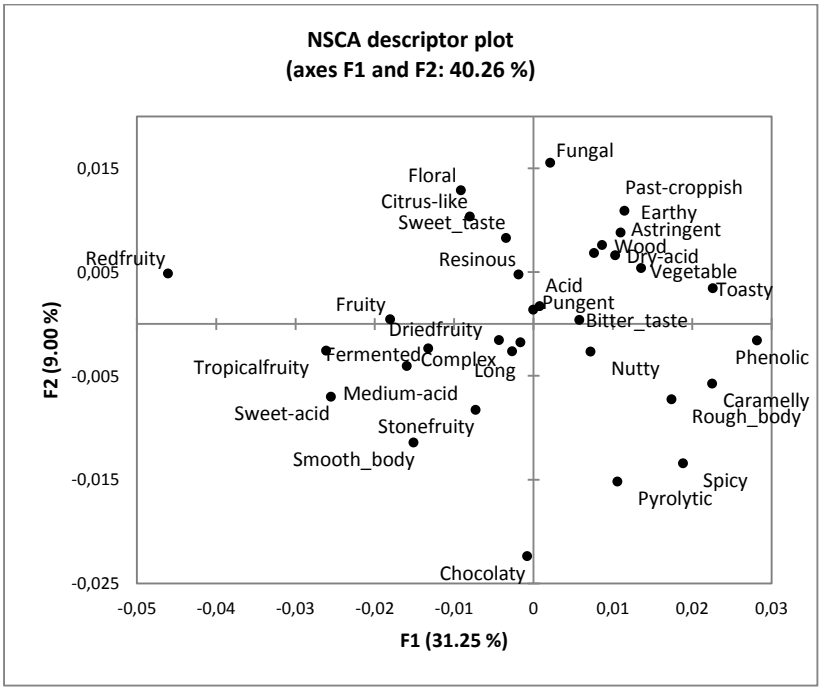

Figure 2. Non-symmetric correspondence analysis (NSCA) map representing the projection on F1 and F2 of descriptor categories for 22 coffee samples, evaluated using the descriptive cupping method.

Beyond flavour profiling, this study also aimed at understanding natural coffees as a product class, including the investigation of links between flavour and other variables. The correlations between the descriptor categories and quantitative data such as attribute intensities, quality scores and some raw bean parameters (colour, bean density) were thus explored using MFA. The RV coefficient (indicative of correlation between the variable

groups) between descriptor categories and quality scores was 0.639, and between descriptor categories and attribute intensities it was 0.598, showing there is some correlation between the descriptors and the other types of data. Figure 3 shows the MFA map for the variables studied. Attribute intensities and quality scores are correlated between them (RV coefficient of 0.761) and are located at the positive end of F1. In this case, attribute intensities and quality scores are directly correlated with a natural coffee's fruitiness and sweetness, and inversely correlated with the presence of phenolic characters. General fruitiness and sweetness are in turn correlated with a higher raw bean density and a darker, redder/yellower raw bean colour. This colour in the raw natural beans may be due to a slower drying rate, which allows for more intense pulp fermentation.

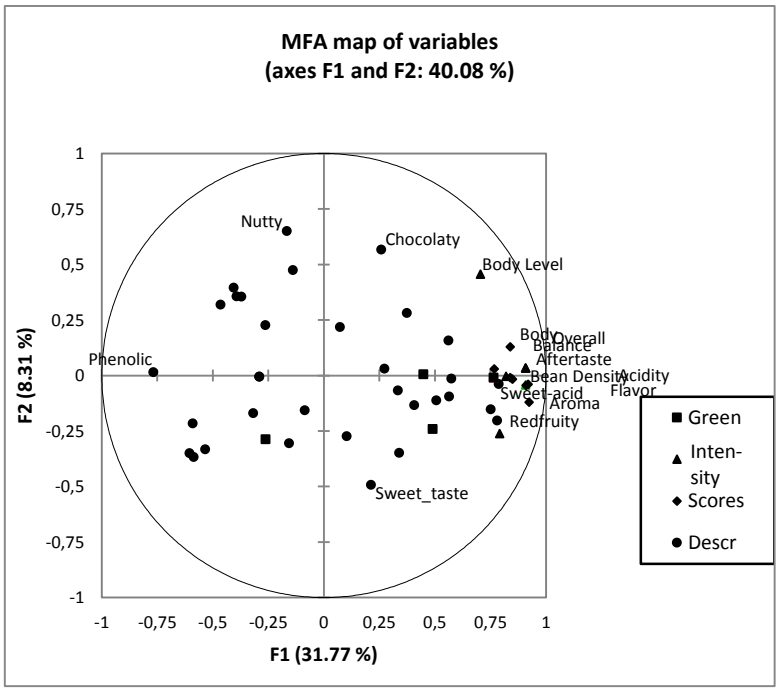

Figure 3. Multifactorial analysis (MFA) map representing the projection on F1 and F2 of descriptor categories (circle), attribute intensities (triangle), quality scores (diamond) and green bean parameters (square) for 22 coffee samples, evaluated using the descriptive cupping method. Only key labels are shown, to improve readability.

CONCLUSION

Descriptive Cupping was able to differentiate between different origins of natural coffee. This study suggests cuppers can be used to provide objective flavour profiling data. This approach appears as a useful methodology to measure variation in cup profile coming from genetic, geographical, varietal or technological factors, which implies a number of coffees is needed in order to provide context. Using these data analysis techniques, the grader's cupping "style" and criteria can also be examined. Further research is needed to compare descriptive cupping data to descriptive sensory analysis, and to other sensory, analytical or categorical variables. Descriptive Cupping was applied in this study to characterise the flavour of natural coffee, which shows a wide variation depending on the origin. In naturals, the main variation, between phenolic/caramelly and fruity characters may be partly due to drying duration, which has a geographical aspect due to local farming practices and coffee drying practices, including drying batch size.

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Cup Quality of Arabica Coffee Variety with Low Caffeine Content

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SUMMARY

This work was carried out at Agronomic Institute (IAC) to study the beverage quality of selecting arabica coffee varieties with low caffeine content in the beans. To this purpose, the sensory profile of commercial varieties, Ethiopian acessions and several intraspecific hybrids, totalizing twenty eight genotypes, was evaluated according to the SCAA protocols. It was observed that commercial varieties show a typical cup quality of arabica coffee, while some selecting hybrids have showed a complex sensory profile, with aroma and flavor characteristics similar to herbs, mixed spices and berries, often with an extremely pleasant and rare sensory perception that could be classified as an exotic coffee. Beans from F_2 and F_1BC_1 hybrids between Ethiopian accessions and Brazilian commercial varieties have showed outstanding cup quality with an unique sensory attributes, emphasizing that the flavor and aroma are affected by the variety. Additionally, it was observed some specific sensory attributes among plants from the same progeny wich produce an unique flavour profile, indicating the need for individual plant assessment for the future selection of arabica coffee variety with low caffeine content in the beans and differentiated intrinsic cup quality.

INTRODUCTION

Coffea arabica is the widely specie cultivated and consumed around the world, due mainly to its intrinsic beverage quality. Aroma and flavor of roasted coffee beans are some of the key attributes related to cup quality, but the development of these attributes during the roasting depends on the precursors present in the green beans whose quantity and quality are inherent to variety, environmental conditions, ripening stage and post-harvest processing. Considering that the aroma and taste of coffee are affected by the genetic constitution of the variety, it is necessary to characterize the sensory profile of the progenies during breeding process aiming to select new varieties with better cup quality. The identification of arabica coffee variety with a low caffeine content in the beans at IAC germplasm bank is resulting of a long term research conducted since 1959. Nowadays some selected genotypes are unique in the world and undoubtedly will constitute an important alternative for the coffee breeding to obtain coffees with differentiated intrinsic quality and exotic sensory profile, according to the specialty coffee market requirement. At the same time this will attend the needs of consumers sensitive to caffeine without interfering in the coffee flavor by any artificial decaffeinated method. Several authors have considered that genetic constitution of the variety is a primordial factor to determine the taste, aroma and other sensory attributes of the beverage, but they pointed that the selection of new coffee variety requires additional evaluations for quality attributes and others desirable traits under various field management and processing techniques.

MATERIALS AND METHODS

The experiment was carried out at Agronomic Institute (IAC), Campinas, Sao Paulo, Brazil, during the 2012/2013 crop year. It was evaluated the beverage quality of several arabica coffee progenies with low caffeine content in the beans from different F_2 and F_1BC_1 hybrids artificially obtained by crossing of Ethiopian accessions with Brazilian commercial varieties. The varieties IAC 045125, Mundo Novo IAC 374, Catuai Vermelho IAC 81 and Obata IAC 1669-20 were used as control. Full ripe coffee fruits were submitted to semi washed process (pulped natural coffee) and the parchment coffee was sun dried until the grain reached moisture content of 11%. After hulling the green coffee beans were classified by size into screens with circular and oblong perforations to determine the predominant bean size. Coffee beans sized up to 15 and without defective beans were submitted to sensory analyses according to Specialty Coffee Association of America (SCAA) procedures. The sensory attributes were assessed and rated on a six to ten-point scale, where the lowest value for the fragrance/aroma, flavor, acidity, body, aftertaste, balance and overall perception correspond to a very poor quality and the highest value correspond to a outstanding quality. The uniformity, cleanliness and sweetness were also evaluated by cumulative score and the results reported in this work are the average of five cup of each genotype from the same progeny.

RESULTS AND DISCUSSION

The results showed that twelve of the selecting hybrids (F_2 and F_1BC_1) were excellent coffees according to the SCAA scale (Table 1), where the highest cumulative score was over 85 out of 100 points (IAC 14/2008, IAC 20152-6, IAC 04/2008, IAC 06/2008, IAC 01/2008, IAC 20289, IAC 20275, IAC 20286, IAC 20300, IAC 20299, IAC 20271 and IAC 20298), expressing better cup quality than its parentals. It was observed that the hybrids IAC 14/2008, IAC 20152-6, IAC 04/2008, IAC 06/2008 and IAC 20289 descendant of the 'Mundo Novo', 'Catuaí Vermelho' and 'Obatã' varieties present high potential to produce differentiated coffees due its intriguing cup quality that reflect the junction of low caffeine content, complex sensory profile and higher SCAA score, which ones can be constitute a new hybrid variety in the future. All commercial variety (Mundo Novo, Catuaí Vermelho and Obatã) showed a typical sensory profile of arabica coffee, while some hybrids showed a complex sensory profile, with aroma and flavor characteristics similar to herbs, earthy, mixed spicy and berry, causing an extremely pleasant and rare sensory perception, being classified as an exotic coffee. Beans from some hybrids between Ethiopian accessions and commercial varieties have showed outstanding cup quality with an unique sensory attributes, emphasizing flavor and aroma, which distinguished them as exceptional complexity, revealing the high genetic potential of Ethiopian genotypes for the cup quality improvement in Brazil. The findings of this study demonstrate the existence of quality diversity among selecting hybrids in organolepic attributes and caffeine content, that can be exploited in the genetic improvement of coffee quality.

Table 1. Caffeine content of the coffee beans (%), SCAA score and main sensory characteristics of different F_2 and F_1BC_1 selecting hybrids and control varieties of *Coffea arabica*.

Genotype	Caffeine content (%)	SCAA score	Sensory characteristics
Control varieties:			
IAC 045125	0,03 - 0,44	83,00	Fruity, aromatic herbs, exotic
Mundo Novo IAC 374	0,82 - 1,23	84,50	Fruity, common flavour
Catuai Vermelho IAC 81	1,02 - 1,19	80,00	Chocolate, common flavour
Obata IAC 1669-20	0,95 - 1,07	82,50	Fruity, common flavour
Hybrids (F_2) derived from 'Mundo Novo' x 'IAC 045125':			
IAC 14/2008	0,17 - 1,13	87,00	Tobacco, herbal, spice, exotic
IAC 20136-17	0,19 - 1,36	83,00	Fruity, floral
IAC 11/2008	0,24 - 1,23	81,50	Fruity, caramel
IAC 20/2008	0,06 - 1,21	81,00	Fruity, earthy
IAC 10/2008	0,80 - 1,18	83,50	Fruity, citric
IAC 20130-36	0,79 - 1,24	81,50	Fruity, caramel
Hybrids (F_2) derived from 'Catuaí Vermelho' x 'IAC 045125':			
IAC 20152-6	0,04 - 1,17	87,00	Red fruits, honey
IAC 09/2008	0,19 - 1,39	84,50	Fruity, caramel
IAC 04/2008	0,62 - 1,32	85,50	Fruity, honey
Hybrids (F_2) derived from 'Obatã' x 'IAC 045125':			
IAC 06/2008	0,10 - 1,26	85,00	Fruity
IAC 07/2008	0,08 - 1,27	82,00	Chocolate, caramel
IAC 20141-27	0,15 - 1,30	82,50	Fruity
IAC 01/2008	0,79 - 1,37	86,50	Citric, floral
IAC 20250	0,94 - 1,53	82,50	Fruity
Hybrids (F_1 BC_1) derived from 'Mundo Novo' x 'IAC 045125':			
IAC 20289	0,03 - 1,09	86,50	Aromatic herbs, complex, exotic
IAC 20275	0,77 - 1,17	86,00	Fruity
Hybrids (F_1 BC_1) derived from 'Catuaí Vermelho' x 'IAC 045125':			
IAC 20274	0,24 - 1,18	81,00	Aromatic herbs, floral, exotic
IAC 20286	1,06 - 1,18	86,00	Citric, floral, aromatic herbs
IAC 20285	1,15 - 1,26	84,00	Dark chocolate, tobacco
Hybrids (F_1 BC_1) derived from 'Obatã' x 'IAC 045125':			
IAC 20272	0,25 - 1,09	80,00	Fruity, herbs, exotic
IAC 20300	0,84 - 1,24	88,00	Fruity, citric
IAC 20299	1,05 - 1,46	87,50	Yellow fruits
IAC 20271	0,82 - 1,29	86,00	Fruity, woody, spice
IAC 20298	0,99 - 1,34	85,00	Fruity, honey, floral

This confirms that simultaneous selection for low caffeine content and good cup quality is possible using Ethiopian genotypes. Additionally, it was observed some quality variation among plants of the same progeny which produce an unique sensory profile, indicating the need for individual assessment of plants for the future selection of arabica coffee variety with low caffeine content and differentiated intrinsic cup quality. Moreover, considering the Genotype × Environment interaction effects on coffee quality, would be necessary to evaluate these genotypes during at least more two crop years under specific environments, field management and processing techniques, avoiding inappropriate conclusions about the intrinsic cup quality of this hybrids.

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Genotype and Environment Interaction in Chemical Composition and Sensory Quality of Natural Coffees

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SUMMARY

This study was conducted with the objective of analyzing the combined effects of genotype, altitude, and slope exposure on the levels of trigonelline, caffeine, sucrose, 3-CQA, 4-CQA and 5-CQA present in green coffee processed using the dry method. Specifically, this study sought to verify the relation between the levels of the chemical compounds analyzed and the sensorial quality of the coffee beverage in the cup. Samples of coffee (*Coffea arabica* L.) of the genotypes Yellow Bourbon and Acaiá were collected in different environments and evaluated over the course of three harvests. It was possible to verify that Yellow Bourbon cultivated above an altitude of 1.200 m presented in green coffee a tendency towards higher levels of trigonelline, 5-CQA, and 3-CQA, while the roasted beverage had an average sensorial cup quality score of around 90 points. Acaiá cultivated below an altitude of 1.200 m presented in green coffee a tendency towards lower levels of trigonelline, 5-CQA, and 3-CQA while the roasted beverage sensorial cup quality scores were below 85 points. From the results obtained, it was possible to establish the relation between the chemical composition of the green coffee and the sensorial quality of the roasted coffee beverage as a function of altitude and genotype.

INTRODUCTION

The quality of the beverage, represented by the flavor and aromas formed in the roasted coffee, is directly associated with the chemical composition of the green coffee. On the other hand, it is known that some of the chemical compounds present in the green coffee are influenced by diverse factors, from the choice of plant species and/or variety up through the selling of the coffee. Among these factors, the genotype and environment as well as post-harvest processes are considered fundamental in obtaining a quality final product. However, studies that explore the combination of certain chemical compounds present in green coffee resulting from the interactions between genetic, environmental, and technological factors involved in the production process and, more specifically, the relation of these compounds to coffee quality descriptors, still lack more detailed analyses. Therefore, this study sought to analyze during three consecutive harvests, the combined effect of genotype, altitude, and slope exposure on the levels of trigonelline, caffeine, sucrose, 3-CQA, 4-CQA and 5-CQA present in green coffee processed using the dry method. Specifically, this study sought to verify the relation between the levels of the chemical compounds analyzed and the sensorial quality of the resulting coffee beverage.

MATERIAL AND METHODS

Samples of coffee (*Coffea arabica* L.) were collected during three harvests (2009/10, 2010/11 and 2011/12) from commercial farms located in the municipality of Carmo de Minas, Minas Gerais, Brazil (Fig. 1).

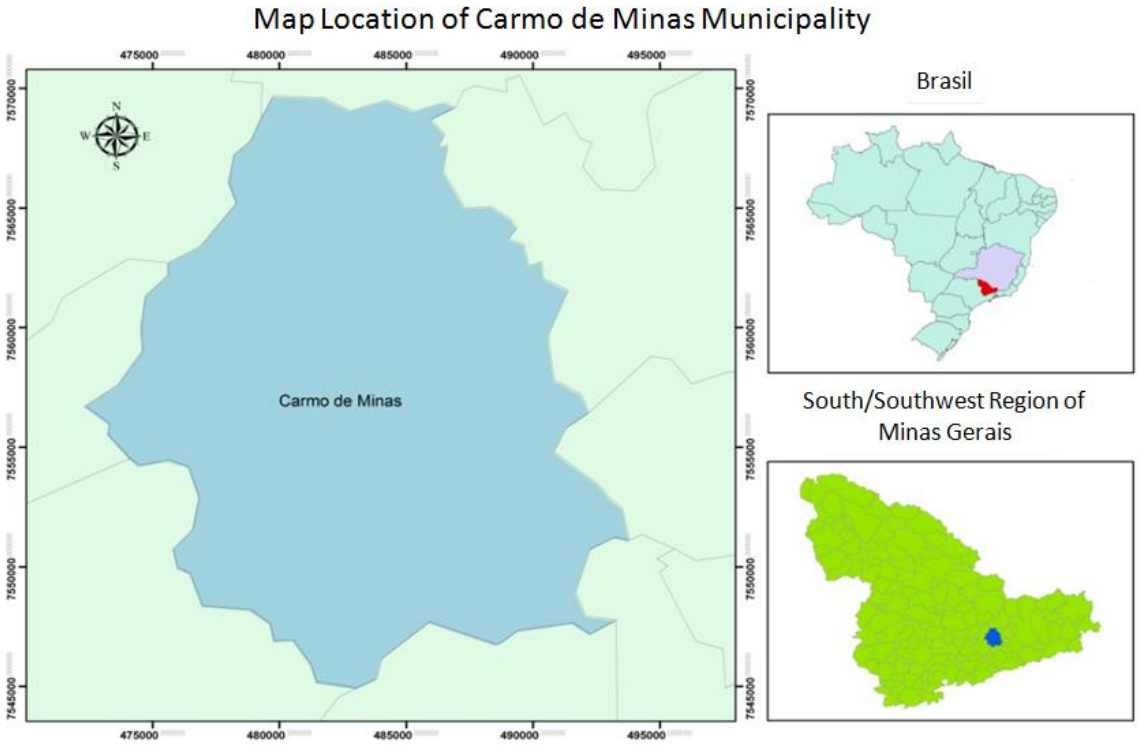

Figure 1. Location of the municipality of Carmo de Minas, in the south/southwest region of the state of Minas Gerais in Brazil.

The experimental design was based on the study of the interaction between environmental, genetic, and processing variables (Table 1). The area of coffee cultivation was stratified into three altitude classes and two slope exposure groups resulting in a combination of six variable environments. For each of the environments only mature fruit was harvested and two genotypes were used: Yellow Bourbon (yellow fruits) and Acaiá (red fruits). For all of the combinations involving environment and genotype, three separate samples were collected and processed using the dry method, therefore totaling 36 samples per harvest. All of the harvest, processing and drying procedures were completed following [3]. The chemical analyses were performed on the green coffee using High-Performance Liquid Chromatography (HPLC). Following a methodology adapted from [5], the levels of caffeine, trigonelline, and, for the chlorogenic acids (CGA), the isomers of the caffeoylquinic acids (3-CQA, 4-CQA e 5-CQA) were simultaneously determined. For sucrose, the levels were determined in a separate analysis, using a methodology adapted from [6]. The sensorial analysis was completed by trained and qualified specialty coffee judges, using the methodology provided by the Specialty Coffee Association of America – SCAA.

Table 1. Experimental design (12 treatments with three separate field samples taken).

Environment		Genotype
Altitude (m)	**Slope Exposure***	
<1000	Sunny	Yellow Bourbon
		Acaiá
	Shaded	Yellow Bourbon
		Acaiá
1000-1200	Sunny	Yellow Bourbon
		Acaiá
	Shaded	Yellow Bourbon
		Acaiá
>1200	Sunny	Yellow Bourbon
		Acaiá
	Shaded	Yellow Bourbon
		Acaiá

**Sun (NE, N, NW and W) and Shaded (E, SE, S and SW)*

The statistical analyses were completed using the R statistical program, and average values from the harvest were used for the results obtained in the chemical and sensorial analyses. Multidimensional scaling (MDS) associated with the Biplots, was used with the objective of making the data more accessible to visual inspection and to less-limited exploration. This type of analysis allows for the rearranging of the variable distribution, thus facilitating the detection of lower significant dimensions in order to explain similarities or differences. However, it was decided to maintain all of the variables with the intent of analyzing the relation between the compounds trigonelline, caffeine, sucrose, 3-CQA, 4-CQA and 5-CQA present in green coffee, with the sensorial quality of the roasted coffee beverage by means of the variable score.

RESULTS AND DISCUSSION

Figure 2 refers to the Biplot with MDS of the genotypes, altitude classes and slope groups for the variables trigonelline, 3-CQA, 4-CQA, 5-CQA, caffeine, sucrose, and the final roasted beverage score. The highest levels of trigonelline, 5-CQA and 3-CQA together with the highest scores were the principle factors responsible for the formation of GI. On the other hand, the lowest levels of these same compounds and the lowest scores contributed in an expressive way to the formation of GIII, thus forming two contrasting groups. The other variables 4-CQA, caffeine, and sucrose present high similarities between themselves with little contribution to the formation of the grouping. The genotype Yellow Bourbon cultivated above 1.200 m and processed using the dry method (GI) presented a tendency towards higher levels of trigonelline, 5-CQA, 3-CQA and a notable expression of the sensorial quality of the coffee beverage, with a total score of around 90 points. For the dry process, the genotype Yellow Bourbon cultivated below an altitude of 1.200 m presented a sensorial beverage quality similar to Acaiá cultivated at altitudes above 1.200 m on sunny and shaded slopes (GII). In the natural coffee produced from Acaiá cultivated below 1.200 m, as well as the genotype Yellow Bourbon below 1,000 m, on the sunny slope (GIII) there was a tendency towards lower levels of trigonelline, 5-CQA, 3-CQA and a sensorial quality with scores below 85 points.

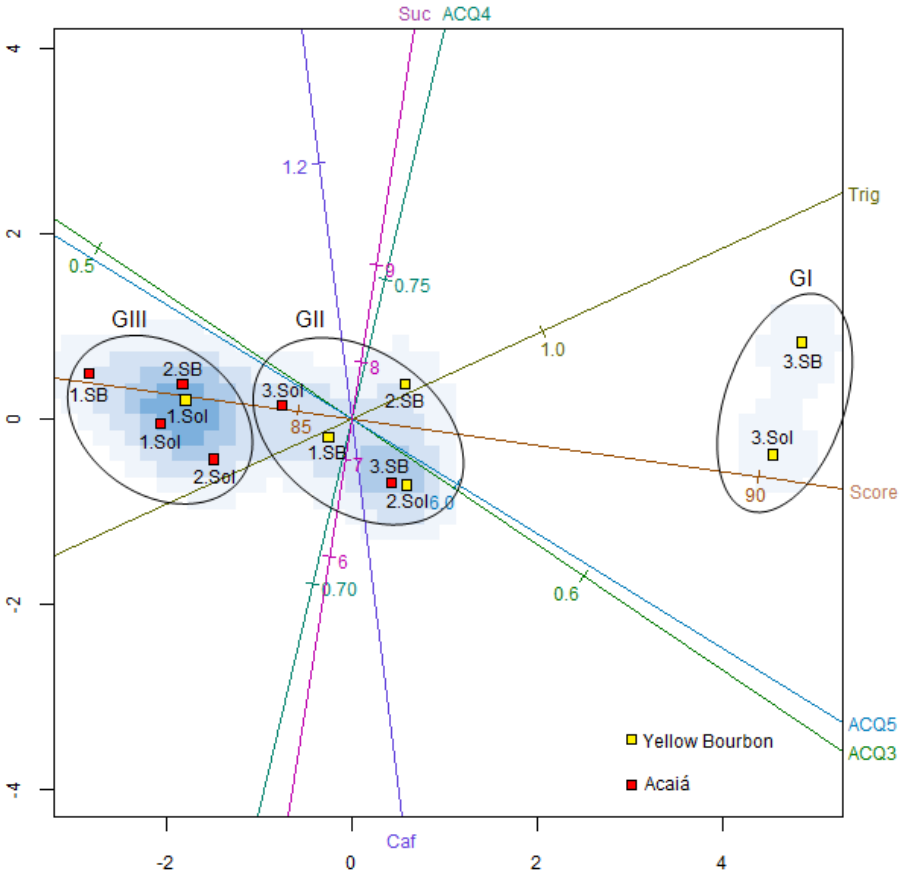

Figure 2. Biplot with multidimensional scaling of the genotypes Acaiá and Yellow Bourbon, cultivated at altitudes (1. <1,000m, 2. 1,000-1.200m and 3. >1.200m) in combination with sunny slopes (Sol) and shaded slopes (SB) and processed using the dry method, for the variables trigonelline (Trig), 3-CQA (ACQ3), 4-CQA (ACQ4), 5-CQA (ACQ5), caffeine (Caf), sucrose (Suc) and total beverage quality score (Score), being GI (Group I), GII (Group II) e GIII (Group III).

The results encountered reveal compounds that, in specific levels present in the green coffee, were able to establish a relation to the sensorial quality of the roasted beverage. In general, various elements can be discussed in relation to quality, such as sensory and chemical aspects and cultivation systems, among others. All, however, fall under the general rule of plant genetics and development: that a plant's final characteristic depends on genetic constitution or genotype, on the environmental conditions to which the genotype is submitted and on the interaction between them. Environmental factors, such as lower temperatures found in higher altitudes, associated with physiological events such as longer periods of grain formation, are related in literature and supply indicators to explain the differences found in the chemical variables. The effect of temperature is noted, principally, between the phases of granulation and maturation of the coffee fruits and, according to [15], the prolonding of these phases caused by milder temperatures, is directly related to the higher relative accumulation of dry material in the coffee seeds. However, even though variations in sensorial quality of the coffee beverage have been described as a function of genotype and altitude, its relation to certain compound groups lacks sufficient research.

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Modelling Espresso Coffee Preparation

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SUMMARY

As known, the quality of espresso coffee is highly affected by several variables such as water temperature, relative pressure, grinding grade, etc. Also, the quality of EC is often considered linked to the experience of the *barista* rather than the effect of chemical and physical behaviours. In spite a wide literature of the quality of EC, the modelling of the changes in quality attributes of EC and the variance of extraction conditions with its effect on properties of the brews has not been the subject of deep experiments. In this paper the effect of grinding grade, weight of coffee ground and the pressure applied on the surface of coffee ground were modelled. Results showed as grinding grade had the main effect for all quality attributes, while for total solids and volume of foam, both grinding grade and the weight of ground into the filter showed a significant effect. In general, changing grinding grades from level 6 to 7, the amount of caffeine in cup from 97 mg to 79 mg, the total solids from 47 to 30 mg/mL and the foam index from 12% to 3%. Furthermore, taking the variability of grinding grade (for each grinding level) into account significant variations were observed. For instance, for grinding level 6, extraction times from 19.72 to 23.31 s were necessary to obtain 25 mL of EC. These differences propagate on total caffeine in cup from 79 to 119 mg and total solid from 39 to 52 mg/mL.

INTRODUCTION

The quality of coffee brew may be generally defined as the ability to transform the ground coffee into an enjoyable beverage. Among the types of coffee beverages prepared in the world, espresso coffee (EC) is the most popular and appreciated, due to its peculiar sensorial attributes such as the “body” of coffee and the amount and persistence of the *crema* which traps the volatilized aroma compounds. The preparation of EC is based on the percolation of hot water at high temperature through a limited amount of manually pressed ground coffee. Several variables affect the quality of EC: some of these such as varieties, degree and type of roasting, mixture of roasted coffee) are controlled at industrial level while other are under the control of the espresso coffee technicians also called “barista”. Severini et al. extensively studied the changes of quality of espresso coffee at commercial scale.

Particularly, as reported from Illy and Navarini the traditional preparation of espresso coffee brew may be summarized in three key steps: 1. Grinding of roasted coffee; 2. Dosing and tamping; 3. Percolation. These phases of espresso coffee preparation, are still believed to be essentially linked from the experience of the *barista* rather than a consequence of physical and chemical behavior during percolation of water through the ground coffee cake. Ground coffee shows in general particle size range of 0.2 – 650 μm but the type of distribution functions and its amplitude (i.e. the particle size distribution) is affected by several factors such as the mixture of coffee beans (i.e. the variability of mechanical properties of each coffee varieties), the type of grinder, the type of burrs of the grinder, burr wear. The weight of coffee ground is recognized in the range of 6 and 8 g but an upper limit of 9 g were reported from

Romani et al.. Moreover, since the dosing is performed with semi-automatic devices (i.e the dosing chamber) based on gravitational force, it is reasonable to suppose a variation of the weight of coffee ground due to different grinding grade, moisture content as well as a loss of standardization of the dosing chamber during its life time. The pressure on the upper surface of coffee cake (tamping) is a steps which influences the microstructure properties as the porosity and the pathway of water during percolation; the pressure applied may vary from few kilograms to 20 kg. The phase of brewing (percolation) is usually performed with heat water at 92-94°C and a relative pressure of 9±2 bar. An ideal flow rate of 1 mL/s should be obtained but different ranges were reported and a total volume in the range of 15 to 50 mL with a regular espresso of 25-30 mL is commonly served. In spite with a wide literature on the effects of some process variables on the quality of espresso coffee, the analysis of the variance of particle size distribution, of the dose of ground coffee and of the pressure applied on the upper surface of coffee cake and their effects on the quality of espresso coffee have not studied sufficiently. Moreover, very few researchers focused their attention on the modeling of espresso coffee attributes during percolation and the majority of these were focused on the kinetic of caffeine. Under these considerations this paper had two main aims: 1. to study the effects and of grinding, dosing and tamping on the quality of espresso coffee: 2. to model the variance of the above variables during brewing. These aims are in agreement with the need to standardize the process conditions under the complete control of the *barista* with the aim to improve the quality of each individual brews.

MATERIALS AND METHODS

Materials

Whole coffee beans were supplied by ESSSE caffè S.p.A. (Anzola, dell'Emilia, Bologna, Italy).

Grinding

Coffee beans were grind by an automatic grinder (Mazzer, Italy) with 8 levels of grinding: 1. for the finest point and 8 for the coarsest. Five sieves (600, 400, 250 and 180 mm) were used to fractionate 100 g of ground coffee. The sieves were shaked until constant weight.

Brewing

EC conditions were fixed as follow: water temperature of 92°C; 9 atm of relative pressure, and TT mm of holder filter diameter. The brew were prepared with a EC machine mod V220 (Vibiemme, Italy). In all cases the coffee brews were prepared using commercial water "Leggera" (Gaudianello, Italy).

Experimental Design

The grinding grade, the weight of coffee ground and the pressure applied on the upper surface of coffee ground were studied modulating their at three levels. Grinding level of 6, 6.5 and 7 were selected as fine, coarse-fine and coarse grind respectively. These levels were chosen on the basis of preliminary measures of the particles size distribution of different grinding grades. The pressure on the coffee cake was changed by using weights of 0.750 kg, 1.5 kg and 2.250 kg to press the upper face of the ground coffee into the filter. The doses of coffee were fixed at 6, 7 and 8 g of coffee ground. For all experiments 24 s of extraction were used. Particularly, each espresso was divided in three sub-extractions corresponding to the first 8 s,

from 9 to 16 s and from 17 to 24 s of extraction, respectively. Each extraction was repeated in triplicate for a total of 243 espresso coffee samples.

Physical and chemical analysis

pH was measured at room temperature by a pH-meter mod. Basic 20 (Crison, Allen) while acidity was measured by titration with NaOH 0.1 N until pH of 7. Foam index was defined as the volume fraction (%) of foam referred to the total volume of espresso coffee. Persistence of foam is the time (s) necessary for appearing of the liquid phase below the *crema* layer. Consistence of foam is the time (s) for the disappearing of 0.5 g of sucrose. Total solids was measured by oven-drying about 5 mL of EC at 105°C until constant weight.

The quantitative analysis of caffeine in EC samples was performed by a Waters Breeze 1525 HPLC binary pump equipped with a C18 Column. The mobile phase was water 74%, acetic acid (1%), methanol (25%) previously filtered of 0.45 μm. The detector was set at 254 nm. Injection volume was of 20 μL of coffee previously filtered at 0.20 μm and the concentration was calculated by using a regression equation of external caffeine standard (Sigma Aldrich).

Sensory Analysis

The sensory properties of EC were analyzed by an electronic nose mod. α-Fox, sensory array 2000 (Alpha M.O.S., France), with an auto-sampler HS 100 (Alpha M.O.S., France). 1 mL of coffee brew was placed in vials of 10 mL and maintained at 86°C in agitation at 300 rpm for 10 min before the injection.

Statistics

Three empirical models were used to fit experimental data of volume of liquid phase (V), volume of foam (F), pH, acidity (A), total solids (S):

1. Changes of volume of liquid phase was modeled with a logistic function: $\boldsymbol{Vt = \log_e\{1+\exp[k(t-tc)]\}}$ **(eq. 1)**, where *k* is the rate constant (mL/s) and *tc* marks the time at which the volume intensifies.
2. Changes in total solids (mg), acidity (mL NaoH/mL), and caffeine (mg/mL) were modeled with a first kinetic order: $\mathbf{A_t=A_0*\exp(-k*t)}$ **(eq. 2)**, where A_0 is the initial value of the dependent variables, *k* is the rate constant (s^{-1}) and *t* is the time (s).
3. Changes in volume foam were modeled with the following equation: $\mathbf{F_t=F_0*[1-\exp(-k*t)]}$ **(Eq.3),** where *F* is the volume of foam, *k* is the rate constant and *t* is the extraction time (s).

A mixed non linear regression was used to study the effect of each independent variables on the quality of espresso coffee. Model parameters were estimated as a group (batch behavior) combining all experimental data. Then, the fits were repeated on the separate series of grinding grade, weight of ground coffee and pressure, to assess where the major variation of the data resides. The goodness of fitting was evaluated by the explained part (R adj.) while the normality of data was assessed by the Shapiro-Wilk test. Furthermore, a PCA was performed to highlight potential differences in terms of aroma compounds of the EC obtained in different brewing conditions.

RESULTS AND DISCUSSION

The changes in volume of espresso coffee during extraction time, showed a clear sigmoidal behavior; also a large variation was observed at each sampling time indicating that the studied variables affect the percolation of water trough coffee cake (data not shown). In order to study where this variance resides, the experimental data were first fitted with eq.1 as a group (batch behavior) and then they were pooled for each of the process variables studied and newly fitted (individual effect). The results are reported in figure 1 where the explained parts ($r_{adj.}$) clearly shows as the most important increase (0.96) was obtained when the data were pooled for grinding grade indicating as this variable was the most significant for the percolation process while the dose and the pressure on the upper surface of ground coffee did not contain further information to explain the variation in volume of EC. Also, when the variables were combined with the aim to analyze eventual interactive effects none further improvement of the explained part was observed. Fig. 2 shows the changes in EC volume as a function of time and the fits obtained by eq 1. From results, the higher the grinding grade (the particle size increases) the significantly higher the extraction rate; it is reasonably to suppose that as the greater particle size is as the porosity of coffee cake increases improving the free pathway for the percolation of water.

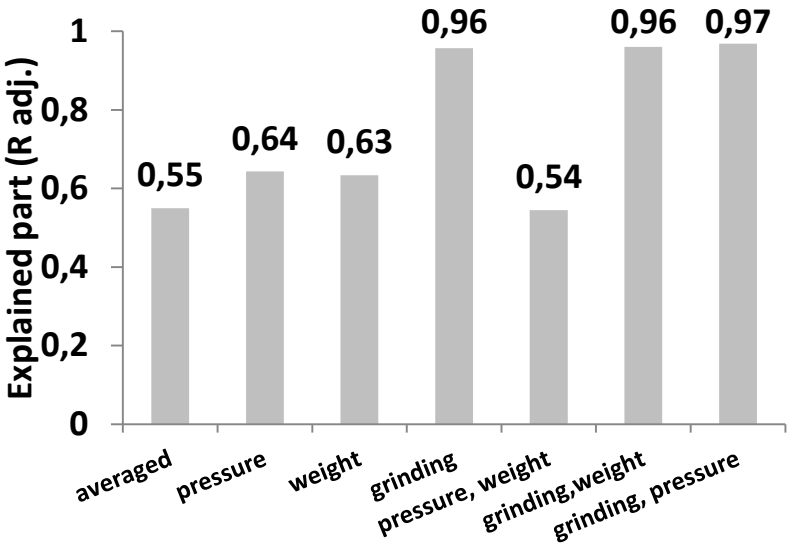

Figure 1. Explained part (R adj) of different model fits describing the effects of grinding grade, weight and pressure on the change of volume of espresso coffee. The horizontal axis indicates the variable free to vary while the other were estimated as a group.

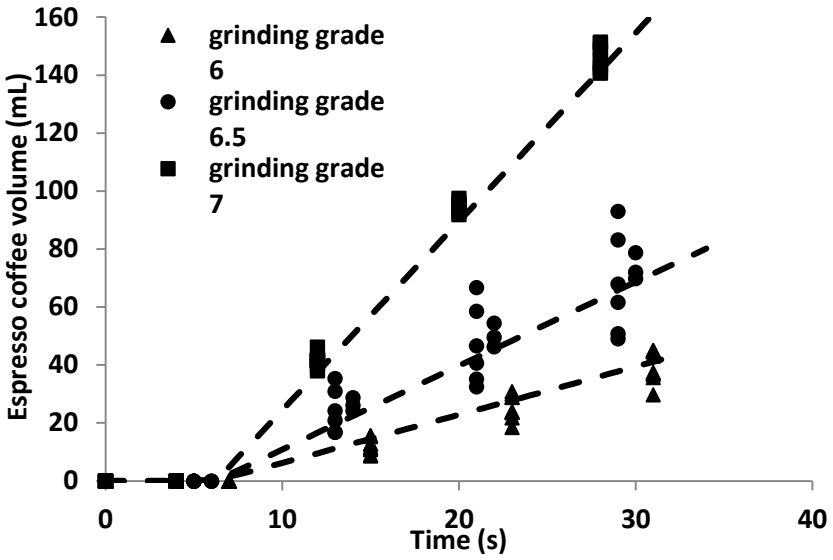

Figure 2. Changes in volume of EC samples obtained using coffee ground at different grinding grade. Dashed lines are the fits obtained with the eq. 1.

However, figure 1 clearly shows additional variation inside each grinding grade that means a large variability of particle size distribution also when a constant grinding level was used. In the next analysis, the data were fitted for each individual coffee brew obtained, using the same grinding grade (random effect) by which an average explained part of 0.96 was obtained. The results of the separated series showed rate constants of 1.66±0.19, 2.89±0.59 and 6.51±0.26 mL/s respectively for grinding grades of 6, 6.5 and 7 confirming two considerations: 1. the rate flow of water through coffee cake is significantly affected by the particle size; 2. the internal variability in particle size of ground coffee obtained with a typical grinder used at the bar is high and affect the percolation process. From the estimated rate constants it was possible to estimate the extraction time necessary to obtain an espresso coffee of 25 mL that is the amount commonly recognized for a regular EC. Ranges of 19.72-23.31, 13.4-17.1 and 9.93-10.24s were estimated for EC prepared with grinding grade of 6, 6.5 and 7 respectively. The same nonlinear mixed effect analysis was used for each quality attributes highlighting as the grinding grade was the only variable affecting the quality of EC except for total solids and foam volume which were influenced from grinding grade and the weight of coffee. However, in all cases explained parts of the variation of experimental data were in the rage of 0.81 – 0.96 (data not shown) indicating as the changes of the quality attributes studied in this paper may be explained by the equation above reported. Among these, the foam on the top of liquid phase is one of the most important attributes for the judge of consumers. In figures 3 the estimated values of foam index as a function of time was reported pooling the data for grinding grade and a constant weight of 7 g.

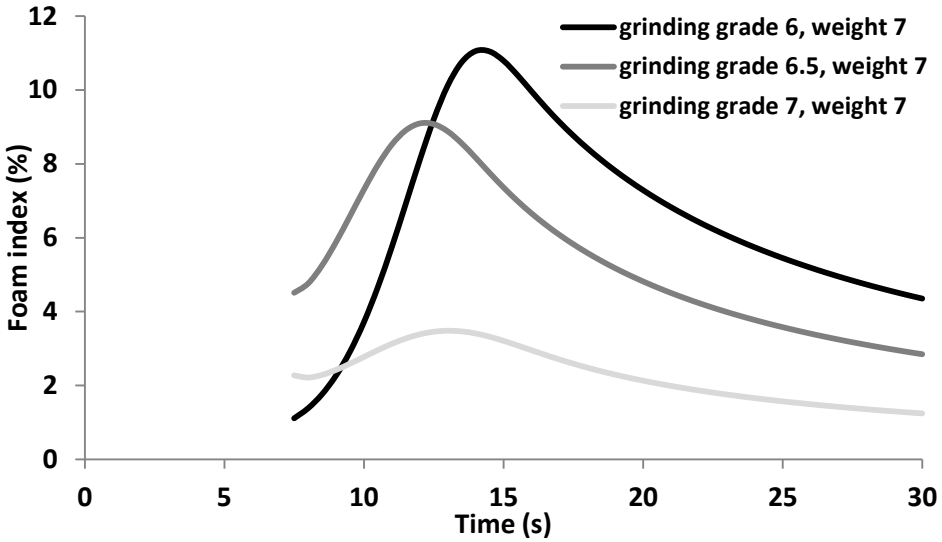

Figure 3. Changes of foam index of espresso coffee samples as a function of time.

It is clearly shown the difference in terms of developed foam when a coffee at different grinding levels was used. The curves have a somewhat of bell-shaped showing a maximum value between 10 and 14 s. The result indicates that during the first few seconds of extraction, both foam and liquid phase are produced until a critical time of ~ 12 s after which only the liquid phase is extracted leading to a reduction of the volume fraction of foam. Moreover, significant differences in terms of maximum foam index values were observed during the entire extraction time with values of ~ 11, ~ 7 and ~ 1.15% respectively, when grinding grades of 6, 6.5 and 7 were used to prepare a regular coffee of 25 mL. The results would suggest an effect of the grinding grade on the extraction mechanisms of the chemical compounds considered as involved in the foam formation, such as oil, protein, polysaccharides, etc.. Fig. 4 shows the changes in caffeine concentration of EC obtained with different grinding grade and the fits obtained by eq. 2 pooling all data for each grinding grade. At first, the changes in caffeine concentration during brewing may be well explained by a first kinetic model. In accordance with our results Spiro et al. modeled the extraction of caffeine from ground coffee infusion in water with a first order kinetic. By increasing the particle size of the ground coffee, a significant decrease of caffeine concentration was observed. Also, from the nonlinear mixed analysis it was observed as no statistical difference were observed in terms of rate constant but only in the initial value of caffeine concentration. This result suggests as the changes in grinding grade did not influence the mechanism of extraction of caffeine but only the percolation rate of water. However, this was sufficient to modify the amount of caffeine in cup; indeed taking into a regular coffee of 25 mL, caffeine content of ~97, ~107, ~79 mg, were estimated by using grinding grade of 6, 6.5 and 7 respectively. A serving regular coffee prepared with coarse ground coffee (i.e. big particle sizes) contains a lower content of caffeine while only slight difference resulted from the use of the lower grinding grades. Furthermore, by considering the internal variability of each grinding grade above discussed high differences in total caffeine content of a cup of 25 mL were estimated (Table 1). The table also reports the caffeine content of validation experiments in which some regular coffee were prepared by using different grinding grade.

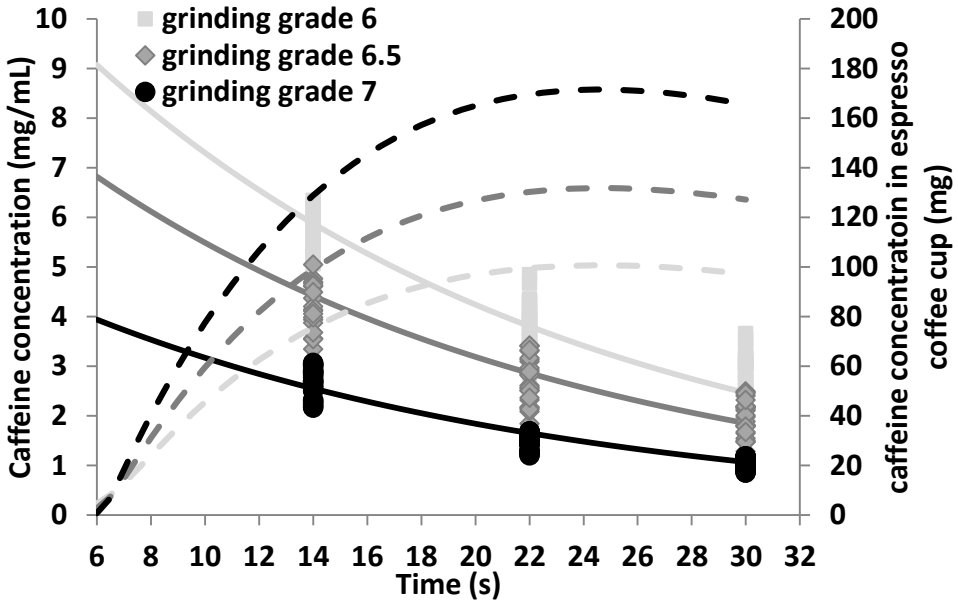

Figure 4. Changes in caffeine concentration of espresso coffee prepared by using ground coffee from different grinding level. Solid lines refer to the caffeine concentration (mg/mL) as a function of time. Dashed lines represent the amount of caffeine in cup during extraction time.

Table 1. Variability of caffeine content of regular EC of 25 mL obtained from different grinding grade.

Grinding grade	Estimated caffeine content for a regular EC of 25 mL	Interval Low	Interval High	Experimental data of regular EC of 25 mL
6	97.6	78.9	119.7	104.4±5.23
6.5	107.38	64.4	161.3	100.35±4.07
7	79.55	67.4	91.9	80.29±6.7

Again, the low standardization of particle sizes observed when a common grinder of bar is used, causes a great difference in caffeine content assumed from the consumers.

Finally, since the grinding grade was the most important variable, an additional series of EC of 25 mL were prepared by using coffee ground at grinding grade of 6, 6.5 and 7 and the volatile compounds were analyzed by electronic noise (Fig. 5). The first PC allows explaining the 98% of the variability of the signals. The figure clearly proved as the grinding grade affects the type of volatiles. The non linear regression mixed analysis allowed showing in detail the changes in quality attributes of espresso coffee during the extraction time and the effects of some variables commonly under the control of *barista*. The grinding grade of coffee beans had a great effect on all attributes of espresso coffee; the weight of ground coffee affected only the total solids and the foam index while the force used to press the ground into the filter did not have any effect. The use of a coarse ground coffee, significantly increases the flow rate of water through the coffee cake, resulting in a reduction of total solids, foam index, caffeine content in cup. Moreover, it was proved as the internal variability of particle size makes significantly varies the quality of EC. That means that the type of grinder, the material of the burrs of grinder, burrs wear, etc., should be monitored with care whit the aim to guarantee high the quality of EC served every day.

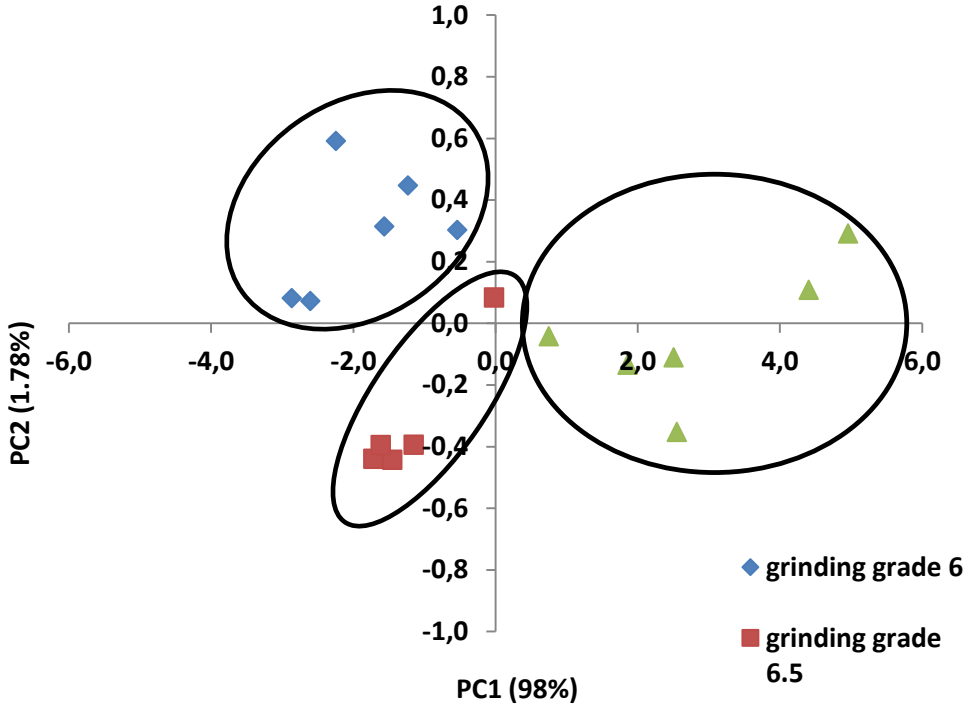

Figure 5. PCA chart depicting the difference in terms of aroma attributes of brews obtained from coffee of different grinding grades.

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No Evidence of Dehydration with Moderate Daily Coffee Intake: a Counterbalanced Cross-Over Study in a Free-Living Population

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SUMMARY

It is often suggested that coffee causes dehydration and its consumption should be avoided or significantly reduced to maintain fluid balance. The aim of this study was to directly compare the effects of coffee consumption against water ingestion across a range of validated hydration assessment techniques. In a counterbalanced cross-over design, 50 male coffee drinkers (habitually consuming 3-6 cups per day) participated in two trials, each lasting three consecutive days. In addition to controlled physical activity, food and fluid intake, participants consumed either 4 x 200 mL of coffee containing 4 mg/kg caffeine (C) or water (W). Total body water (TBW) was calculated pre- and post-trial via ingestion of Deuterium Oxide. Urinary and haematological hydration markers were recorded daily in addition to nude body mass measurement (BM). Plasma was analysed for caffeine to confirm compliance. There were no significant changes in TBW from beginning to end of either trial and no differences between trials (51.5 ± 1.4 vs. 51.4 ± 1.3 kg, for C and W respectively). No differences were observed between trials across any haematological markers or in 24 h urine volume (2409 ± 660 vs. 2428 ± 669 mL, for C and W respectively), USG, osmolality or creatinine. Mean urinary Na^+ excretion was higher in C than W ($p= 0.02$). No significant differences in BM were found between conditions, although a small progressive daily fall was observed within both trials (0.4 ± 0.5 kg; $p< 0.05$). Our data show that there were no significant differences across a wide range of haematological and urinary markers of hydration status between trials. These data suggest that coffee, when consumed in moderation by caffeine habituated males provides similar hydrating qualities to water.

INTRODUCTION

Maintenance of fluid balance is essential to sustain human life. Water intake balances fluid losses to achieve adequate hydration of bodily tissues. Although there are widespread guidelines in scientific literature and media for achieving optimal hydration status and about the effects that various caffeinated beverages may have on fluid balance, there is no clear consensus about how much fluid an individual should consum. Despite this, it has been suggested that caffeinated beverages should not be included in daily fluid requirement guidelines and that a glass of water should be consumed with every cup of coffee or tea to ensure hydration is maintained.

Caffeine (1, 3, 7-trimethylxanthine) is a naturally occurring methylxanthine which can be found in coffee, tea and chocolate. Caffeine acts as an adenosine receptor antagonist to reduce fractional sodium reabsorption in both the proximal tubule and distal nephron. When consumed in large doses (≥500 mg), caffeine elicits a diuretic effect, yet a low to moderate dose of caffeine may not indue this same effect. Furthermore, it has alo been suggested that

whilst caffeine causes acute diuresis, regular caffeine consumption may lead to a tolerance developing against its diuretic effect.

It is estimated that 1.6 billion cups of coffee are consumed worldwide every day, thus it is of interest to know whether coffee contributes to daily fluid requirement, or whether it causes low-level chronic dehydration. In the present study, our aim was to directly compare the effects of a moderate intake of coffee in caffeine-habituated adults against equal amounts of water across a wide range of hydration markers, including the gold standard TBW measure.

MATERIALS AND METHODS

Fifty-two healthy non-smoking males aged 18-46 y, who were classified as moderate coffee drinkers, were accepted to participate in the study. Each participant underwent two experimental trials whereby they were provided with equal amounts of coffee (C) or water (W) in addition to a standardise diet of carefully calculated individual food and fluid intakes (Fig. 1).

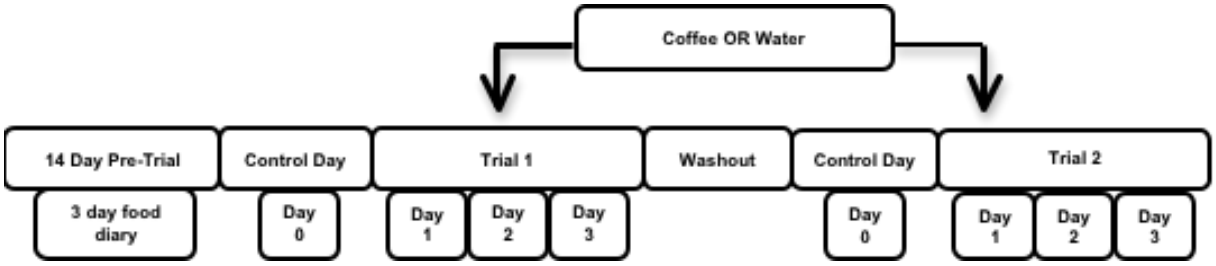

Figure 1. Study Design Overview.

Each participant was provided with a mug marked at 200 mL. During trial C, participants received four pre-weighed containers of Nescafé Original coffee to provide 4 mg/kg BM of caffeine per day (2.3 ± 0.4 g Nescafé Original per cup) and were instructed to make the beverage with boiled tap water to the 200 mL marker in the mug provided. During trial W, participants were instructed to consume 200 mL tap water in the same mug. Total test beverage intake was 800 mL/day. Blood and urine samples were collected daily and analysed for markers of hydration status, including: haematocrit, total body water, indirect measures of renal function, osmolality, sodium, potassium, USG and 24h urine volume. Serum caffeine was analysed for complicance.

RESULTS AND DISCUSSION

Haematological markers did not differ between conditions across all measures: serum osmolality, haematocrit, total plasma protein, serum sodium, serum potassium ($p< 0.05$). Student's t-test analysis showed no significant differences between conditions in the delta change from day 1 to day 3 for all haematological measures.

Renal function was normal throughout each trial as assessed by urine creatinine, serum creatinine and BUN. Neither urine or serum creatinine differed between conditions or time points ($p> 0.05$).

Twenty four hour urine volume, USG, urine osmolality or urine creatinine did not differ between conditions ($p> 0.05$). Urinary Na^+ was not different between trials days, however mean Na^+ excretion was significantly higher on both days in the coffee trial than the water trial ($p= 0.02$). K^+ concentration was significantly higher in C than W on day one only. K^+

concentration were significantly higher on day 2 in both conditions ($p= 0.02$), but no between-condition difference was found.

Neither urine void volume nor urine void USG were between conditions; $p= 0.86$ and $p= 0.95$, respectively.

Mean body mass did not differ between the two conditions ($p= 0.45$), however a small but progressive daily fall in BM occurred within both conditions ($p< 0.05$). Mean decrease in BM from day 1 to day 3 across both trials was 0.39 ± 0.5 kg.

With acknowledgement of the study's limitations, results suggest that coffee did not result in dehydration when provided in a moderate dose of 4 mg/kg BW caffeine in four cups per day. Thus, these data suggest that coffee, when consumed in moderation by caffeine habituated males contributes to daily fluid requirement and does not pose a detrimental effect to fluid balance. The advice provided in the public health domain regarding coffee intake and hydration status should therefore be updated to reflect these findings.

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CYP1A2 phenotyping: Caffeine and Paraxanthine in Human Saliva by Validated HPLC Method

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SUMMARY

The drug metabolising enzyme cytochrome P450 1A2 (CYP1A2) primarily found in the liver, contributes to the metabolism of a number of therapeutic medicines including methylxanthines. It is also involved in the bioactivation and detoxification of carcinogens, particularly heterocyclic amines. Caffeine, due to its relative ease of administration and safety, is the most widely used probe to measure CYP1A2 activity (phenotype). Over 95% of caffeine is metabolised in the liver with the majority converted to paraxanthine (81.5%) therefore the 'gold standard' to estimate an individual's CYP1A2 activity is the systemic clearance of caffeine following administration of a known dose of caffeine. CYP1A2 activity has shown a large degree of inter- and intra-individual variability, up to 40-fold and 2-fold differences, respectively, among healthy individuals. Significant inter-ethnic differences in CYP1A2 activity have been reported, with diet, lifestyle and genetic factors believed to contribute. The present work is aimed at *in vivo* studying caffeine metabolism by using a validated HPLC method and by determining the paraxanthine/caffeine concentration ratio. Saliva has been selected as a matrix since it offers both the ease of sampling and avoidance of the complex caffeine metabolism pathways as in the case of other matrices like urine and plasma.

INTRODUCTION

CYP1A2 is part of the cytochrome P450 (CYP) family of drug-metabolizing enzymes. The CYP1A2 gene is found in a cluster with CYP1A1 and CYP1B1 on chromosome 15. In contrast to the few variant genes identified, the inter-individual variation in CYP1A2 activity as assessed by, e.g., *in vivo* caffeine metabolism is extensive and no polymorphic site in the CYP1A2 gene that can unequivocally be used to predict the metabolic phenotype in any individual has yet been identified despite quite ambitious sequencing efforts.

The SNP rs762551:C > A is the most well-studied genetic variant in CYP1A2. It is the sole variant of the CYP1A2*1F haplotype and found with other variants in several haplotypes (*1J, *1K, *21, and others that have not been confirmed). The AA genotype was considered 'fast' caffeine metabolizer and the C allele 'slow'; however, this is an oversimplification because, the fast metabolizer phenotype for rs762551AA is only observed under induction by smoking or high habitual coffee intake. The latter induction effect has been reported in studies where high habitual coffee intake was considered deriving from regular daily intake of three or more cups of coffee or from more than two cups of coffee a day, without details on coffee preparation. In both the above mentioned studies, CYP1A2 phenotyping has been performed

by administering 100 mg caffeine dose and by determining 4 h later, the ratio of paraxanthine and caffeine concentrations in plasma or in unstimulated whole saliva. In the present study, we examined the effect of habitual coffee (100% Arabica *espresso*) consumption on CYP1A2 enzyme activity of 18 healthy, no-smokers, Italian subjects, by administering caffeine via regular *espresso* coffee (3 occasions). Intra- and inter-individual variations have been determined and compared with reported data.

MATERIALS AND METHODS

Study Protocol

In total 18 healthy (no-smokers and habitual 100% Arabica *espresso* coffee drinkers) Italian participants (13 male and 5 female) were recruited to the study: mean ± SD age 41.2 ± 7.6 years; mean weight 71.9 ± 12.5 kg; BMI 23.3 ± 2.6 kg/m2. None of the subjects had chronic or acute use of any medications and none of the female participants was pregnant or used oral contraceptives. As far as self-declared daily coffee consumption is concerned, the panel includes 1 subject with 1 cup, 11 subjects with 2-3 cups, 4 subjects with 4-5 cups and 2 subjects with > 5 cups.

Caffeine (74 ± 9mg) was administered (3 occasions) through a regular *espresso* coffee (iperespresso® medium roasting illycaffè spa) prepared by means of a Francis Francis X7.1 home iperespresso® coffee machine, after a period of 12 hours of abstinence from caffeine ingestion (coffee, tea, chocolate, energy drinks) and 24 hours of abstinence from a group of food that inhibit or induce enzyme activity.

Unstimulated whole saliva samples (2-3 mL) were collected at 5 min pre dose and 15, 30, 45, 60, 90, 120, 180 and 240 min post dose by spitting directly into plastic vials and stored at 4°C till sample preparation. The sampling was carried out in the morning starting from 8-9 am up to reaching the final post dose time. During this period volunteers were asked to abstain from food ingestion.

To avoid coffee contamination in saliva, volunteers were asked to rinse the mouth with water for at least 2 min before sampling, especially for the first point.

Sample preparation and Analysis

Benzotriazole (internal standard), caffeine and paraxanthine were purchased from Sigma-Aldrich (Steinheim am Albuch, Germany), ethyl acetate, formic acid, acetonitrile were analytical grade (Sigma-Aldrich, Steinheim am Albuch, Germany).

Caffeine and paraxanthine concentration were quantified via UHPLC method, a 1290 Infinity system was used equipped with a a 4.6 mm × 150 mm, 2.7 μm 120 SB-C18 Poroshell column (Agilent Technologies, Waldbronn, Germany). 1 mL of saliva was extracted with 4 mL of ethyl acetate, vortex for 5 minutes and centrifugated for 10 min at 4000g. The organic layer was separated and evaporated under a steam of nitrogen, then reconstituted in mobile phase before injection.

The mobile phases were aqueous formic acid (0,1%) and acetonitrile, flow rate is 1 ml/min, starting at 90% of aqueous phase, reaching 73% at 6 min and then back to initial conditions. Wavelenght for caffeine and paraxanthine quantification was 273 nm.

For calibration curves analytes were added at known concentrations to caffeine-free saliva, to monitor matrix effect. Peak height ratio of analyte and internal standard were plotted and concentrations chosen based on literature data.

Linearity of calibration curves was good, being r^2 for caffeine 0.99 and paraxanthine 0.97; limit of quantification and detection for the analytes were similar to those reported in literature [8]: caffeine LOQ 0,05 μg/mL and LOD 0,02 μg/mL; paraxanthine LOQ 0,07 μg/mL and LOD 0,02 μg/mL.

Pharmacokinetics and Statistical Analysis

The maximum concentration (C_{max}) and the time to C_{max} (T_{max}) were obtained by inspection of the concentration–time data.

The structural base model used is a one-compartment open model with first-order absorption and first-order elimination.

The analytical solution of the model:

$$C_{saliva}(t) = \frac{D\,K_a}{V(K_a - CL/V)}\left[\exp(-CL/V\,t) - \exp(-K_a t)\right]$$

was used to calculate pharmacokinetics parameters: rate of absorption (Ka), clearance (CL) and volume of distribution (V). The parameters of the model were estimated by nonlinear mixed effects modelling procedure using software NONMEM.

Each subject covariate (weight – WT, age, sex – SX, coffee consumption) was tested against the base model. Significance of the effect on CL and V was evaluated by the likelihood ratio test (OFV = -2 × ln(likelihood), the difference in OFV between the base model and model with covariate is approximately chi2 distributed (with one additional parameter a difference bigger than 3.84 is significant at the 5% level of significance).

Continous covariates were modeled using a linear (1) and power (2) relationship as follows.

$$\theta = \tilde{\theta}\left(1 + \theta_p(x_p - \bar{x}_p)\right) \quad (1)$$

$$\theta = \tilde{\theta}\left(x_p / \bar{x}_p\right)^{\theta_p} \quad (2)$$

where $\tilde{\theta}$ is the typical population parameter estimate and θ_p is the effect of covariate x_p centred to the typical value (median) $\bar{x}_p$ in the studied population. Categorical covariates (sex, coffee consumption) were assigned values of 0 and 1 and were modelled to estimate proportional change in the pharmacokinetic parameter value:

$$\theta = \tilde{\theta}\left(1 + \theta_p x_p\right)$$

Coffee consumption was dichotomized, that is each category was tested against (2-3 cups/day). In the second step categories were grouped to two groups (≤3 cups/day and >3 cups per day) – covariate CONSBI. Covariate analysis was performed in two steps: in the first

step each covariate was tested against the base model (1-linear, 2-power model). Strong effects of covariates put in evidence in this step were incorporated into the model and in the second step, the remaining covariate effects were tested against this new model to get the final model.

RESULTS AND DISCUSSION

The followed protocol, very well tolerated by volunteers, differently from those typically adopted in CYP1A2 phenotyping studies, uses the oral administration of caffeine dose through a well-defined *espresso* coffee. Due to the use of saliva as matrix, this choice is very challenging as far as possible residual caffeine in the oral cavity is concerned. In fact it is well known that coffee compounds present in *espresso* coffee persist in the oral cavity after drinking [9]. The C_{max} is particularly sensitive to possible residual caffeine persisting in the oral cavity and we found an excellent agreement with literature data only when rinsing with water was intensified and prolonged up to at least 10 min before sampling. By using the protocol, a C_{max} = 4.6 ± 1.5 µg/ml (min. 0.9 – max. 15.3 µg/ml) was found (T_{max} = 30 min), however when an additional and insistent rinsing was recommended (on 4 volunteers) C_{max} = 2.1 ± 0.7 µg/ml (min. 0.9 – max. 3.2 µg/ml) was determined. The latter is in excellent agreement with C_{max} of 2.47 ± 0.39 µg/ml (in plasma) reported after oral dose of a ready-to-drink coffee beverage containing a caffeine dose close to that of the present work [11]. For this reason, experimental data obtained 15 min post dose cannot be considered for further pharmacokinetics calculations.

Paraxanthine/caffeine concentration ratio

The molar concentrations of caffeine (1,3,7-trimethylxanthine or 137X) and its metabolite paraxanthine (1,7-dimethylxanthine or 17X) in saliva at 4 h post dose, were used to estimate the activity of CYP1A2 in terms of 17X/137X ratio.

Table 1 reports the whole set of experimental data. The median value (0.50) determined on the present group of habitual *espresso* 100% Arabica coffee drinkers is consistent with that of groups of coffee drinkers from different Countries or ancestries.

The intra-individual variation in CYP1A2 activity (% coefficient of variation, CV) ranged from 3.1 to 31.0% whereas the inter-individual CV was 29.8%. These findings are in excellent agreement with literature data.

In Table 2, this ratio is reported for the different groups which were clustered on the basis of coffee consumption and compared with literature data. The comparison with an Australian population of European ancestry phenotyped by using saliva as a matrix and under analytical conditions very close to that of the present work reveals that both 115 not heavy coffee consumers (< 2 cups) and 17 heavy coffee consumers (≥ 2 cups) showed a CYP1A2 activity higher than that estimated on 17 coffee consumers ((≥ 2 cups) of the present study as clearly shown by the median values (in parentheses minimum and maximum values). By selecting from our volunteers those consuming ≥ 4 cups (6 subjects) the median value approaches that of the heavy coffee consumers ((≥ 2 cups) of the Australian population of European ancestry but it is still lower. Interestingly, median CYP1A2 activity of the present group of habitual *espresso* coffee Italian drinkers of 2 cups or more is in between that of Australian no heavy and heavy coffee drinkers of South Asian ancestry.

Table 1. 17X/137X at 4 h post dose determined in three different occasions. Average, standard deviation, coefficient of variation and coffee consumption are reported.

Subject	Occasion			Average	St. Dev.	CV (%)	Coffee Consumption (cups)
	1	2	3				
1	0,65	0,69	0,66	0,67	0,02	3,12	2-3
2	0,63	0,65	0,68	0,65	0,03	3,85	4-5
3	0,48	0,61	0,88	0,66	0,20	31,07	4-5
4	0,34	0,35	0,45	0,38	0,06	16,01	2-3
5	0,52	0,88	0,82	0,74	0,19	26,06	2-3
6	0,57	0,57	0,67	0,60	0,06	9,57	2-3
7	0,52	0,56	0,38	0,49	0,09	19,42	2-3
8	0,84	0,65	0,54	0,68	0,15	22,43	> 5
9	0,39	0,34	0,32	0,35	0,04	10,30	2-3
10	0,46	0,3	0,57	0,44	0,14	30,62	2-3
11	0,52	0,38	0,63	0,51	0,13	24,57	4-5
12	0,24	0,33	0,43	0,33	0,10	28,51	2-3
13	0,37	0,24	0,28	0,30	0,07	22,44	2-3
14	0,37	0,41	0,38	0,39	0,02	5,38	1
15	0,54	0,42	0,49	0,48	0,06	12,47	2-3
16	0,57	0,85	0,64	0,69	0,15	21,22	> 5
17	0,25	0,26	0,3	0,27	0,03	9,80	2-3
18	0,72	0,75	0,43	0,63	0,18	27,90	4-5

By comparing our data (mean values) with those reported for Serbs and Swedes population, phenotyped by using plasma as matrix [6], it comes out a very good agreement in spite of different adopted protocol and analytical conditions. In agreement with literature data, higher CYP1A2 enzyme activity was observed in heavy coffee consumers (> 3 cups) compared to not heavy coffee consumers (≤ 3 cups) and both groups not statistically different from those of both Serbs and Swedes populations. However, it has to be stressed that the two groups of Italian *espresso* drinkers are not (p 0.01) or very weakly (p 0.05) statistically different, and moreover, in this comparison, 3 cups consumption is included in the heavy coffee consumer groups of both Serbs and Swedes populations but it is included in the no heavy coffee consumers of the present Italian groups of coffee drinkers.

Table 2. 17X/137X ratio at 4 h post dose. Saliva and Plasma refer to matrix used in the Reference. Data related to Saliva columns are reported as number of subjects, median in bold and min. max. interval. Data related to Plasma columns are reported as number of subjects and mean ± standard deviation in bold. A different clustering is highlighted in the case of Italian subjects.

	Saliva		Plasma		Ref.
Population	**< 2 cups**	**≥ 2 cups**	**< 3 cups**	**≥ 3 cups**	
Australians (European ancestry)	(115) **0.57** (0.19– 1.35)	(17) **0.73*** (0.22 – 1.22)			[7]
Australians (South Asian ancestry)	(145) **0.42** (0.12– 1.06)	(8) **0.76*** (0.21– 0,68)			[7]
Serbs			(54) **0.39 ± 0.14**	(18) **0.50 ± 0.11**	[6] #
Swedes			(72) **0.48 ± 0.13**	(42) **0.55 ± 0.16**	[6] ##
Italians		(17) **0.50** (0.27 – 0.74)	**≤ 3 cups** (12) **0.45 ± 0.15**	**> 3 cups** (6) **0.64 ± 0.10**	

** Italians consumer of ≥ 4 cups (6) **0.66** (0.51 – 0.69)*
 # p (95% CI of the mean difference) 0.003
p (95% CI of the mean difference) 0.02

By focussing on coffee consumption these findings suggest that coffee beverages typically consumed in the Countries taken into consideration for comparison, may be very different from an *espresso* coffee of pure Arabica. In particular the determined CYP1A2 activity seems to be related to the caffeine content of coffee beverages being that of *espresso* 100% Arabica apparently lower than that of coffee beverages habitually consumed in Australia, Serbia and Sweden. From this point of view, when *espresso* coffee Italian style (100% Arabica) is habitually consumed it seems more appropriate to define "moderate coffee consumption" a consumption of 3 -5 cups. However, in view of the possibility to modulate the caffeine content during *espresso* brewing (*ristretto* contains less caffeine than *lungo*) it is not possible to draw a clear line between no heavy and heavy consumption by just considering the numbers of cups without any detail.

Pharmacokinetics

In order to ascertain the reliability of the 17X/137X ratio at 4 h post dose, pharmacokinetics approach was followed. In Table 3, results from base model (without covariate effect) are reported.

Table 3. Pharmacokinetic parameters (RSE – relative standard error) of caffeine in saliva following espresso (approx 75 mg caffeine)

Parameter	Typical value		Interindividual variability (CV%)		Interoccasion variability (CV%)	
	Estimate	RSE (%)	Estimate	RSE (%)	Estimate	RSE (%)
CL (L/h)	5.79	8.8	29.6%	38.5	22.9	46.3
V (L)	27.0	8.8	37.7%	47.7	30.5	47.3
Residual error (%)	29.3	11.3				

Absorption ($t^{1/2}$ = 1 – 10 min.) is very rapid as expected. Clearance and volume of distribution are in agreement with literature data when similar caffeine dose was orally administered. Half-life, $t^{1/2}$, (3.23 ± 1.20 h) is consistent with that reported in previous studies. Inter-individual and inter-occasion variability are in good agreement with those observed by using the 17X/137X ratio.

When covariate effects were studied, in the first step the effects of age, coffee consumption and weight were significant at the 5% level. The effect of weight using a power relationship (CLWT – 5) resulted in highest drop in OFV which was highly significant (p=0.000192) (see Table 4). With coffee consumption we have three additional degrees of freedom DF) as we have three more parameters (there are four categories). In the second step none of the remaining covariates was significant at the 5% level, however, you can note that CONSBI is close (p= 0.061927) (see Table 4). Finally, we have a backwards elimination step where we pull each covariate out of the model and again check if this is significant applying a more stringent criterion (1% significance level) due to multiple comparisons issue. We make this step as there may be some co-linearity between the covariates. In our example this step however is irrelevant as only one covariate entered the model.

Multivariate analysis (against the final model) performed to estimate relative effects of coffee consumption on CL (relative to 2-3 cups/day) suggest that subjects who drink ≥ 4 cups/day have on average 21.3% higher caffeine clearance compared to those subjects who drink ≤ 3 cups/day. However, by likelihood ratio test, this effect is not significant.

Table 4. Covariates analysis.

Model	ΔOFV	DF	p – value
First step			
CL-AGE (linear)	5.062	1	**0.024**
CL-AGE (power)	4.998	1	**0.025**
CL-CONSBI	5.502	1	**0.019**
CL-CONSUM	10.671	3	**0.014**
CL-SX	2.986	1	0.084
CL-WT (linear)	13.398	1	**<0.001**
CL-WT (power)	13.909	1	**<0.001**
V-AGE (linear)	1.700	1	0.192
V-AGE (power)	1.767	1	0.184
V-CONSBI	0.092	1	0.762
V-CONSUM	2.032	3	0.566
V-SX	0.869	1	0.351
V-WT (linear)	2.943	1	0.086
V-WT (power)	2.938	1	0.086
Second step			
CL-AGE (linear)	0.264	1	0.607
CL-AGE (power)	0.154	1	0.695
CL-CONSBI	3.485	1	**0.062**
CL-CONSUM	6.588	3	0.086
CL-SX	1.088	1	0.297
V-AGE (linear)	1.772	1	0.183
V-AGE (power)	1.842	1	0.175
V-CONSBI (linear)	0.094	1	0.760
V-CONSUM (linear)	2.083	3	0.555
V-SX	0.862	1	0.353
V-WT (linear)	2.955	1	0.086
V-WT (power)	2.950	1	0.086

The general mechanism of CYP1A2 induction is well known and includes ligand-dependent activation of aryl hydrocarbon receptor (AHR). Caffeine is known as CYP1A2 inducer, and in spite of its low affinity to AHR, other mechanisms have been suggested to explain its inducing activity. The planarity of caffeine molecule has been suggested as a structural feature which make not surprising its nature of selective substrate/inducers of the CYP1A2 protein. Thanks to its planarity, caffeine is able to form hydrophobically bound π-molecular complexes with many aromatic ring-containing molecules including coffee polyphenols like chlorogenic acid and caffeic acid. It may be speculated that caffeine in complexed form could favourably interact with AHR more than caffeine alone.

It has been presumed that other compounds present in coffee (for instance polycyclic aromatic hydrocarbons) may be at least partly responsible for the CYP1A2 inducing effect however, it cannot be excluded a priori that coffee polyphenols could play some role. In fact, dietary polyphenols have been indicated as AHR ligands.

As far as coffee consumption is concerned, it may be suggested that when it is used as a covariate in CYP1A2 phenotyping studies, the number of cups is a not appropriate metric in

the lack of details on preparation, and possibly, on coffee product (instant, roasted and ground, blend, etc.).

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Contribution of Stimulant Foods for Habitual Daily Intake of Methylxantines in Rio De Janeiro

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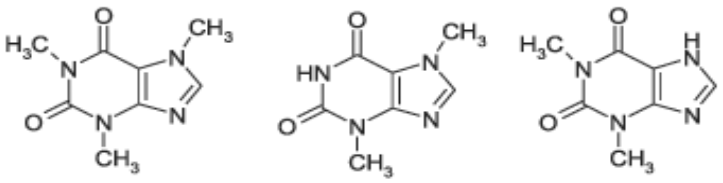
SUMMARY

The present work aimed to evaluate the contents of methylxanthines in the most commonly consumed food sources in the city of Rio de Janeiro and to estimate their contribution to the daily dietary intake of these compounds. For this, stimulant foods and beverages were analyzed using HPLC-DAD-reverse phase and a questionnaire evaluating the frequency of methylxanthines food sources consumption was applied to a 2120 people sample living in the different regions areas of Rio de Janeiro.

INTRODUCTION

Caffeine, theobromine and theophylline (Fig. 1) are chemical compounds known as methylxanthines. Caffeine is the most abundant methylxanthine in foods, followed by theobromine, with theophylline present only in lower concentrations. These substances are particularly found in coffee (*Coffea sp*), tea (*Camelia sinensis*), mate (*Ilex paraguariensis*), cocoa (*Theobroma cacao*), guaraná seeds (*Paulinia cupana*) and cola seeds (*Cola nitida*). Also, these compounds can be added to soft drinks, energy drinks and medications.



A B C

Figure 1. Chemical structures of caffeine (A), theophylline (B) and theobromine (C).

Methylxanthines are the most consumed stimulant substances worldwide, especially caffeine, exerting important effects on human central nervous system, increasing alertness and reducing fatigue. Like Caffeine, theobromine acts by stimulating diuretic activity but its action on the central nervous system is weaker, about one-third of caffeine's. Theophylline is used as a bronchodilatador drug.

Many studies have linked consumption of foods rich in methylxanthines with medicinal properties, which have been attributed both to these substances and to the polyphenols contained in such foods. Coffee is the primary source of caffeine among foods and due to its psychoactive effects, there has been prejudice against its use, even though other foods containing caffeine and other methylxanthines, such as tea, chocolate and soft drinks are often highly consumed by the population.

The aims of this study were to evaluate the levels of methylxanthines in the most commonly consumed food sources in the city of Rio de Janeiro and to estimate their contribution to the daily dietary intake of methylxanthines so that consumer education strategies can be developed.

MATERIALS AND METHODS

Assessing methlyxanthines intake

To assess the type of food sources of methylxanthines consumed and frequency of consumption, a questionnaire was applied to a 2120 people sample living in Rio de Janeiro, including children, teenagers, adults and elderly. The questionnaires were also used to evaluate the contribution of foods to methylxanthines intake in Rio de Janeiro urban area.

Food samples selection and acquisition

Using information contained in the questionnaires from this study and from *Interscience* data base (Brazil), commercial samples of the most commonly consumed stimulating foods in Rio de Janeiro were purchased in supermarkets, totaling 215 samples as follows: ground and roasted coffee (n=40), soluble coffee (n=10), instant cappuccino (n=10), white tea in sachet (n=9), green tea in sachet (n=9), black tea in sachet (n=9), toasted mate tea in sachet (n=6), white tea in bulk (n=7), green tea in bulk (n=7), black tea in bulk (n= 7), toasted mate tea in bulk (n=7), industrial white tea (n=3), industrial green tea (n=3), industrial black tea (n=3), industrial mate tea (n=3), green mate (n=8), cola soft drinks (n=4), guarana soft drinks (n=4), energy drinks (n=5), natural guarana (n=5), syrup guarana (n=4), chocolate powder (n=13), milk chocolatey drink (n=5), cocoa powder (n=8), milk chocolate sweets (n=10), dark chocolate sweets (n=10).

Beverages preparation

Beverages that were not ready to drink were prepared according to traditional methods used in Brazil. When available, the preparation instructions on the products' labels were followed. In all cases, ultrapure water (MilliQ, Millipore USA) at 95ºC was used for drinks preparation. Coffee drinks were prepared at 10% (weight/volume), using four different methods: manual percolation with cloth strainer, manual percolation with filter paper, electric coffee maker with filter paper, and espresso coffee. These results were considered when calculating methylxanthines intake according to the questionnaire answers; instant coffee and instant cappuccino were prepared dissolving the powders at concentrations of 4% and 15%, respectively; green, black, and white tea, as well as toasted mate tea were prepared infusing one tea bag (1.6g) or 3.2g of bulk tea in 200mL of water; green mate was prepared according to the method for preparation of "chimarrão" as described by Mazzafera; for preparation of chocolate drink, 30g of cocoa powder were diluted in 200mL of water.

Extraction and clarifications

Ready to drink green, black, white and mate teas as well as natural guarana and milk chocolatey drink were directly clarified for chromatography with Carrez solutions and filtered in paper filter Whatman nº1, as described by Farah *et al.*. Solid samples (dark and milk chocolate bars and sweets) were defatted prior to extraction using Soxhlet method and methylxantines were extracted from powders with methanol at 40% prior to clarification; soft drinks and energy drinks were degassed using an Ultrasonic Cleaner (Bransonic, USA), followed by clarification.

Chromatographic analysis of methylxanthines

Methylxanthines were analyzed by HPLC, using DAD (Shimadzu, model LC-10-AD, Kyoto, Japan), operating at 272nm, ODS-C18 column (Rexchrom: 5µM, 250 x 4.6 nm, Regis Technologies, Morton Grove, IL) and methanol solution at 40% as mobile phase at flow rate of 1mL/min, according to Farah *et al.*.

Statistical analysis

The content of each methylxanthine in foods was presented as mean ± standard deviation. Chromatographic results were analyzed by *GraphPad Prism*, version 5.0 (San Diego, CA, USA) using non pared *t*-test to compare pairs of samples and analysis of variance (ANOVA) to compare three or more samples. Results were considered significant when $p \leq 0.05$. Data from questionnaires were analyzed using software *CSPro* version 4.1. (USA).

RESULTS AND DISCUSSION

Methylxanthine analyses

Regarding the mean contents of methylxanthines in foods (Table 1), caffeine was the only methylxanthine detected in all evaluated food samples (Fig. 2); theobromine was identified in mate, cocoa derivatives, cola soft drinks, guarana soft drinks and energy drinks (Fig. 3); small amounts of theophylline were identified in samples derived from cocoa (Fig. 4).

Soluble coffee beverages presented the highest contents of caffeine (mean of 269.0 ± 11.7mg/100mL), probably because of the high percentage of Robusta coffee in the blends. Espresso coffee, beverages prepared using electric coffee maker, and dark chocolate followed. Guarana soft drink presented the lowest levels of caffeine (0.5 ± 0.3mg/100mL) (Fig. 2). Dark chocolate presented the highest mean contents of theobromine and theophylline (1036.8 ± 136.4mg/100g and 7.8 ± 2.1mg/100g, respectively), followed by other type of chocolate products (Figg. 3 and 4). Mate tea, energy drinks, cola soft drink and guarana soft drink also contained low amounts of theobromine (Fig. 3).

Table 1. Caffeine, theobromine and theophylline contents in stimulant foods.

	Methylxanthines content (mg/100g or mL)			
Beverages	**Caffeine**	**Theobromine**	**Theophylline**	**Total Methylxanthines content**
Cloth strainer coffee	63.2±8.6	Nd	Nd	63.2
Filter paper coffee	56.7±12.6	Nd	Nd	56.7
Coffee maker beverage	119.6±18.7	Nd	Nd	119.6
Espresso coffee	196.4±39.3	Nd	Nd	196.4
Soluble coffee drink	269.0±11.7	Nd	Nd	269.0
Instant capuccino	31.4±3.0	12.1±3.9	Nd	43.5
White tea in bulk	13.3 ± 7.1	Nd	Nd	13.3
White tea in sachet	6.6 ± 1.2	Nd	Nd	6.6
Green tea in bulk	19.7 ± 6.2	Nd	Nd	19.7
Green tea in sachet	6.6 ± 5.3	Nd	Nd	6.6
Black tea in bulk	23.2 ± 8.3	Nd	Nd	23.2
Black tea in sachet	11.2 ± 6.7	Nd	Nd	11.2
Green mate	21.7±3.0	7.3 ±1.6	Nd	29.0
Toasted mate tea in bulk	13.0 ± 6.7	2.4 ± 1.1	Nd	15.4
Toasted mate tea in sachet	6.5 ± 3.6	2.4 ± 1.1	Nd	8.9
Ready to drink white tea	2.3 ± 0.4	Nd	Nd	2.3
Ready to drink green tea	2.5 ± 0.4	Nd	Nd	2.5
Ready to drink black tea	4.5± 1.5	Nd	Nd	4.5
Ready to drink mate tea	11.5 ± 0.7	1.0 ±0.1	Nd	12.5
Chocolate drink	2.4 ± 0.8	288.7±81.6	3.7±1.3	294.8
Cocoa drink	185.5±52.3	1752.7±728.0	4.9±0.8	1949.3
Milk chocolatey drink	25.2±12.7	296.2±121.2	3.3±1.8	324.7
Cola soft drinks	8.3 ± 1.2	0.3±0.3	Nd	8.6
Guarana soft drinks	0.7± 0.2	0.4±0.1	Nd	1.1
Energy drinks	35.2 ± 0.8	2.2±0.07	Nd	37.4
Natural guarana	3.0 ± 2.7	0.5±0.3	Nd	3.5
Syrup guarana drink	11.0 ± 2.5	1.1±0.1	Nd	12.1
Dark chocolate	83.1 ± 12.6	1036.8 ± 136.4	7.8 ± 2.1	1127.7
Milk chocolate	25.2 ± 12.7	296.2 ± 121.2	3.3± 1.8	324.7

Average methylxanthines content (mg/100g or mg/100mL) in stimulant foods commonly consumed by a sample of the population living in Rio de Janeiro (n=2120). Results are expressed as mean ± standard deviation.

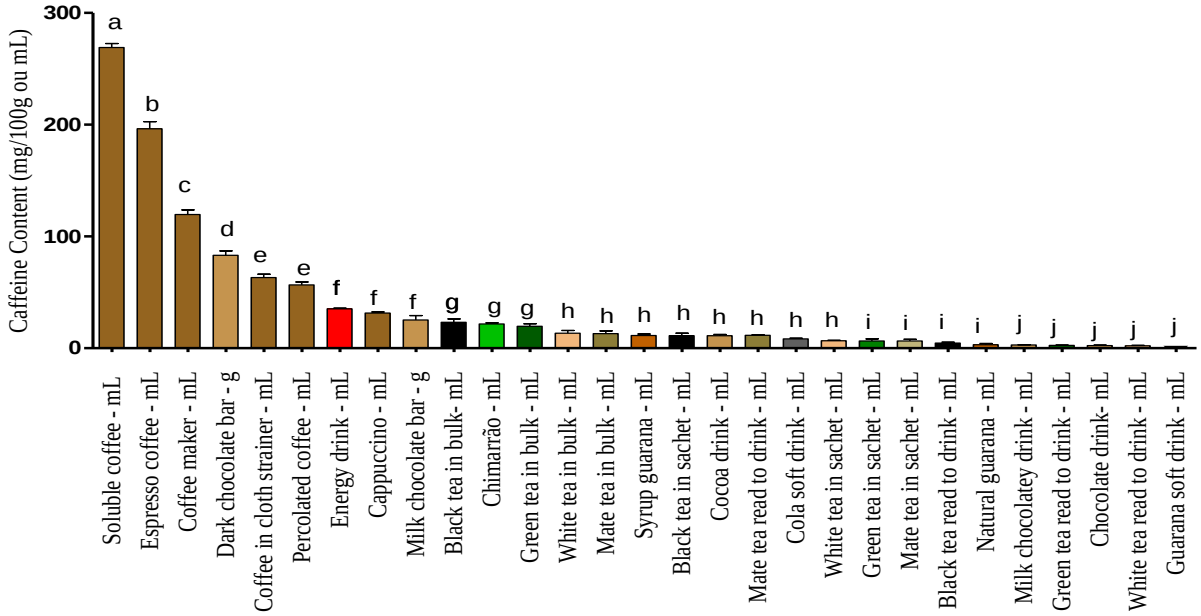


Figure 2. Average caffeine content (mg/100g or mg/100mL) in stimulant foods commonly consumed by a sample of the population living in Rio de Janeiro (n=2120). Results are expressed as mean ± standard deviation.

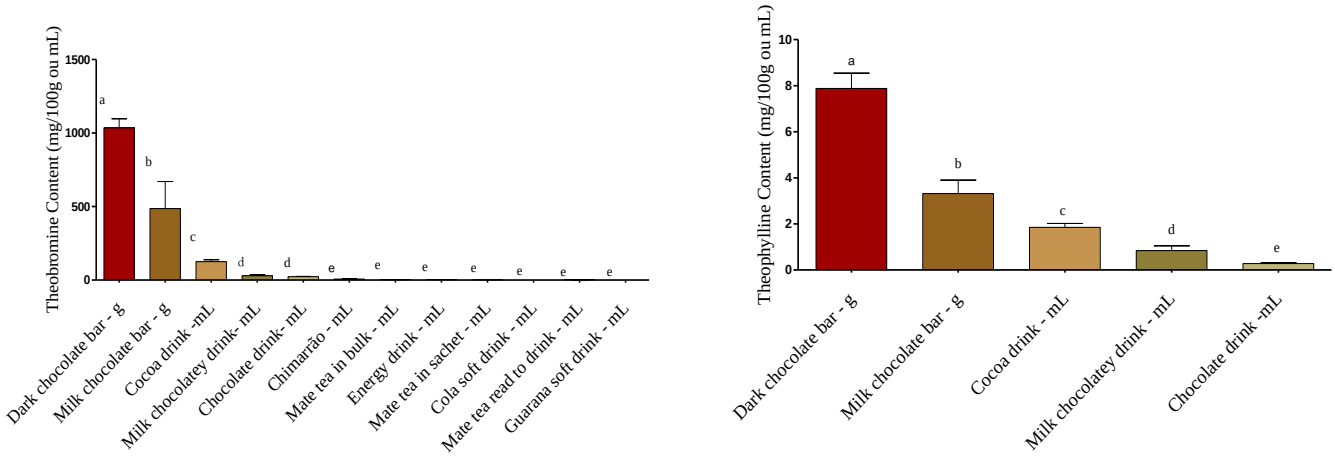


Figure 3. Mean theobromine content (mg/100g or mg/100mL) in stimulant foods commonly consumed by a sample of the population living in the city of Rio de Janeiro (n=2120). Results are expressed as mean ± standard deviation and were considered significant when $p \leq 0.05$. Figure 4. Mean theophylline content (mg/100g or mg/100mL) in stimulant foods commonly consumed by a sample of the population living in the city of Rio de Janeiro (n=2120). Results are expressed as mean ± standard deviation and were considered significant when $p \leq 0.05$

When comparing the contents of caffeine considering traditional consumed servings, differences between coffee and other foods decrease considerably (Fig. 5).

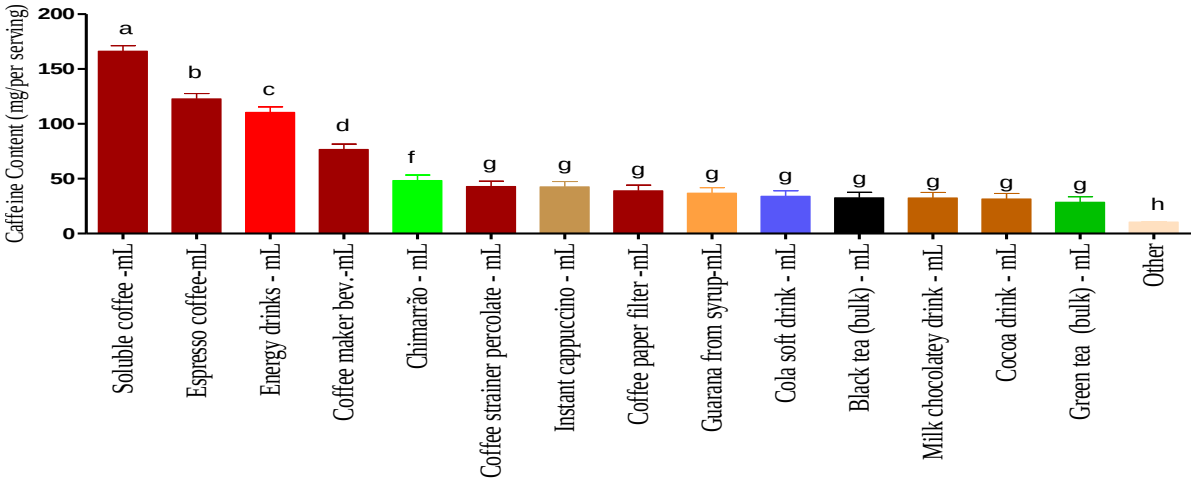


Figure 5. Mean caffeine content (mg/per serving) in stimulant foods commonly consumed by the population living in the city of Rio de Janeiro. Results are expressed as mean ± standard deviation. Differences were considered significant when $p \leq 0.05$. Serving sizes: coffee beverages: 60mL, energy drinks: 250mL, teas: 120mL, cappuccino: 240mL, soft drinks: 350mL, chocolate drinks: 240mL).

Methylxanthines intake

Data collected in Rio de Janeiro survey showed that a variety of stimulating foods are consumed daily by all groups investigated, and their contribution to total consumption of methylxanthines was significant, varying according to age groups.

Filtered coffee was the main contributor to total intake of methylxanthines in male adults (n=490) and elderly (n=220) (17.8 and 16.8% respectively). In male adults, filtered coffee was followed by energy drinks (9.0%) and espresso coffee (8.9%). In male elderly, filtered coffee was followed by dark chocolate bar (16.4%), coffee with milk (13.5%) and espresso coffee (12.6%). The main difference between male adults and elderly was that energy drinks were more consumed by male adults, while black and green teas were more consumed by male elderly.

Among female adults (n=530) and elderly (n=240), on the other hand, dark chocolate bar was the main contributor to total intake of methylxanthines, corresponding to 18.6% and 18.4%, respectively. In female adults, dark chocolate bar was followed by milk chocolate bar (10.5%), mate tea read to drink (9.6%) and espresso coffee (8.7%), while in female elderly, dark chocolate bar was followed by filtered coffee (18.4%), coffee with milk (14.8%) and instant coffee (12.6%). While in male adults and elderly, coffee and chocolate were the main contributors to methylxanthines intake, in female adults and elderly, not only chocolate but other foods were important contributors to methylxanthines intake, such mate tea read to drink and cola soft drinks.

Teenagers and children presented similar consuming behavior, with just a few differences. For male (n=150) and female (n=170) teenagers, dark chocolate was the main contributor to total intake of methylxanthines, about 16.9% and 19.1%, respectively, followed by milk chocolate (14.8% and 11.2%, respectively). Among female teenagers, chocolate products presented the highest contribution to total intake of methylxanthines and it was in accordance with female adults pattern. For male teenagers, energy drink was the third (8.7%) contributor

to total consumption of methylxantines, and its contribution is in agreement with male adults. Among male (n=160) and female (n=160) children, chocolate products and cola soft drinks were the main contributors to total intake of methylxanthines (about 70 and 68%, respectively). Coffee with milk was the seventh contributor to total intake of methylxanthines in both groups, corresponding to 6.0 and 6.6% in male and female children, respectively.

Based on the present results, if one compares the caffeine and theobromine contents in milk-chocolate drinks (up to 4 mg/200mL serving and up to 60mg/200mL serving, respectively) with the caffeine content of milk with 10% coffee, as Brazilian usually drink (10-12 mg/150mL serving), it is possible to note that methylxanthine contents are quite similar and even considering the milder effect of theobromine on central nervous system, children can consume a considerable amount of methylxanthine daily. Therefore, there would not be a logical justification from the stimulation point of view for restraining children, as it is commonly observed, from consuming milk with 10% coffee.

Regarding the total daily consumption of different methylxanthines, while in adults and elderly caffeine was the main methylxanthine consumed, among children and teenagers (average of 215.5 and 233.1mg/day, about 58% and 56%, respectively), theobromine was most consumed (average of 224.8mg/day about 62% and 60%, respectively), as expected (Fig. 6).

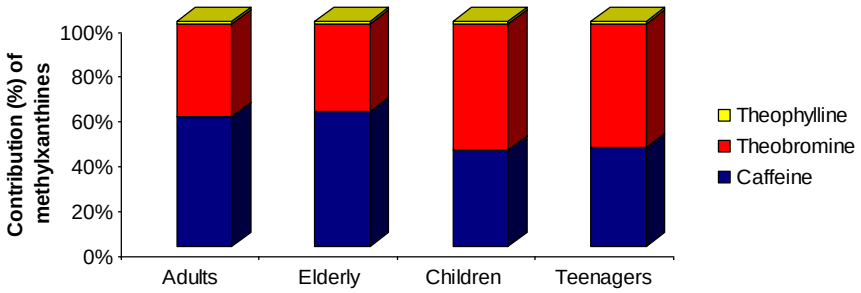


Figure 6. Contribution of caffeine, theobromine and theophylline for total consumption of methylxanthines by adults, elderly, children and teenagers. Results are expressed as percentage.

Finally, male elderly and adults were the main daily consumers of caffeine (about 257.3 and 240.7mg/day, respectively), followed by female elderly (209.0mg/day), female adults (191.7mg/day, male and female teenagers (about 171.0mg/day and 153mg/day). Female and male children were the groups that consumed less caffeine, about 121 and 123mg/day, respectively.

There are no official daily recommendations or limits for caffeine or methylxanthines intake by adults and elderly. However, Nawrot et al. suggested that levels of up to 300mg caffeine /day are not associated with undesirable effects in adults. Regarding children, although there are no recommendations for methylxanthines intake, in Canada, a maximum limit of ingestion of 45-85mg of caffeine/day is officially recommended, depending on age. Based on data from the present study, Brazilian male and female children living in the city of Rio de Janeiro are consuming on average, 123.0 and 128.1mg of caffeine/day, respectively, which is equivalent to three times the maximum ingestion recommended in Canada. Therefore, future investigations are needed in order to evaluate how the higher consumption of caffeine affects Rio de Janeiro children comparing to Canadians.

In conclusion, soluble coffee beverages presented the highest contents of caffeine, whilst guarana soft drinks, the lowest and dark chocolate bar presented the highest contents of theobromine and theophylline. Considering the contribution of different stimulant foods for total intake of methylxanthines, coffee was the main contributor among male adults and elderly while dark chocolate was the main contributor among female adults and elderly. Chocolate products were the main contributor for total intake of methylxanthines among children and teenagers of both genders. The present results show that foods different from coffee may play an important role for total consumption of stimulant substances in all age groups living in the city Rio de Janeiro and the population should be aware of it. Moreover, the implications of "high" consumption of methylxanthines by Brazilian children based on recommendations for Canadian children must be evaluated.

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Evaluation of Hepatic Cytochrome P450 (CYP) 1A Enzymes Involvement in the Metabolism of Caffeine and Chlorogenic Acid from Coffee

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SUMMARY

The aim of the study was to evaluate the effects of orally administered 5-caffeoylquinic acid (5-CQA) and caffeine solutions as well as regular and decaffeinated coffee extracts on the activities of CYP1A1/2 in the mouse liver. Although treatments with plain caffeine and regular coffee increased CYP1A1/2 activity, only administration of plain caffeine led to a consistent dose-dependent induction pattern. Liver constitutive activity of CYPA1/2, however, remained unaltered in mice treated with 5-CQA and decaffeinated coffee. These results were consistent with the notion that caffeine is an inducer of (and possibly a substrate for) CYP1A enzymes and additionally suggested that these enzymes are unlikely to play a role in the metabolism of 5-CQA and other chlorogenic acids (CGA).

INTRODUCTION

Coffee is not only one of the most consumed beverages worldwide, but also one of the valuable primary products in the world trade. Unsurprisingly, therefore, the effects of coffee consumption on human health have been studied extensively over the past decades. Regular consumption of coffee at certain amounts have been associated with a reduced risk of chronic illnesses such as liver and colon cancer, type-2 diabetes mellitus, and Parkinson and Alzheimer diseases. Coffee beverages contain high amounts of caffeine and phenolic compounds, particularly chlorogenic acids, and are major sources of these substances in the human diet.

Chlorogenic acids (CGA) and their derivatives, along with caffeine and its metabolites, have been singled out as the bioactive components of coffee that are responsible for several of the putative beneficial health effects of this beverage. Among CGA, caffeic acid derivatives (caffeoylquinic and dicaffeoylquinic acids) are the most abundant and best-studied coffee phenolic constituents. To elucidate the effects of coffee on health, it is needed to investigate, among other topics, CGA and caffeine metabolism after coffee intake and their pharmacological actions.

It has been demonstrated that plain caffeine is metabolized predominantly in the liver and that cytochrome P450 enzymes of 1A subfamily (CYP1A1/2) contribute to over 95% of these oxidation reactions. Nonetheless, the biotransformation of caffeine from coffee beverages, and how caffeine interacts with other components of the food matrix have not been completely unveiled. Moreover, there are relatively few investigations on CGA metabolism and on how these compounds are eliminated from the body and in some instances, there is a lack of consistency between results from different studies. It remains to be elucidated, for instance, whether CGA and their metabolites are substrates for and/or inducers of phase I enzymes, including the monooxygenases of the subfamily 1A. CYP1A enzymes seem to be involved in the metabolism of other phenolic compounds such as flavonoids. CYP1A2 is known to take part in the biotransformation of a variety of widely used drugs such as the pain killers phenacetin and paracetamol, the anticoagulant R-warfarin, the beta-blocker propranolol, and the bronchodilator theophylline, which is a metabolite of caffeine. The antidepressants amitriptyline and fluvoxamine, and the antipsychotic drugs clozapine and haloperidol are also substrates for CYP1A2.

The effects of caffeine and CGA present in the coffee matrix on CYP1A1/2 activities have not been thoroughly evaluated either in humans or in animals. As far as the authors are aware, there are no reports on the effects of these two coffee components on the activity of CYP1A enzymes in the mouse liver. Since CYP1A1/2 takes part in the metabolism of caffeine and several commonly used drugs, a potential CYP1A inducing effect of coffee and its constituents would indicate that consumption of coffee beverages might influence the clearance and effects of these xenobiotic compounds. Therefore, this study was undertaken to investigate whether 5-caffeoylquinic acid (or chlorogenic acid, the main CGA in coffee) and caffeine solutions, as well as regular (caffeinated) and decaffeinated coffee extracts induce CYP1A1/2 activities.

MATERIALS AND METHODS

Animals

Female *Swiss Webster* mice, aged 12-14 weeks, from the Oswaldo Cruz Foundation (FIOCRUZ) Central Animal House breeding stock were used. The animals were housed in standard plastic cages with stainless steel covers and white wood shavings as bedding, and kept in animal facilities under controlled temperature (23 ± 2° C), air relative humidity (approximately 70%) and light/dark cycle (12h/cycle). All mice had free access to filtered tap water and a rodent commercial chow (Nuvital CR1, Nuvilab®, Curitiba, PR, Brazil) throughout the experiment.

Extracts and solutions

Coffee extracts were prepared from green regular and decaffeinated *C. canephora* beans. Infusions were made by adding 100mL of hot water (90° C) to 20g of ground coffee (20% w/v). After 5 minutes, infusions were filtered using a paper filter. Aliquots of each extract were stored in the freezer at -20°C for further CGA and caffeine analysis. Anidrous 5-cafeoilquinic acid (98%, C3878, Sigma-Aldrich Chem. Co) and caffeine (C3878, Sigma-Aldrich Chem. Co) were used to prepare solutions administered to mice.

Treatment

Mice were treated orally (gavage) once a day for three consecutive days. Nine different treatment groups received solutions as follows: 1) water (control, n=6); 2) 5-CQA aqueous

solution (100mg/ kg bw/ d, n=4); 3) 5-CQA aqueous solution (200mg/ kg bw/ d, n=5); 4) caffeine aqueous solution (100mg/ kg bw/ d, n=8); 5) caffeine aqueous solution (200mg/ kg bw/ d, n=5); 6) regular aqueous coffee extract (100mg of CGA and 50 mg of caffeine/ kg bw/ d, n=5); 7) regular aqueous coffee extract (200 mg of CGA and 100 mg of caffeine/ kg bw/ d, n=4); 8) decaffeinated aqueous coffee extract (100mg of CGA/ kg bw/ d, n=5); 9) decaffeinated aqueous coffee extract (200mg of CGA/ kg bw/ d, n=4). Twenty-four hours after last dosing mice were euthanized by cervical dislocation.

Preparation of liver microsomal fraction (LMF)

Immediately after euthanasia, mouse liver was quickly removed, weighed, individually wrapped in aluminium foils and kept frozen in liquid nitrogen until the LMF preparation. LMF was prepared as described in details by De-Oliveira *et al*. (1997). All procedures were performed at low temperature (0–4 °C). Briefly, livers were homogenized in 4× (w/ v) ice-cold buffer (Tris-HCl 100mM/ KCl 150mM; pH=7,4) and centrifuged at 9000×*g* for 30 min (at 4 °C). The supernatant was filtered and then ultracentrifuged at 100,000×*g* for 60 min (at 4 °C). The pellet was re-suspended in buffer solution (Tris-HCl 100mM/ KCl 150mM; pH=7,4) and ultracentrifuged again at 100,000×*g* for 60 min (at 4 °C). This second pellet was suspended in 2 mL of a freezing buffer solution (K_2HPO_4 100mM/ 20% v/ v de glycerol/ EDTA 1mM; pH=7,4). LMF was divided into aliquots in Nunc® cryo-tubules that were stored at -196°C until further analysis.

Measurement of protein concentration

LMF protein concentration was determined using Bradford reagent and bovine serum albumine as standard. The method was adapted to a microplate and absorbance was read at 595nm in a spectrophotometer Spectramax Plus® (Molecular Devices, USA).

Measurement of monooxygenase activities

Ethoxy-(EROD) and methoxy-(MROD) resorufin-*O*-dealkylases were assayed essentially as described by Burke *et al*. (1985) except for the use of a NADPH regenerating system as reported by De-Oliveira *et al*. (1997). Adaptation to the microplate (96 wells) method was performed as described by Kennedy & Jones (1994) [20] with a few modifications [21]. 50 mM pH 7.8 potassium phosphate buffer, substrate (for a final concentration of 5µM) and LMF (0.025 mg protein) were added to each well. After a 10-min incubation period at 37°C in a water-bath with shaking, reaction was started with the addition of the NADPH-regenerating system. After a 10 min-reaction period, acetonitrile was added to stop the reaction. The amount of resorufin was measured in a fluorescence plate reader (Spectramax Gemini XS®, Molecular Devices, USA) with excitation and emission wavelengths set at 530 and 590 nm, respectively. Results were expressed as *p*moles of resorufin/ mg protein/ min.

Statistical analysis

Data are presented as mean ± SE. Comparisons between two group means were made by Student's *t*-test. The induction factor (IF) was the ratio between mean activity determined for the testing solution group and the activity measured for respective control group. The Prism for Windows software (version 6.0, GraphPad Software Inc.) was used for statistical calculations. Differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Treatment with coffee extracts, 5-CQA, caffeine or β-naphthoflavone did not cause any change in body weight compared to control groups. The treatment with coffee extracts or constituents did not affect liver absolute or relative (ratio of liver weight to body weight) weights either.

As shown in Table 1, the activities of reactions (EROD and MROD) catalyzed predominantly by CYP1A subfamily enzymes (EROD and MROD activities) were induced by the positive control β-naphthoflavone (a known aromatic hydrocarbon receptor ligand). Activities of EROD and MROD in β-naphthoflavone treated mice (80 mg/ kg bw/ d, 3x) were 296% and 590% higher than the activities measured in the control group mice (treated with the vehicle, corn oil). These results were expected since β-naphthoflavone - a flavonoid - is known to be a potent inducer of CYP1A subfamily enzymes.

The administration of 5-CQA and decaffeinated coffee had not discernible effect on CYP1A (EROD and MROD) activities (Table 1). Caffeine in the tested doses, on the other hand, caused a small to moderate induction of CYP1A activity (IF = 1.6 – 1.9) in the mouse liver. Administration of regular coffee at the highest (200 mg/ kg bw/ d, 3x) but not at the lowest dose (100 mg/ kg bw/ d, 3x) also produced inductions of EROD (IF= 1.9) and MROD (IF= 1.7) activities. Since regular coffee contains caffeine, and decaffeinated coffee did not cause any change in CYP1A activity, it seems fair to think that enzyme induction was due to the presence of caffeine in the extract.

Chen *et al*. (1996) observed similar results when assessing the effect of tea consumption on the activity of CYP1A enzymes. When green tea (2%) and black tea (2%) were given to male rats as the sole source of drinking fluid for 21 days, an induction of MROD activity was observed in liver microsomes (IF = 2.4 and 2.7, respectively). Treating rats with caffeine (0.04%) also resulted in an increase in the MROD activity (IF = 1.9), but decaffeinated green tea (0.8%) did not cause such an induction. Rats treated with green tea (2%) or caffeine (0.055%) as the sole source of drinking fluid for 1, 3, and 7 days also showed comparable induction (from 1.7- to 2.1-fold) of the MROD activity. The induction was also shown by intragastric administration of caffeine (100 mg/kg). In addition, the concentrations of tea polyphenols and caffeine were also measured in plasma of the rats. Close correlation of the increase in the MROD activity was observed only with the plasma caffeine level ($r = 0.736$, $n = 10$, $p = 0.015$), not with the combined tea polyphenol level ($r = 0.058$, $n = 6$, $p = 0.913$). The referred study established that caffeine is an inducer of CYP1A2 enzyme and demonstrated that caffeine, not tea polyphenols, is the component in tea responsible for the induction of this isoform.

The present data together with literature information shows a pattern of induction of subfamily 1A enzymes by caffeine consistent with the dose offered, which is in accordance with Goasduff *et al*. (1996), who concluded that caffeine increases its own metabolism in a dose-dependent manner.

CONCLUSION

In conclusion, the intake of plain caffeine and regular coffee led to an increase in the activity of liver CYP1A1/2 compared to that of control group, with a consistent dose-dependent induction pattern observed for caffeine. Treatment with 5-CQA and with decaffeinated coffee extract caused no discernible increase in the activities of CYP1A catalyzed reactions (EROD and MROD). Caffeine has been reported to be a substrate for liver CYP1A enzymes and

results presented here are consistent with the notion that caffeine also induces the activity of these phase 1 enzymes. Data also suggested that, as far as mice are concerned, 5-CQA and other CGA do not induce the activity of CYP1A enzymes.

Since CYP1A subfamily takes part in the clearance of a number of commonly used drugs and are involved in the metabolic activation of a variety of chemical carcinogens (e.g. PAH: polycyclic aromatic hydrocarbons) these findings suggest that consumption of high amounts of caffeine and regular coffee (but not decaffeinated coffee) may have some effect on the kinetics of drugs and toxicants.

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New Findings of Oxidized Component-Reduced Coffee Containing Higher Levels of Chlorogenic Acids

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SUMMARY

Coffee chlorogenic acids (CGAs) are the major polyphenols in coffee beans. CGAs contribute to reduce high blood pressure. The high temperature of the coffee bean roasting procedure decomposes CGAs, and produces several other ingredients such as hydroxyhydroquinone (HHQ). HHQ inhibits the blood pressure improving effects of CGAs. Therefore, we developed a manufacturing process (oxidized component-reducing process; OC-R process) to produce ready-to-drink (RTD) coffees that contain high levels of CGAs and low levels of HHQ. The RTD coffee, which was manufactured using the OC-R process (OC-R coffee), reduced high blood pressure, and had improved taste and flavor features; that is, OC-R coffee had a smooth aftertaste and rich retronasal aroma.

The improved taste and flavor characteristics of the OC-R coffee were confirmed by sensory evaluation by a consumer panel (N=118). To determine the properties that underlie these features, we identified the components reduced by the OC-R process. Seven components were identified: HHQ; catechol; pyrogallol; 4-(furan-2-ylmethyl)-5-methylbenzene-1,2-diol; 2-acetylpyrrole; 2-acetyl-1-methylpyrrole; and 2-ethylpyridine. The impact of each of these seven components on the aftertaste and retronasal aroma was evaluated sensorially. The findings suggest that HHQ, catechol, pyrogallol, and 2-acetyl-1-methylpyrrole have negative effects on the aftertaste and retronasal aroma of coffee, whereas 2-acetylpyrrole and 2-ethylpyridine do not.

INTRODUCTION

Polyphenol is a generic term for compounds containing phenolic hydroxyl groups. Most plants contain some polyphenols. Tea leaves are well-known to contain polyphenols called catechins. Polyphenols have attracted considerable attention due to their wide variety of health benefits.

Chlorogenic acid (CGA) is another plant polyphenol with positive effects on health. CGA, present in high amounts in green coffee beans, is a generic term for hydroxycinnamic acid esters. CGAs in coffee beans generally comprise caffeoylquinic acids, di-caffeoylquinic acids, and feruloylquinic acids. Coffee CGAs comprise a large number of compounds. Roasting produces good coffee aroma. During the roasting process, CGAs decompose due to the high temperature, and other components are produced, such as hydroxyhydroquinone (HHQ). HHQ is not volatile. CGAs improve high blood pressure in rats and humans, whereas HHQ inhibits the blood pressure-improving effects of CGAs, based on studies of humans and animals.

We are working to produce coffees with health benefits, such as decreasing high blood pressure. To take advantage of the effects of CGAs to reduce blood pressure, we developed a manufacturing process to produce functional ready-to-drink (RTD) coffee beverages with high levels of CGAs and reduced levels of HHQ. This oxidized component-reducing process (OC-R process) effectively reduces only oxidized components such as HHQ without affecting CGAs and other components that contribute to coffee taste and aroma. Thus, coffee manufactured using the OC-R process not only has health benefits, but also has improved taste and flavor. In the present study, we evaluated the taste and flavor features of our RTD coffee.

MATERIALS AND METHODS

Chemical formulation of CGAs

We defined CGAs as a group of nine chlorogenic acid compounds, as shown in Fig. 1.

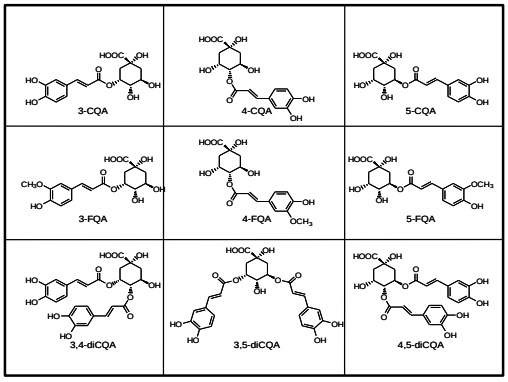

Figure 1.Chemical formulation of chlorogenic acid analogues.

Chemical formulation of HHQ

The chemical formulation of HHQ is shown in Fig. 2.

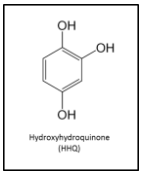

Figure 2. Chemical formulation of hydroxyhydroquinone.

Preparation of the coffee samples

Coffee beans that were roasted to the middle-light degree were granulated to the appropriate particle size. The granulated coffee beans were placed into the extracting apparatus and extracted with boiled water. The obtained extract was cooled to room temperature. For the OC-R coffee, the extract was filtered with an adsorption-filter and microfiltration membrane. For the control (Normal) coffee, the extract was filtered with only a microfiltration membrane. The pH of the extracts was adjusted to 6.3 with 10% sodium bicarbonate solution. The obtained coffee was diluted to 2.1 degrees Brix (˚Bx). The prepared coffee solution was heated to 75˚C and poured into 185-g cans. The headspace of the cans was filled with nitrogen

gas, the lids were quickly placed on the cans, and the cans were sealed. The canned coffees were sterilized by autoclaving at 124˚C for 10 min.

Sensory evaluation 1

The taste and flavor features of the OC-R coffee were evaluated using a paired comparison method. One hundred eighteen men and women who drink canned coffee on a daily basis were selected to serve as a consumer panel. The aftertaste and retronasal aroma of the OC-R coffee and Normal coffee were compared by the selected panel, who were blind to the identity of each coffee sample. The investigator serving the samples was not blind to the identity of the samples. Samples were served in 50-ml cups labeled "P" and "Q". The samples were rated using two 5-level rating scales according to the following criteria:

Standards for aftertaste

- The aftertaste of sample "Q" is smoother than that of sample "P".
- The aftertaste of sample "Q" is somewhat smoother than that of sample "P".
- The aftertaste of both samples is the same.
- The aftertaste of sample "P" is somewhat smoother than that of sample "Q".
- The aftertaste of sample "P" is smoother than that of sample "Q".

Standards for retronasal aroma

- The retronasal aroma of sample "Q" is richer than that of sample "P".
- The retronasal aroma of sample "Q" is somewhat richer than that of sample "P".
- The retronasal aroma of both samples is the same.
- The retronasal aroma of sample "P" is somewhat richer than that of sample "Q".
- The retronasal aroma of sample "P" is richer than that of sample "Q".

Identification of components reduced by the OC-R process

Liquid chromatography-time of flight-mass spectroscopy (LC-TOF-MS) analysis was performed on four samples: OC-R canned coffee, Normal canned coffee, and two Japanese commercial canned coffees named "1 and 2". Each sample was diluted 1:19 in ultrapure water. The diluted samples were then filtered through an ultra-filter with a pore diameter of 10,000 Da. The obtained samples were analyzed under the LC-TOF-MS conditions shown in Table 1.

Table 1. The LC-TOF-MS conditions

Analytical equipment	ACQUITY UPLC (Waters), LCT Premier XE (Waters)
Column	ACQUITY UPLC T3 column (2.1 × 50 mm)
Condition of LC	Linear gradient of 0.1% Formic acid aq and Acetonitrile
Ionization Mode	ESI positive, Negative
Iteration count with same condition on same samples	2

The obtained chromatogram was analyzed in detail according to following procedure. First the chromatograms of the samples were compared with the chromatogram for the solvent, and information regarding the predominant peaks was extracted. The m/z ratios and peak intensities for the reproducible peaks were then corrected. Peaks that were obtained as ESI-

positive and ESI-negative were combined. The peak information to be compared among the samples was extracted and aggregated among the samples. The intensities of the aggregated peaks were then corrected. The features of the peaks for each sample were extracted by calculating the relative intensities of the peaks for each sample. Finally, the components were estimated by comparing the information about molecular mass and retention time of each peak with those of known standards.

Sensory evaluation 2

The aftertaste features of the components reduced by the OC-R process were evaluated by a scaling analysis. The evaluation was conducted by a panel of seven trained analysts according to a 1 through 5 grading scale. The samples were prepared by adding each of the components identified by LC-TOF-MS. Each component was added so that the total amount of the component in the sample was the same as that in Normal coffee. The evaluation was conducted by regarding the aftertaste intensity of the OC-R coffee as '1', and that of the Normal coffee as '5'. The panelists were first asked to sip a sample and keep it in their mouths for 3 s before swallowing. Next, they were asked to rate the aftertaste intensity level by comparing it with that of the OC-R coffee and Normal coffee. Six samples were each evaluated by seven panelists.

Sensory evaluation 3

The retronasal aroma features of components reduced by the OC-R process were also evaluated by a scaling analysis. The evaluation was conducted similarly to that of sensory evaluation 2. In this evaluation, however, a '5' was used to indicate the same retronasal aroma intensity as the OC-R coffee, and '1' was used to indicate the same retronasal aroma intensity as the Normal coffee.

RESULTS

Sensory evaluation 1

The sensory evaluation results relating to aftertaste are shown in Fig. 3. The aftertaste of the OC-R coffee was rated by 83% of the subjects to be the same as or smoother than that of the Normal coffee, with 30% rating it as somewhat smoother, and 11% rating it as smoother. No one answered that the aftertaste of the Normal coffee was somewhat smoother than the OC-R coffee. The sensory evaluation results relating to retronasal aroma are shown in Fig. 4. The retronasal aroma of the OC-R coffee was rated by 89% of the subjects to be similar to or richer than that of the Normal coffee, with 17% rating it as somewhat richer than that of the Normal coffee, and 19% rating it as richer than the Normal coffee. No one answered that the retronasal aroma of the Normal coffee was somewhat richer than the OC-R coffee. Based on these results, the OC-R coffee was considered to have smoother aftertaste and a richer retronasal aroma than the Normal coffee.

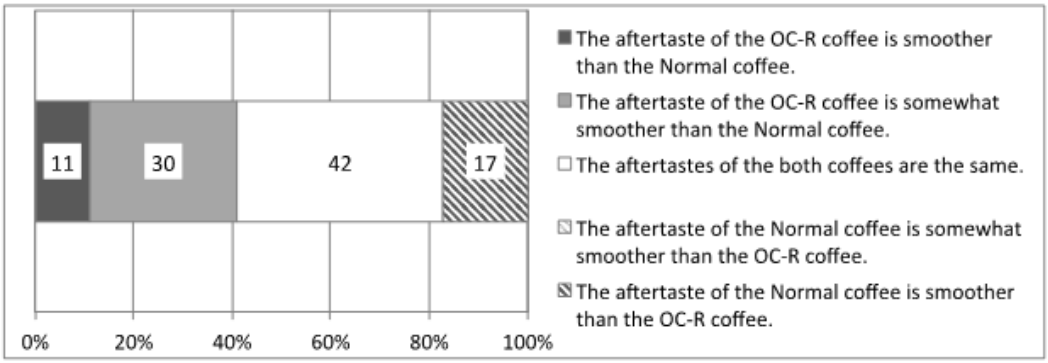

Figure 3. Results of the paired comparison evaluation of aftertaste between the OC-R coffee and Normal coffee.

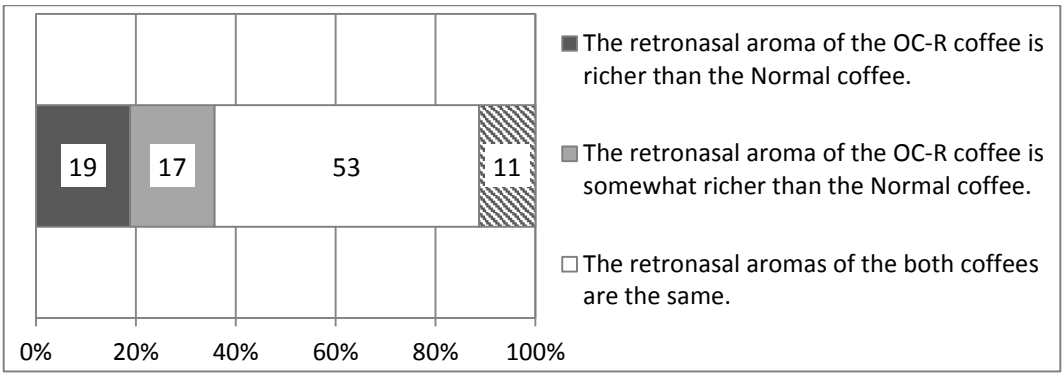

Figure 4. Results of the paired comparison evaluation of retronasal aroma between the OC-R coffee and Normal coffee.

Identification of components reduced by the OC-R process

A total of 73 components were identified from LC-TOF-MS. The seven components that were reduced by the OC-R process were identified by comparing the peak intensity of each identified peak between the OC-R coffee and the other coffees. The seven identified components were HHQ; catechol; pyrogallol; 4-(furan-2-ylmethyl)-5-methylbenzene-1,2-diol; 2-acetylpyrrole; 2-acetyl-1-methylpyrrole; and 2-ethylpyridine. The chemical formulas of these components are shown in Fig. 5.

The analytical results of the LC-TOF-MS are shown in Fig. 6. The peak intensity of the LC-TOF-MS for each component in the OC-R coffee was remarkably lower than that for each component in the Normal coffee and the two Japanese commercial canned coffees.

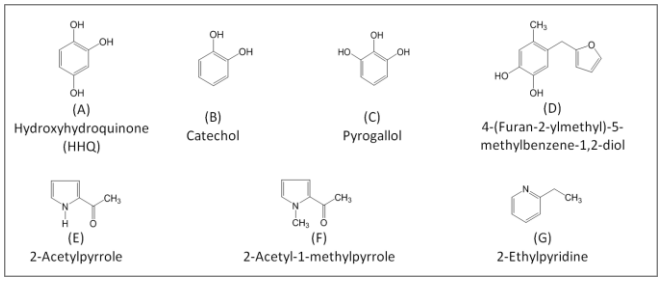

Figure 5. Chemical formulas of the identified seven components that were reduced by the OC-R process.

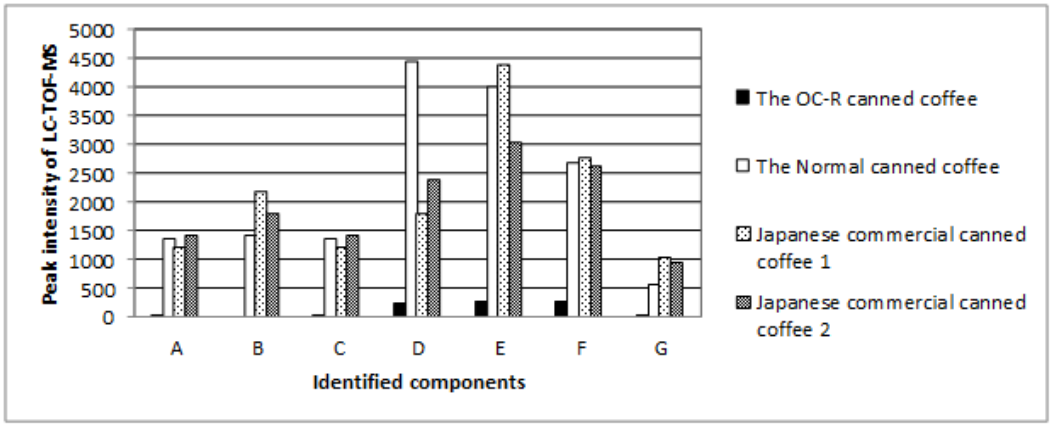

Figure 6. Peak intensities of LC-TOF-MS for the seven identified components in the four samples.

Sensory evaluation 2

The effects of the identified components on the aftertaste of the OC-R coffee were assessed by sensory evaluation. Evaluation of 4-(furan-2-ylmethyl)-5-methylvenzen-1,2-diol was not performed because we could not obtain a pure sample. The sensory evaluation results are shown in Figure 7. The bar chart shows the mean rating values of the seven trained panelists, and the error bars indicate the standard errors for each sample. The addition of HHQ, catechol, pyrogallol, and 2-acetyl-1-methylpyrrole to the OC-R coffee increased the aftertaste intensity of the OC-R coffee, whereas the addition of 2-acetylpyrrole and 2-ethylpyrridine had little effect on the aftertaste of the OC-R coffee.

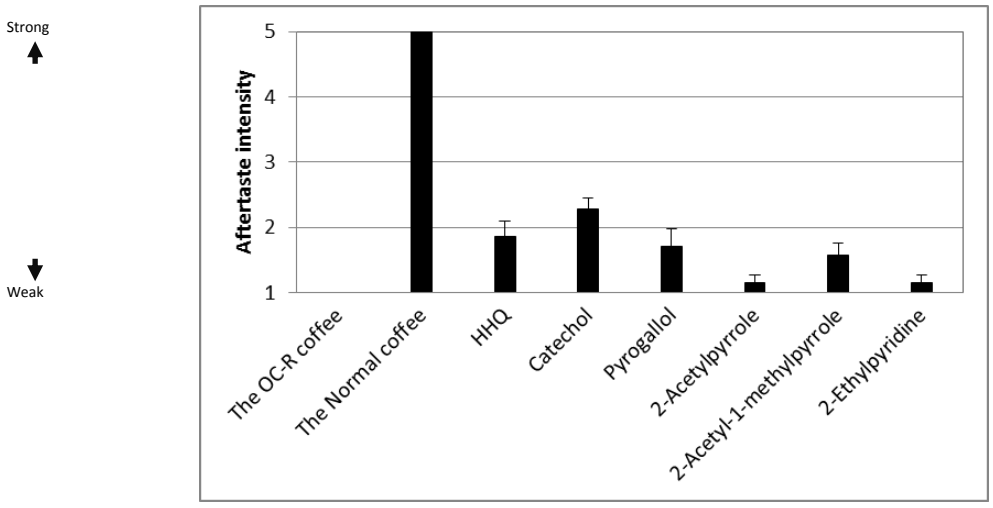

Figure 7. Sensory evaluation results of the effects of the components reduced by the OC-R process on the aftertaste.

Sensory evaluation 3

The effects of the components reduced by the OC-R process on the retronasal aroma were also evaluated sensorially. The evaluation procedure was almost the same as that for aftertaste; the intensity of the retronasal aroma for each sample was rated from 1 through 5, but with 5 indicating the same retronasal aroma intensity as OC-R coffee and 1 indicating the same retronasal aroma intensity as the Normal coffee. The results are shown in Figure 8.

Contrary to the previous sensory evaluation result, the addition of HHQ, catechol, pyrogallol, and 2-acetyl-1-methylpyrrole to the OC-R coffee weakened the retronasal aroma, whereas the addition of 2-acetylpyrrole and 2-ethylpyridine had little or no effect on the retronasal aroma of the OC-R coffee.

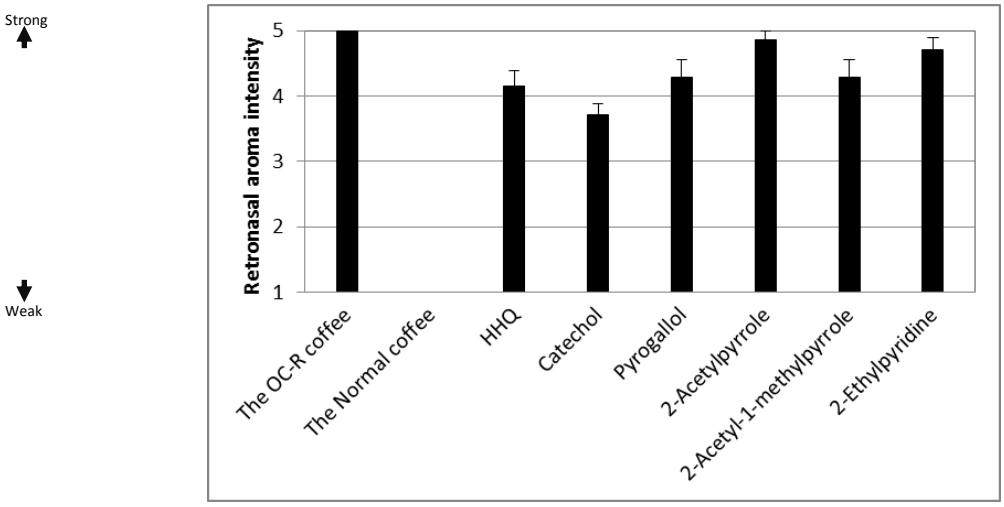

Figure 8. Sensory evaluation results of the influence of the components reduced by the OC-R process on the retronasal aroma.

DISCUSSION

The smooth aftertaste and rich retronasal aroma of the OC-R coffee were confirmed by sensory evaluation by a consumer panel comprising subjects who drink canned coffee on a daily basis. The results suggest that the OC-R process enhances these features, based on a comparison of the tastes and flavors between the OC-R coffee and Normal coffee.

The effects of the components reduced by the OC-R process on the aftertaste and retronasal aroma were evaluated. The aftertaste of the OC-R coffee was more intense following the addition of any of four identified components. These four components were HHQ, catechol, pyrogallol, and 2-acetyl-1-methylpyrrole. Conversely, when any of these four components were added to the OC-R coffee, the retronasal aromas of the samples were weakened. Based on these results, these four components affect not only the aftertaste but also the retronasal aroma of coffee. Interestingly the results of the sensory evaluations revealed that components that affect the aftertaste of coffee also affect the retronasal aroma. These findings suggest that the aftertaste and retronasal aroma of coffee interact molecularly or sensorially.

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Trial Running of Solar Drying House on Arabica and Robusta Coffee Parchment and Cherry: Influencing on the Drying Time and Flavors

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SUMMARY

Drying method is determine to beans quality and flavor profile of coffee. Beside, an apropriate drying method may decrease to cost of production. Moisture content of coffee parchment, after washing and draining (*dripping*) is between 50 - 55 %. In the end of drying process, the moisture content of green coffee should be between 10 – 12,5 %. Sundrying is the best method for coffee at dry area with sufficient sun-shine. Sunshine radiation at coffee estate is ranging from 3.000 - 4.500 watt-hour/m^2. Utilization of sunshine radiation may be icreased by solar dryer technology adoption. Indonesian Coffee And Cocoa Research Institute (ICCRI) had construct a "Solar Drying House" for coffee. The Solar drying house is suitable for small coffee estate, about 200 hectares. The roof of Solar drying house were formed of fiber-glass, the half down part wall were made of armoured concrete, and the half upper part were glass-iron framed windows. Trial running of the Solar drying house had been done during 2013 coffee season. Materials for the trial were Robusta coffee chery, and coffee parchment of both Arabica and Robusta. When the windows of drying house were opened, maximum temperature of drying room was about 47 – 55 ^oC, and minimum relative humidity about 20 – 32%, those were reached at 11.00 to 14.00 pm. On the surface of dried coffee, average temperature were about 37 ^oC - 52 ^oC, and minimum relative humidity was 30 %. Observation of moisture content reduction was approached by weighing a certain volume of dried coffee. The method is easy, cheap and adequately consistence, especially for sundrying or solar drying, becouse of the coffee bean is rarely possible overheated or overdried. When the windows were opened, drying time of Arabica parchment were 9 days (30 kg/m2 thicness) – 17 days (60 kg/m2 thicness). The Arabica parchment had been dry when the weight were 4,39 – 4,49 kg/10 liters, where moisture content the Arabica coffee beans were 10,5 – 12,9 % approximately. Drying time of Robusta parchment were 7 days (30 kg/m2 thicness) – 13 days (60 kg/m2 thicness). The Robusta parchment had been dry when the weight were 4,44 – 4,69 kg/10 liters, where moisture content the Robusta coffee beans were 10,40 – 13,7 % approximately. Drying time of Robusta chery were 8 days (30 kg/m2 thicness) – 10 days (60 kg/m2 thicness). The Robusta chery had been dry when the weight were 4,49 – 4,79 kg/10 liters, where moisture content the Robusta coffee beans were 9,60 – 13,4 % approximately. Thickness of dried coffee had a significanly effect on drying time and flavors of the coffee, but then the turning frequency of dried coffee had a not-significantly effect on drying time and flavors of the coffee. SCAA cupping score of Arabica coffee reaching to 83,8, and Robusta coffee reaching to 79,96, when their parchments were dried at 45 kg/m2 thickness. If Robusta chery dried at 45 kg/m2 thickness, the cupping score of the coffee beans were reached to 79,71. There are some oromatic taints arising from Robusta chery, those are winy and fruity.

INTRODUCTION

The production of coffees with a high standard of quality depends on various factors, such as crop management and harvesting, processing, drying and storage procedures (Borem, et. Al., 2012). A coffee cherry has a relatively thick skin, which encloses the parchment (Weiss & Buchinger). The coffee chery was a product that brings some peculiarities, such as the high proportion of water, approximately 60% (wb), and lack of uniformity. Therefore, the drying process is a fundamental phase to avoid the attack of microorganisms and the fermentations which can hazard the quality of the coffee (Alves et al., 2012**).** Drying method is determine to beans quality and flavor profile of coffee (Borem, et. Al., 2012). Drying is, without doubt, the key point in the production of natural specialty coffees, because the presence of the shell and the mucilage with high sugar content, reduces the drying rate and increases the risk of undesirable fermentations occur, affecting their quality (Isquierdo et al, 2012). The drying rate is influenced mainly by the temperature of the drying air. However, if coffee bean temperature exceeds 40°C during drying, coffee quality is harmed (Isquierdo et al, 2012). An apropriate drying method may decrease to cost of coffee production.

The quality of coffee (cup) did not change over the range of drying temperature (40 -60 ^{o}C) (Weiss & Buchinger). Moisture content of coffee parchment, after washing and draining (*dripping*) is between 50 - 55 %. In the end of drying process, the moisture content of green coffee should be between 10 – 12,5 %.

Sun drying of coffee parcments/cheries is the most widespread method in a lot of coffee producing countries due solar irradiance being very high for the most of the year. Sundrying is the best method for coffee at dry area with sufficient sun-shine. Sunshine radiation at coffee estate is ranging from 3.000 - 4.500 watt-hour/m^2. On the other hand, sun drying has its inherent disadvantages owing to the unpredictable weather conditions, which often lead to quality deterioration (Hii and Ong, 2012). Solar drying is a posible raplacement for sun drying or for standard dehydration processes. However, this is only possible when care is taken during the design, construction and testing of the solar dryer for the coffee parchment/cherry. Utilization of sunshine radiation may be icreased by solar dryer technology adoption. There are some types of solar dryers, those are tent dryer, box dryer, seesaw dryer, cabinet solar dryer, active ventilated cabinet solar dryer, cabinet dryer with back-up heating. The other types of solar dryer is greenhouse dryers (Natural convection greenhouse dryer, greenhouse dryer with forced ventilation), tunnel dryer, and in-house dryer. Some types of solar dryer had been used for coffee. A large scale combined biomass and solar drying had been used for drying coffee parchment in Indonesia. A medium scale coffee drying, tunnel type, had been used in Kenya, that's designed by Thailand Development Research Institute. A Small scale solar cabinet dryer had been developed for a farmers cooperative in Zimbabwe" (Weiss & Buchinger).

In this trial, coffee parchments and cheries were dried at an in-house solar dryer type. Objetives of the trial are to obtain influencing of thickness and turning freqwency on the drying time and the flavors of Arabica and Robusta coffee parchment and chery.

MATERIALS AND METHODS

Indonesian Coffee And Cocoa Research Institute (ICCRI) had construct a "Solar Drying House" for coffee. The Solar drying house is suitable for small coffee estate, about 200 hectares. The roof of Solar drying house were formed of fiber-glass, the half down part wall were made of armoured concrete, and the half upper part were glass-iron framed windows (Figure 1.). Inside dimension of the sollar drying house was 24 m in length, 18 m in width,

and the height was 2 m of back side and 3 m of front side. Total drying area was 432 m^2 (24 m X 18m). Trial running of the Solar drying house had been done during 2013 coffee season. Materials for the trial were Robusta coffee chery, and coffee parchment of both Arabica and Robusta. The Arabica coffee cheries were admixture of some varieties from Andungsari Experimental Garden-Bondowoso, ICCRI. While, Robusta coffee cheries were acquried from Sumber Asin Experimental Garden-Malang. To get coffee parchment, the coffee cheries were processed by full-wash method. During drying period, the treatments were thickness of coffee parchments/cheries and turning frequency. The thickness treatments of dried coffee parchments/cheries were 5 cm (30 kg/m2), 7,5 cm (45 kg/m2), and 10 cm (60 kg/m2). The turning frequency treatments were 1 turning/hour, 1 turning/2 hours and 1 turning/3 hours. The experiments made use of Rondomized Complete Block Design by 3 replications.

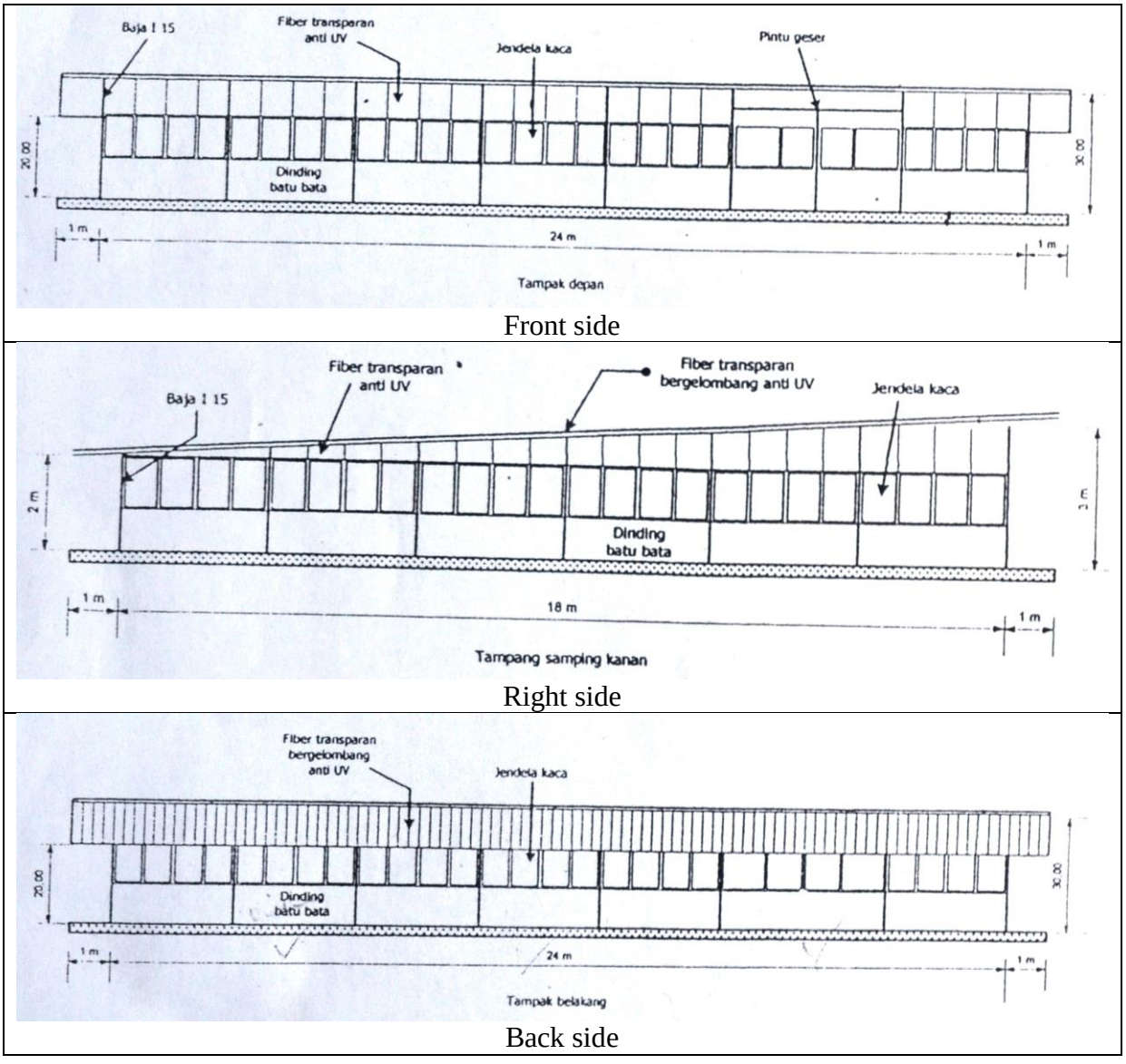

Figure 1. Sketch of coffee drying house.

Drying process were observed by measuring of the bulk density of dried coffee parchments/cheries. The coffee parchments/cheries had been dried if no reduction of the bulk density. After dehulling, the green coffee samples were roasted at medium level (Agtron Scale at 65#). From each sample, 5 (five) cups of coffee were prepared and tested. The coffee cup taste was evaluated by idetifying its fragrance, aroma, flavor, aftertaste, acidity,

body, uniformity, balance, clean cup, sweetness and overall/preference. This procedure was developed by Specialty Coffee Association of America [SCAA. 2009]. The cupping test involving 3 specialists at Sensory Laboratory of ICCRI.

RESULTS AND DISCUSSION

When the windows of drying house were opened, maximum temperature of drying room was about 47 – 55 ^{o}C, and minimum relative humidity about 20 – 32%, those were reached at 11.00 to 14.00 pm. On the surface of dried coffee, average temperature were about 37 ^{o}C - 52 ^{o}C, and minimum relative humidity was 30 % (Figure 1.). The drying rate is influenced mainly by the temperature of the drying air. However, if coffee bean temperature exceeds 40°C during drying, coffee quality is harmed (Isquierdo et al, 2012).

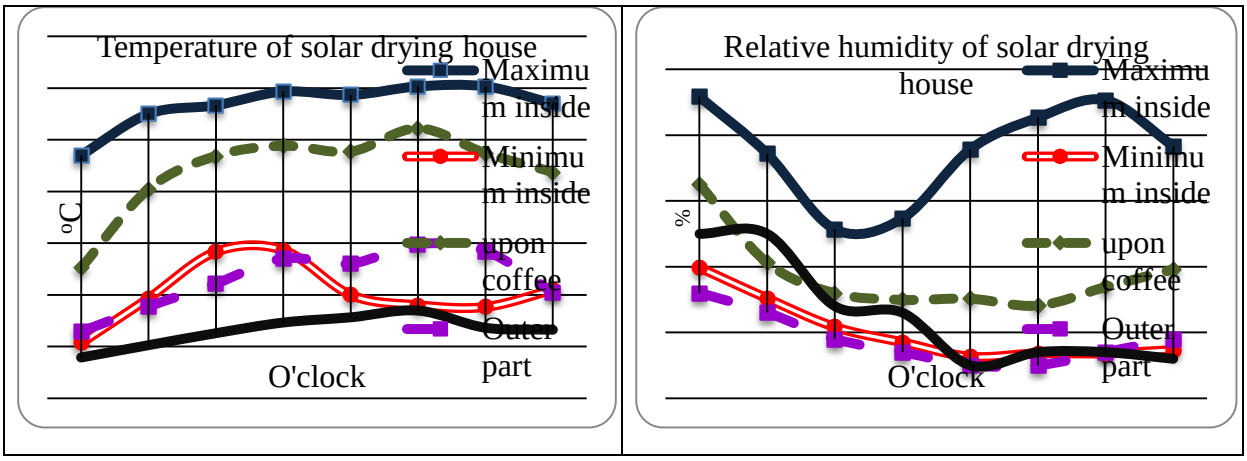

Figure 1. Temperature and Relative humidity profile of solar drying house.

Observation of moisture content reduction was approached by weighing a certain volume of dried coffee (bulk density measurement). The method is easy, cheap and adequately consistence, especially for sundrying or solar drying, becouse of the coffee bean is rarely possible overheated or overdried. When the windows were opened, drying time of Arabica parchment were 9 days (30 kg/m2 thicness) – 17 days (60 kg/m2 thicness). The Arabica parchment had been dry when the weight were 4,39 – 4,49 kg/10 liters, where moisture content the Arabica coffee beans were 10,5 – 12,9 % approximately. The drying house capacity was 108 – 115 tons of wet parchment/harversting season, where the Arabica harvesting season was 75 days. Drying time of Robusta parchment were 7 days (30 kg/m2 thicness) – 13 days (60 kg/m2 thicness). The Robusta parchment had been dry when the weight were 4,44 – 4,69 kg/10 liters, where moisture content the Robusta coffee beans were 10,40 – 13,7 % approximately. For Robusta parchment, the drying house capacity was 110 – 120 tons of wet parchment/harversting season, where the Robusta harvesting season was 60 days. Drying time of Robusta chery were 8 days (30 kg/m2 thicness) – 10 days (60 kg/m2 thicness). The Robusta chery had been dry when the weight were 4,49 – 4,79 kg/10 liters, where moisture content the Robusta coffee beans were 9,60 – 13,4 % approximately. For Robusta chery, the drying house capacity was 97-155 tons of chery/harversting season.

Thickness of dried coffee had a significanly effect on drying time and flavors of the coffee, but the turning frequency of dried coffee had a not-significantly effect on drying time and flavors of the coffee. SCAA cupping score of Arabica coffee reaching to 83.8, when their parchments were dried at 7,5 cm thicness, about 45 kg of wet parchment/m^2. General characters of the Arabica coffee were spicy, floral, chocolaty, and bright acidity. Harsh taste was detected at Arabica coffee when it was dried at 5 cm thicness, but herbal and green taste

were detected at Arabica coffee when at was dried at 7,5 cm and 10 thicness. Total score of the Arabica coffee beans were included in spesialty standard (SCAA, 2009).

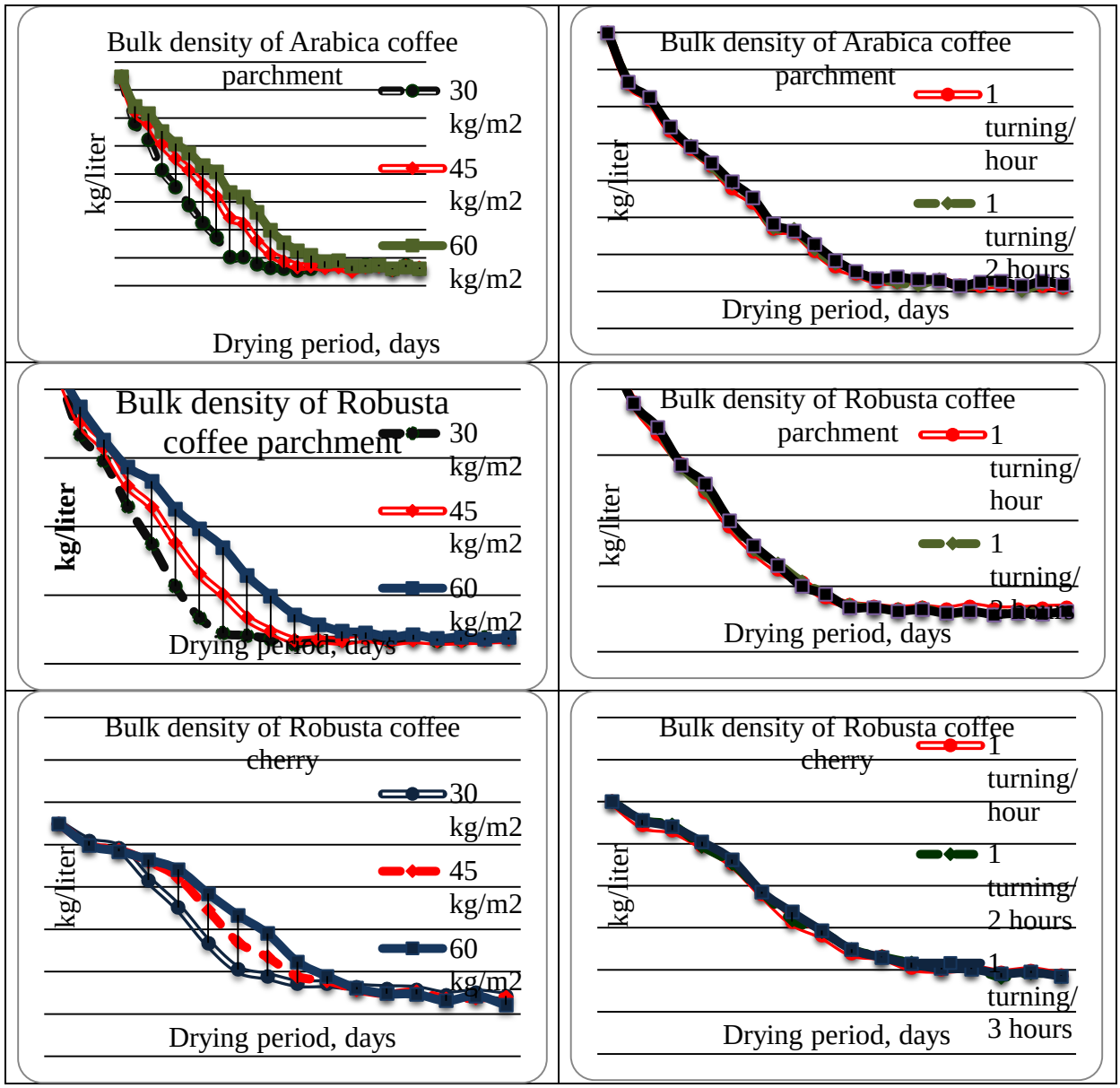

Figure 2. Drying rate of Arabica coffee parchment, Robusta coffee parchment, and Robusta coffee cherry in drying house.

Table 1. Flavor profile of Arabica coffee dried in drying house.

Flavor Atributes	Treatments of Coffee parchment Thicknesses					
	5 cm (30 kg/m^2)		7,5 cm (45 kg/m^2)		10 cm (60 kg/m^2)	
	Average score	Standard Deviation	Average score	Standard Deviation	Average score	Standard Deviation
Fragrance /Aroma	7,38	0,40	7,61	0,35	7,57	0,48
Flavor	7,38	0,32	7,67	0,26	7,49	0,36
Aftertaste	7,46	0,29	7,69	0,28	7,61	0,30
Acidity	7,43	0,25	7,54	0,21	7,58	0,35
Body	7,46	0,25	7,68	0,28	7,74	0,63
Uniformity	10,00	0,00	10,00	0,00	10,00	0,00
Balance	7,47	0,28	7,76	0,22	7,63	0,31
Clean Cup	10,00	0,00	10,00	0,00	10,00	0,00
Sweetness	10,00	0,00	10,00	0,00	10,00	0,00
Overall	7,53	0,26	7,76	0,25	7,63	0,27
Total Score	82,10	1,74	83,72	1,34	83,24	2,21

Note: Based on the Spesialty Coffee Association of America method. Quality scale: 6.00 – 6.75 = Good; 7.00 – 7.75 = Very Good; 8.00 – 8.75 = Excellent; 9.00 – 9.75 = Outstanding. Total Score = 80.00 or more belonged to specialty grade.

Table 2. Flavor profile score of Robusta coffee dried in drying house.

Flavor Atributes	Thicknesses Treatments Of Coffee Parchment			Thicknesses Treatments Of Coffee Cheries		
	5 cm (30 kg/m^2)	7,5 cm (45 kg/m^2)	10 cm (60 kg/m^2)	5 cm (30 kg/m^2)	7,5 cm (45 kg/m^2)	10 cm (60 kg/m^2)
Fragrance /Aroma	7.61	7.54	7.57	7.33	7.40	7.39
Flavor	7.64	7.56	7.69	7.32	7.50	7.40
Aftertaste	7.65	7.51	7.79	7.28	7.54	7.49
Salt/Acid	7.51	7.47	7.49	7.26	7.29	7.36
Bitter/Sweet	7.53	7.46	7.46	7.25	7.36	7.38
Mouthfeel	7.71	7.57	7.90	7.72	7.74	7.65
Uniformity	10.00	10.00	10.00	10.00	10.00	10.00
Balance	7.65	7.50	7.71	7.36	7.46	7.43
Clean Cups	10.00	10.00	10.00	10.00	10.00	10.00
Overall	7.65	7.57	7.79	7.32	7.50	7.50
Total Score	80.54	79.76	81.40	78.85	79.37	78.75

Note: Based on the Spesialty Coffee Association of America method. Quality scale: 6.00 – 6.75 = Good; 7.00 – 7.75 = Very Good; 8.00 – 8.75 = Excellent; 9.00 – 9.75 = Outstanding. Total Score = 80.00 or more belonged to fine grade.

Flavor of Robusta coffee were better when it was dried at parchment shape than chery. When the Robusta parchments were dried at 30 kg/m2 thickness, the SCAA cupping score reaching

to 80.54, suitable for fine Robusta standard (SCAA, 2009). But the Robusta chery dried at 45 kg/m2 thickness, the cupping score were reached to 79,71, still not suitable for fine Robusta standard (SCAA, 2009). When Robusta coffee dried in parchment shape, the general good characters were chocolaty, spicy, caramel, mild, and clean, but with harsh note. Astringent taste was detected at Robusta coffee when it was dried at 5 cm and 7,5 cm thicness. When Robusta coffee cheries were dried, the general good characters were chocolaty, buttery, mild, and sweet taste, also bitter, harsh, and astringent as generals taste of Robusta. But there are some oromatic taints arising from dried Robusta chery, those are winy, fruity, fermented, and stinkers. When coffee were dried In solar tunnel dryer, the visual and organoleptical tests showed no significant difference between the solar dried and sun dried coffee beans. This could be due to the rather similar drying rates between solar and sun drying. Also, when coffee driend in "solar dryer with black transpired air solar collector", the coffee beans dried faster in the solar dryer but still produced an acceptable cup with no serious defects. No OTA (Ochra Toxin A) forming fungi was found in solar dried samples (Hii and Ong, 2012).

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EcoMill®: an Eco-Friendly Technology for Wet Coffee Processing

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SUMMARY

This paper presents results obtained in the development of a technology suited for washing coffee which mucilage was degraded by natural fermentation or by pectinolytic enzymes. With the new technology, called EcoMill®, the specific water consumption is reduced up to 97.5% compared to that used in the traditional wet coffee processing, removing 97.1% of the mucilage contained at the time of starting to wash, with a maximum mechanical damage to the beans of 0.45%. The viscous wastewater can be added to the coffee pulp or dehydrated using solar dryers, getting a product that can be used as organic fertilizer, and control up to 100% of the water pollution due to wet coffee processing. EcoMill® has allowed to account for the first time with a fully environmentally friendly technology for wet coffee processing, including the great advantages, in terms of quality, of mucilage degradation by natural fermentation. The technology showed the same environmentally friendly performance in both controlled and in-farm conditions.

INTRODUCTION

The coffee fruit has two structures that are removed in the wet process, epicarp or pulp and mesocarp or mucilage. Mucilage is removed from the beans once it has been degraded through natural fermentation or with application of pectinolytic enzymes and by mechanical means. When using natural fermentation pulped coffee are let in tanks for 12 to 18 hours to allow the mucilage degradation by micro-organisms and bean enzymes. When commercial pectinolytic enzymes are used, coffee beans can be washed after 1-3 h from the application of the product depending upon the concentration. The volume of water used in coffee washing varies from 4.17 liters/kg of dried coffee (DC) to more than 20 liters/kg of DC, depending of the technology. The detaching of mucilage by mechanical means is produced by a rotor that generates shear stresses on the beans and collisions among them, with water consumption between 0.7 and 1.0 L/kg of DC. Wastewater of coffee washing may be mixed with the pulp, achieving a retention of 60% to 65% of the added volume, controlling 90% to 92% of the pollution generated in coffee washing.

This article presents results obtained with a technology designed for washing coffee which mucilage is degraded by natural fermentation, with very low specific water consumption and a control of up to 100% of the water pollution.

MATERIALS AND METHODS

This research was developed at the National Research Center of Coffee-Cenicafé, located in Chinchina (Caldas), at an altitude of 1,310 m, a mean temperature of 21.5°C, a mean relative humidity of 79.5% and a mean annual precipitation of 2,662 mm.

Equipment description.

EcoMill® technology consists of: a cylindrical tank made of stainless steel, to conduct the mucilage degradation through natural fermentation or by using pectinolytic enzymes, designed to allow the discharge of coffee beans by gravity; a screw conveyor to feed coffee beans with degraded mucilage to the washer; an up-flow vertical coffee washer with radial discharge of fluids. The water used to wash the coffee beans is supplied in sites located on the housing. The models with capacities of 500, 1500 and 3000 kg/h of washed coffee were designed. Figure 1 presents a model with capacity for 3000 kg /h of washed coffee and the technical specifications of the developed models are shown in Table 1.

Figure 1. EcoMill® 3000 model.

Table 1. Technical specifications of EcoMill® models

	Washer					Fermentation tank
	Total height	Rotor diameter	Speed of rotation	Power	Water flow	Volume*
Model	m	m	rpm	kW	L/min	m^3
EcoMill® 500	0.65	0.092	870	1.1	1.5	2.0
EcoMill® 1500	0.75	0.135	870	2.9	5.6	5.6
EcoMill® 3000	1.00	0.135	870	3.7	12.5	7.5
*two hours of operation per day						

METHODOLOGY

EcoMill® 500 and 3000 models were evaluated at Cenicafé using *Coffeea arabica* of the variety Castillo®, with hydraulic separation of coffee fruits before pulping and 16 h of fermentation. For each model were conducted 10 trials. In each trial was registered washed coffee flow (kg/h), initial and final mucilage content (%), mechanical damage to the beans (g) and water consumption (L). From this information was obtained capacity (kg /h of washed coffee) for each equipment, mucilage removal (%), mechanical damage (%) and specific water consumption (L/kg of DC).

EcoMill® 1500 model was evaluated in a farm, operated by personnel assigned by the owner, with separation of floating material before pulping and 13 h of fermentation process, by request of the farmer. The equipment was used in the main harvest of 2012. The following information was obtained in each processed batch: washed coffee flow (kg/h), water flow (L/min) and mechanical damage (%). Additionally, sensorial quality of the processed coffee was evaluated in some batches.

RESULTS AND DISCUSSION

Results obtained in the evaluation of the EcoMill® modules are presented in Table 2.

Table 2. Capacity, mucilage removal and mechanical damage obtained with EcoMill®

	Capacity		Water consumption		Mucilage removal		Mechanical damage	
Model	kg/h	D.E.	L/kg cps	D.E.	%	D.E.	%	D.E.
EcoMill® 500	465.0	34.8	0.34	0.03	97.1	0.91	0.25	0.17
EcoMill® 1500	1535.7	43.0	0.42	0.02	95.9	1.91	0.44	0.25
EcoMill® 3000	2706.7	86.9	0.51	0.02	96.5	0.91	0.45	0.34

Average capacity varied between 465.0 up to 2706.7 kg/h of washed coffee for model EcoMill® 500 and EcoMill® 3000, respectively. The capacity obtained with EcoMill® 1500 was similar to that observed in previous assessments.

The specific water consumption ranged between 0.34 and 0.51 L/kg of DC, notoriously less than that observed with technologies currently in use for washing of the coffee in Colombia, as shown in Fig. 2.

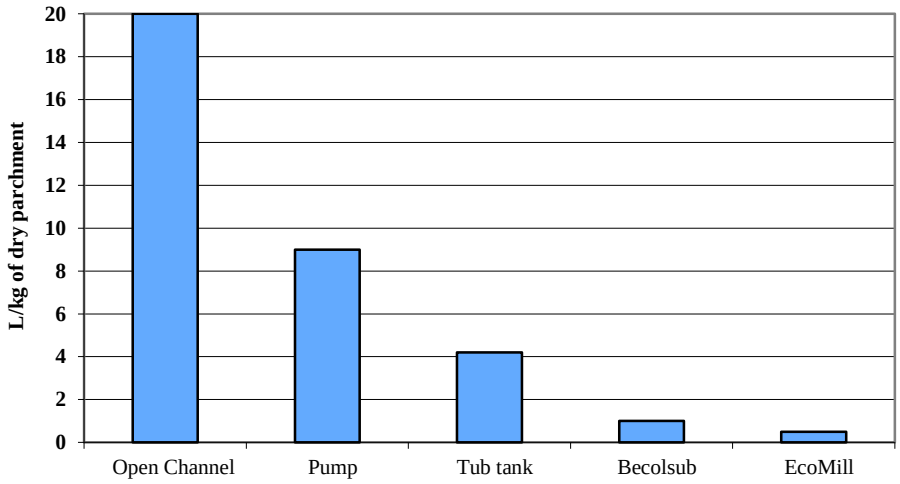

Figure 2. Specific water consumption in washing coffee beans in Colombia.

Due to the low specific water consumption obtained with EcoMill®, the viscous washing wastewater (WW) can be mixed up with pulp, reaching a retention of more than 90% of them (Fig. 3a). The resulting leachate are later added to pulp with lower moisture, obtaining in this way a control of 100% of the pollution generated in the washing process. Also the WW can be dried using solar dryers, getting a product that can be used as organic fertilizer (Fig. 3b).

a

b

Figure 3. Strategies used in the management of the washing wastewaters produced with EcoMill® technology: mixing them with the pulp (3a) and drying using solar dryers (3b).

Mucilage removal with EcoMill® varied between 95.9% and 97.1%. This removal, added to the additional release of mucilage which occurs during the fermentation process, which represents between 20% and 30% of the weight of the total of mucilage presented in pulped coffee beans, totals a mucilage removal of up to 97.9%, which is higher than the average observed in the traditional washing process.

The mechanical damage caused in the EcoMill® machines to the beans is low, similar to the one presented in machines used in coffee pulping [6].

Results obtained with the new technology EcoMill® indicate it is a breakthrough in comparison to those used today, both in process with natural fermentation and with mechanical removal, especially in specific water consumption (reduction of up to 97.5%) and in the control of the pollution produced in coffee washing (up to 100%), using simple wastewater management practices such as mixing them with the pulp or drying them using low-cost solar dryers.

The EcoMill® technology is suitable for coffee growers of all productions to benefit of the great advantages, in terms of quality, of mucilage degradation by natural fermentation, in a fully eco-friendly way.

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Coffee Sustainability in Kenya: Role Played by Improved Varieties

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SUMMARY

Coffee production in Kenya has been sinking since 1980s. The condition has been worsened by climate change phenomenon which has brought new production challenges in recent years. The biggest challenge is the changing dynamics of coffee pests and diseases, for example, Coffee Leaf Rust, which has become of a major concern globally. Variety improvement through breeding is believed to be one of the most sustainable ways of reducing production costs and mitigating climate change. Over the years, considerable success has been made in Arabica coffee breeding to improve yields, quality and to manage some biotic and abiotic stresses. Kenya produces mainly Arabica coffee from five commercial cultivars. These include three traditional varieties namely SL28, SL34 and K7, all of which are also susceptible to major coffee diseases, and two improved varieties namely Ruiru 11 and Batian. Owing to the current production challenges and the rising demand of Kenyan coffee in the world market, improved Arabica coffee cultivars with better quality, higher yield potential, resistance to diseases and tolerant to drought are largely replacing traditional varieties on a large scale in Kenya. This paper highlights the foreseeable role of these improved varieties in reversing the tumbling production trend and ensuring coffee sustainability in Kenya.

INTRODUCTION

In Kenya, coffee was introduced as a cash crop in 1900's by European colonialists, and has remained one of the most important products of the country's agriculture. Over 90% of the total Kenya coffee acreage is under Arabica coffee, while the rest is occupied by Robusta coffee (Omondi *et al*., 2001; Gichimu *et al*., 2010). Production of *C. arabica* is seriously constrained by diseases (Gichuru *et al*., 2008; Gichimu *et al*., 2013). The major diseases are Coffee Berry Disease (CBD) caused by *Colletotrichum kahawae*, Coffee Leaf Rust (CLR) caused by *Hemileia vastatrix* and Bacterial Blight of Coffee (BBC) caused by *Pseudomonas syringae* pv. *garcae* (Gichimu *et al*., 2013). CBD mainly infects the green immature berries, a stage in which it can cause up to 80% crop loss if not controlled and conditions are favourable (Gichimu *et al*., 2014). On the other hand, CLR is a disease of foliage that causes premature leaf fall, yield loss and even death of the tree in severe cases (McDowel and Wolffenden, 2003). BBC causes dark, water-soaked necrotic lesions on leaves, tips and nodes of vegetative and cropping branches culminating in a die-back (Ithiru *et al*., 2013)

Control of the three diseases on susceptible coffee varieties is by intensive spray programmes that accounts for up to 30% of the total cost of production. This is a major constraint to economic coffee production especially to the small-holders who find the use of pesticides beyond their financial and technical capabilities (McDowel and Wolffenden, 2003). In view of the economics and to minimise the chemical input for disease management, the development and cultivation of tolerant cultivars is encouraged as the most effective and viable option. Due to these production challenges and the rising demand of Kenyan coffee in the world market, improved Arabica coffee cultivars with better quality, higher yield

potential, resistance to diseases and tolerant to drought are largely replacing traditional varieties on a large scale in Kenya. This paper highlights the foreseeable role of these improved varieties in reversing the tumbling production trend and ensuring coffee sustainability in Kenya.

KENYAN COFFEE PRODUCTION TREND

Kenya coffee production increased rapidly in ripples in the two decades after independence. Total production for both estates and cooperative sub-sectors rose from 43,778 tons in 1963–64 to 128,941 tons in 1983–84 but fell to below 60,000 tons after 2000. Since then, the downward trend has continued except for a brief spell in 2006/07. The lowest production of 32460 tons was realized in 2008/09 after which the trend reversed as production started rising.

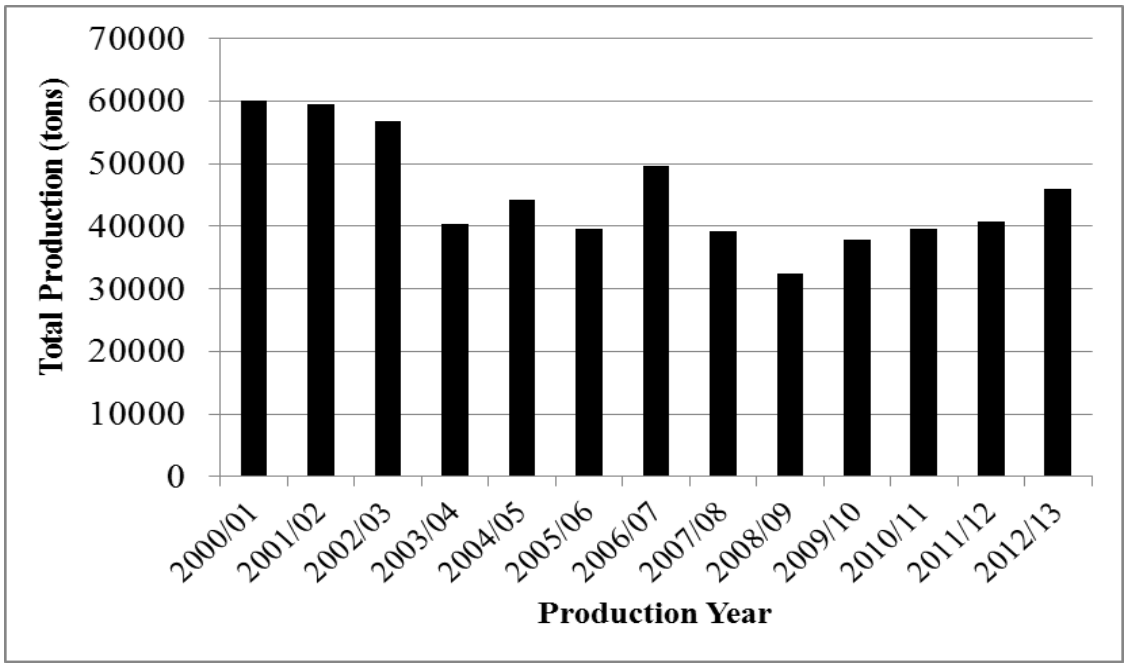

Figure 1. Kenyan Coffee Production Trend.

KENYAN COFFEE CULTIVARS

Coffee breeding in Kenya started in 1920s (Thorold, 1947). Emphasis in selection was primarily for high yields, better bean size and liquor quality (Walyaro, 1983). This saw the selection and subsequent release of the first Kenyan coffee varieties (SL28, SL34 and K7) in 1930s. These cultivars produce high yields of fine quality coffee but are susceptible to CBD, CLR and BBC although K7 has resistance to some races of CLR as well as partial resistance to CBD. A breeding programme for disease resistance started in 1971 after the outbreak of CBD and CLR in the late 1960s. The main breeding goal has been to develop cultivars that combine resistance to diseases with improved yields and quality (Van der Vossen, 1973; Walyaro, 1983). In 1985, the first disease resistant hybrid cultivar, Ruiru 11, that is also high yielding, of fine quality and compact growth was released (Omondi *et al*., 2001). Further research and development culminated to the release of other disease resistant cultivars namely Batian 1, Batian2 and Batian 3. Their unique features include tall stature, true breeding and resistance CBD and CLR. They are also high yielding with good bean and liquor quality (Gichimu *et al*., 2010).

EMERGING CHALLENGES IN COFFEE PRODUCTION IN KENYA

The increase of greenhouse gas emissions (carbon dioxide and methane) in the atmosphere is causing wide changes in atmospheric events, influencing climate change and variability with critical impacts on coffee production (Kimemia, 2010; Gichimu, 2012). These include, shifting of optimal growing zones, changes in rainfall (amount and distribution), changes in dynamics of crop diseases and pests, changes in crop yields and quality, loss of agricultural land due to either rising sea levels and/or desertification (Kimemia, 2010; Gichimu, 2012). For a long time, BBC was restricted to the west of the Great Rift Valley in Kenya (Kairu 1985). However, with the current shifts in weather pattern caused by climate change, the disease is becoming more widespread. For CBD, although there are no physiological races for *C. kahawae* that have been identified, there are recent cases of infection on varieties hitherto considered resistant thus showing some weakened resistance probably caused by changes in climate, increased variation in pathogen virulence and/or pathogenicity (Gichimu, 2012). For *Hemileia vastatrix* recent work by Gichuru *et al*. (2012) using Kenyan isolates found that there are six new races (III, XVII, XXIII, XXXVI, XLI and XLII) carrying three new virulence genes (v1, v7, v8) and possibly v9. This represents a serious threat to CLR resistant varieties including Hibrido de Timor as well as resistant commercial varieties in Kenya.

POTENTIAL OF IMPROVED VARIETIES

Since their release, improved varieties, Ruiru 11 and Batian, have been attracting appreciable demand from farmers indicating that they have a wide acceptance by farmers. This has been attributed to their desirable agronomic characteristics including resistance to CBD and CLR, their high yielding capacity and their good cup quality which makes them attract high demand in the world market. In addition, these varieties have a wide adaptability making them suitable for all coffee growing areas in Kenya. Owing to their tall statured morphology, Batian cultivars have deep and extensive root system which makes them relatively more tolerant to drought than the compact Ruiru 11 cultivar. Both Ruiru 11 and Batian are planted in a relatively closer spacing of 2m x 2m compared to the recommended spacing of 2.75m x 2.75m for traditional varieties. Coupled with their high yields per tree, their closer spacing contributes further to their higher productivity per unit area. These varieties are therefore playing a major role in reversing the tumbling production trend and ensuring coffee sustainability in Kenya.

CONCLUSION

All the Kenyan coffee varieties are potentially high yielding and of good quality if properly managed under suitable production conditions. However, over 90% of Kenyan coffee is of Arabica type and therefore susceptibility to diseases have been the major constraits of coffee production in the country. Other production challenges are associated with climate change and can be managed through climate change mitigation. Adoption of improved disease resistant varieties can therefore play a major role in ensuring coffee production sustainability in Kenya.

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Performance of Coffee Seedlings as Affected by Soil Moisture and Nitrogen Application

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SUMMARY

Nitrogen (N) and soil moisture are the most important factors controlling yield in Colombian coffee crops. Since long-term productivity is contingent on early growth, it is imperative to study these factors in seedlings in order to ensure maximum yield potential of trees.

A greenhouse experiment with four soil water and four N levels was used to determine how these two important variables affect quantifiable parameters representing seedling performance.

Shoot biomass, the most obvious indicator of performance, was increased by both higher soil moisture and higher N application, although the root:shoot ratio diminished with increasing N application. Like shoot biomass, leaf N content also increased with increasing N application, with relatively more leaf N recovered from fertilizer as soil moisture increased. Water use efficiency, in terms of shoot mass produced per unit of evapotranspired water, responded dramatically to N level but was not affected by soil moisture. The strong effect of N application on water use efficiency was affirmed by the higher 13C/12C ratios of plants grown under higher N levels, indicating greater water stress in these plants.

All of these responses were associated with changes in photosynthetic metabolism as a result of acclimation to the imposed conditions. We aim to generate new recommendations for maximizing growth of coffee seedlings, by increasing the use efficiency of critical resources while at the same time reducing economic and environmental impacts attributed to N fertilizers.

INTRODUCTION

- Coffee is an important traded commodity worldwide (DaMatta, 2004). In Colombia, coffee crops are grown by more than 560,000 families mostly smallholders.
- Early growth stages are critical since transplanting small seedlings reduces crop productivity (Salazar, 1996). It is imperative to study the nutritional requirements of seedlings to ensure maximum yield potential of reproductive coffee trees.
- Nitrogen (N) inputs in coffee are mostly derived from synthetic fertilizers (100-300 kg N ha^{-1} yr^{-1}) (Bornemiza, 1982). However, losses by volatilization from broadcast application of urea and NO_3^- lixiviation from excessive precipitation are causing severe environmental and economic impacts.
- Current recommendations suggest applying N under wet soil conditions, but as rain distribution patterns are changing, according to DaMatta and Ramalho (2006).areas where coffee is cultivated will be greatly affected.

- Given the low N use efficiency (NUE) and high costs of N fertilizers, it becomes necessary to seek practices to reduce N losses and increase NUE without compromising crop production.

OBJECTIVE

This study sought to investigate how N application and water availability affect quantifiable parameters representing coffee seedling performance. We aim to generate new recommendations for maximizing growth of coffee seedlings, by increasing the use efficiency of critical resources while at the same time reducing the economic and environmental impacts attributed to N fertilizers.

MATERIALS AND SITE DESCRIPTION

We conducted a greenhouse experiment at the University of California, Davis, using three-month-old seed-grown coffee seedlings in plastic pots containing soil representative of field conditions (Andisol) exhibiting the following chemical properties:

Soil	**OM**	**pH**		**Olsen P**	**NH_4**	**NO_3**	**K**	**Ca**	**Mg**	**CEC**
	%	water	KCl	---------- mg kg^{-1} --------			---------- $cmol_+$ kg^{-1} ----------			
Andisol	16	6.7	5.5	4	2.1	1.2	0.62	9.6	1.45	13.7

Treatments

- Four soil moisture levels (0.1, 0.5, 1 and 5 MPa).
- Four N doses (0, 0.1, 0.2 and 0.4 g $plant^{-1}$).
- Randomized block design: 4x4 factorial and 5 reps.

Measurements

- Dry mass of roots and shoots plus root to shoot ratio.
- Water use efficiency (WUE) in terms of grams of shoot biomass per liter of water evapotranspired.
- Leaf contents of N, ^{15}N and ^{13}C (as an integrative measure of water stress).
- N recovered from ^{15}N-labeled urea
- Stomatal conductance and transpiration in the third fully extended pair of leaves.

RESULTS AND DISCUSSION

- There was a significant effect of the interaction between soil moisture and N on shoot biomass ($p=0.0133$), WUE ($p=0.0087$) and ^{13}C content in the leaves ($p=0.0137$).
- Main effects of soil moisture and N were registered on leaf N content ($p=0.0014$, $p<0.0001$), conductance ($p<0.0001$, $p<0.0001$) and transpiration ($p<0.0001$, $p<0.0001$).
- Root to shoot ratio was only affected by N levels ($p<0.0001$) but no effects of water and N were registered for N recovered from labeled urea.
- Shoot biomass, the most obvious indicator of performance, was increased by both higher soil moisture and higher N rates, although the root to shoot ratio diminished with increasing N application (Fig. 1).

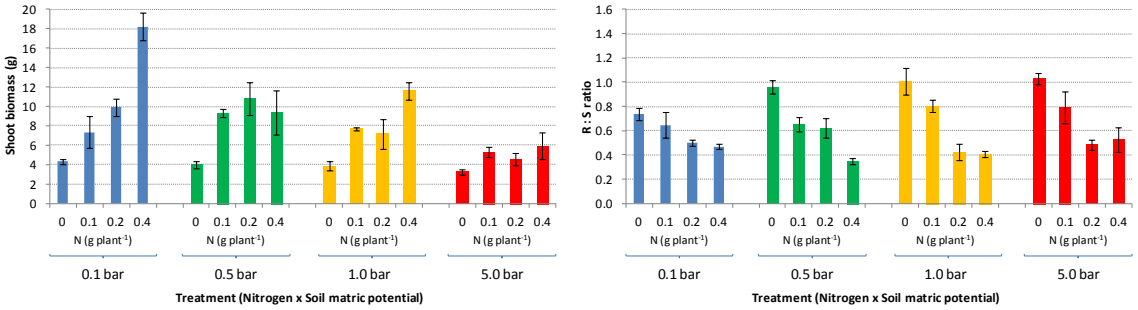

Figure 1. Effect of soil moisture and N doses on biomass allocation in coffee seedlings.

- WUE and ^{13}C discrimination increased dramatically by increasing N doses reaching the highest values at a soil moisture equivalent to 1.0 MPa with the maximum N rate (0.4 g.plant^{-1}) (Fig. 2). Plants grown under higher N levels exhibited greater water stress, as indicated by the higher ^{13}C values.

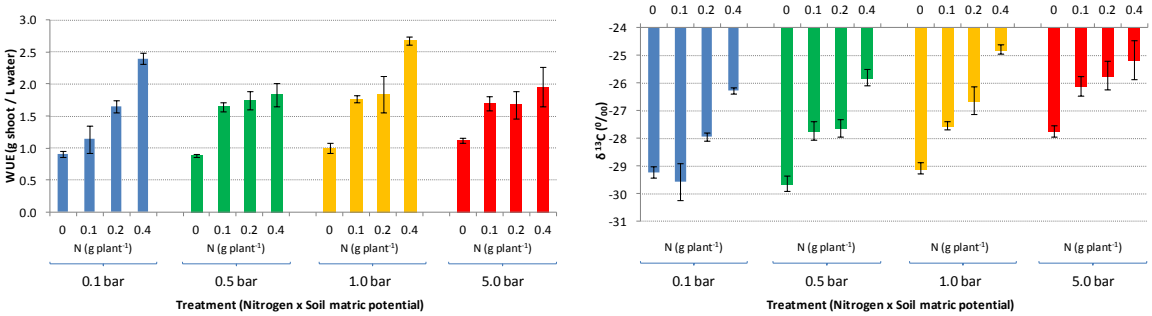

Figure 2. Effect of soil moisture and N doses on WUE in coffee seedlings.

- Leaf N content increased in wetter soil with increasing N application, whereas N recovered from fertilizer was not significantly affected by these two factors (Fig. 3). However, a previous experiment with higher N doses and older plants showed that NUE decreased with increasing N availability regardless of soil water content.

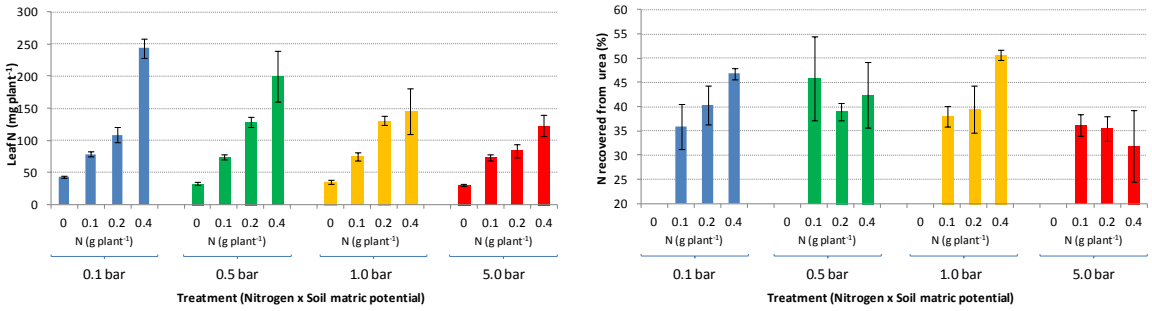

Figure 3. Effect of soil moisture and N doses on leaf N uptake in coffee seedlings.

- All of these responses were associated with changes in photosynthetic metabolism as a result of acclimation to the imposed conditions. Stomatal conductance and transpiration were higher with no N but decreased with increasing N doses; the lowest values were seen with the water stress induced by drier soil conditions (Fig. 4).

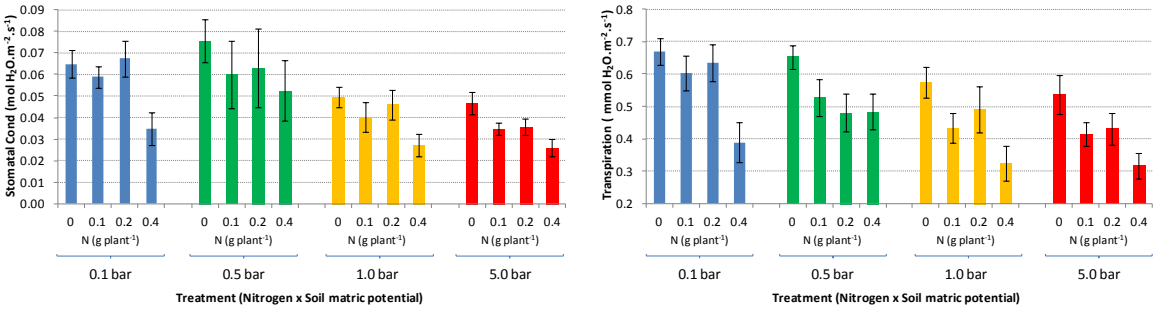

Figure 4. Effect of soil moisture and N doses on physiological traits in coffee seedlings.

CONCLUSION AND IMPLICATIONS

- The response of coffee seedlings to water and N levels was associated with changes in plant traits due to water stress. The water*N interaction did not affect physiological response in terms of conductance and transpiration. Both decreased as water decreased and N increased.
- Shoot growth decreased with decreasing soil water but increased when N increased. Root growth also decreased when N increased, as a strategic response to N availability.
- The increase in WUE and ^{13}C content as a result of increasing N was greater than the similar increase observed by decreasing soil water. Application of 0.4 g N plant^{-1} resulted in the highest values of WUE.
- By increasing N application, leaf N contents increased but NUE decreased. Both were less affected by changes in soil water content. Since NUE is only minimally affected by soil moisture, N applications should be done under less wet soil conditions to reduce environmental impacts.

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The Power Factor in Coffee Harvesting and Processing in Kenya

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SUMMARY

Various forms of energy are expended during the harvesting and post-harvest processing of coffee including transportation and conveyance. Since most of the operations along the coffee processing chain are done manually, challenges like drudgery, shortage of labor and inefficiencies among others exist. It is hence important to extend the mechanization of operations in this area at least to promote interest in coffee farming. Other sources of power include animal draft, water, petrol, diesel and or electricity. However, application of wind powered equipment is only common in other industrial warehouses. The extent to which each type of energy is expended depends on the factory processing capacity, layout, operational procedure and managerial aspects. Besides that, energy is provided to each operation at a cost which is subsequently catered for from the coffee proceeds. Therefore, to enhance the profitability of coffee production, there is need to optimize the usage of energy in every activity between harvesting and storage. For that purpose, process modifications for higher efficiency or even substitution of the expensive sources with any existing cheaper and renewable ones should be considered.

INTRODUCTION

In Kenya, coffee is harvesting by selective picking of ripe coffee cherry at fortnightly intervals. Immediately after harvesting coffee under the conventional processing system, it is transported to the primary processing factories. Coffee processing unit operations at the wet mills include sorting, pulping, pre-grading, fermentation, intermediate washing, final washing, grading, drying and storage stored prior to dispatch to the mills for secondary processing. During primary processing, there is conveyance of coffee from one stage to another. At the end or within the harvesting season, the dry coffee is bagged and loaded onto the transport means to the secondary processor. Coffee harvesting, conveyance, processing and storage requires energy which is apportioned from various sources including human labour (manpower), animal draft, electricity, diesel, petrol, sun, hydropower and gravity. The importance of considering such energy inputs into the harvesting and processing of coffee lies in their cost. That is so except for the sun provision which is available naturally in addition to the utility equipment that go with them. Demand for power during coffee harvesting, processing and inter-stage conveyance within the processing chain together with their respective sources of power is shown in Fig. 1. These are outlined in more details as follows.

HUMAN LABOUR

This is required for harvesting and sorting coffee cherry in the farm and transportation of cherry to the primary processing factory by carrying physically or using wheel burrows, bicycles and carts. At the primary coffee processing factory labour is a vital utility for cherry sorting if not already done at the farm, conveyance of cherry and loading it into the cherry hopper, cherry pulping by the small holder planters, washing of fermented parchment, final

washing and grading, conveyance of clean parchment to the skin drying table, sorting of the coffee defects during skin drying, conveyance of the parchment from the skin to the final drying tables, stirring of parchment from skin drying through to the fully dry state, covering and uncovering the coffee on the final drying tables daily, transfer of the dry parchment to the store in which parchment is ventilated by stirring in some cases followed by sorting, bagging, stacking and finally loading into transport means to the mill. Out of all the processing operations, sun drying is the most labour intensive and requires 56.70 man-hours per ton of cherry processed (Whitaker et al., 1983). However, a coffee drier can reduce labout demand to 26.38 (Whitaker *et al.*, 1983) or at least by a third (Ilsley, 1973). On the other hand, the sorted cherry rejects are just dried while being manually attended from time to time and finally transferred to the store in a fully dry state.

Operation	**Humans**	**Animals**	**F. fuels**	**Electricity**	**Gravity**	**Sun**	**Wind**
Harvesting	■						
Transportation	■	■	■				
Cherry sorting	■						
Cherry feeding					■		
Pulping	■		■	■			
Pre-grading	■		■	■			
Fermentation						■	
1st washing	■			■			
Final washing	■						
Grading	■		■	■			
Skin drying	■					■	
Final drying	■					■	
Storage	■			■			■
Conveyance	■			■	■		
Water supply	■		■	■	■		

Key: ■ *Applied energy in the respective operation*

Figure 1. Chart of power application in coffee harvesting and processing

ANIMAL DRAFT

Animal draft power is relied in some parts of the country for ploughing and transportation. However, it is only in two coffee growing regions that, this commodity is transported to the primary factory using either oxen or donkey towed carts. Besides, it is quite peculiar that these two sources of draft power are hardly found in the same region. Instead, a particular region operates either of them as if by tradition. Donkeys are for instance popular in for this purpose in a region in the rift valley while oxen are found around the Mount Kenya region. However, unlike oxen, donkeys are increasingly being deployed for this and other purposes countrywide.

FORCE OF GRAVITY

Application of this phenomenon starts at the coffee cherry hopper whose surfaces slope towards the pulper at an angle of repose of 45^{o}. Such a design assists feeding of cherry to the pulper with or without water. Besides that, some coffee factories are supplied with water by gravity. Flowing water under gravity advances the pulped coffee through the pre-grader at the specified speed to grade the coffee precisely. Similarly, the different grades of parchment are transferred to their respective fermentation tanks and final washing and grading channel after complete fermentation. In the channel, flowing water grades the parchment and transfers the

different grades separately to the discharge point and to the skin drying tables from here in some cases.

FOSSIL FUELS

Large amounts of coffee cherry are delivered to the factory either by petrol powered motorbikes, tricycles, family cars, and pickups or diesel powered pickups, tractors and lorries. These means of transport are sometimes used in transporting dry parchment and buni to the dry mills. However, motorbikes are rapidly becoming a very popular mode of transport over the others. Diesel engines are also used to power water supply pumps, pulpers and pre-graders though most of them have to a very large extent been replaced by electrical motors. There are also small petrol powered pulpers for the small scale farmer.

ELECTRICITY

Electricity is used in pumping water from the river, pulping, grading, water recirculation, pulp delivery to the disposal site, washing, transfer of parchment coffee from one tank to another and washing in some estates, conveyance to the skin drying tables or mechanical drier, skin and final drying and ventilation of the parchment (air conditioning in ventilated coffee stores mainly by air electrical fans) in storage and security lighting. Some farms also hull dry cherry (Buni) and some grades of parchment (estate curing) towards value addition prior to marketing their coffee.

SOLAR ENERGY

Fermentation of parchment requires warmth as provided by the ground in which the fermentation tanks are built or the ambient environment after absorbing heat from the sun. Drying of coffee on tables in the sun is almost the only option for the entire coffee industry. The intensity of solar energy is controlled by either covering the coffee or heaping it along the longitudinal center of the table accompanied by vigorous stirring.

WIND POWER

Wind power is already being used to ventilate non-coffee storage and ware houses and clean coffee stores in the coffee mills. Otherwise, coffee stores in primary coffee factories are ventilated by conventional natural draught using vents on the roof.

POTENTIAL ENERGY SOURCES.

Hydro powered turbines for pumping water or electricity generation if installed in some rivers with mini falls or canals abstracting water in the upstream though in existence albeit sparingly can also be technically viable. There are systems which can transfer dry parchment coffee from the tables to the store pneumatically using electrical fans but they are yet to be adopted in Kenya. The concept of extending usage of solar energy from pumping water to small scale coffee pulping is another alterative that need to be persued further. As for wind power, priority should be given to generation of electricity. Biogas from bio-digestion of the coffee effluent from pulping (Murthy *et al.*, 2004) and pressing of fresh coffee pulp (Mburu *et al*, 1995 and Wood *et al.*, 2000) is an attractive option for drying coffee. Coffee and parchment husks have been applied in other industrial aspects to an extent which signifies their importance as alternative energy sources. For instance, dry coffee cherry husks used to be carbonized into charcoal briquettes (Mburu and Mwaura 1996)). Parchment husks were also used in industrial boilers, domestic cookers, driers for coffee cherry and charcoal briquettes

(Mburu and Mwaura 1996) and rotary coffee driers (Mburu and Mason, 1993 and Karanja *et al.*, 1994).

DISCUSSION AND CONCLUSION

Although energy is a key input in coffee harvesting and processing, there is very limited information quantifying its application in these cases. However, since it costs when used as such, there is need to optimize its usage by evolution of more efficient operating systems and mechanisms like improved solar drying systems (Ilsley 1973 and Trim *et al.*, 1984). Minimization of some inputs like water by re-circulation and recycling (Wood *et al.*, 2000 and Mburu, 2010) can also save a lot of energy. It is also worth trying to broaden use of solar, wind and hydro power beyond their current applications country wide. Other enterprising alternatives also lie in adopting other renewable energy options like the wet and dry coffee processing wastes as sources of energy to complement those which are commonly used. Other attractive energy saving options includes efficient management resources, adherence to the available technical recommendation and operating at optimal capacity.

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Effects of Single and Combined Drought and Cold Stresses on the Triggering of the Antioxidative Defenses of Icatu Genotype

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SUMMARY

Abiotic stresses related to water availability and cold are major limiting factors in crops production. In most cases, stress responses in plants are investigated by subjecting plants to a single stress type, although this situation does not reflect the conditions at field-level cultivation, where several different limiting conditions may occur simultaneously. Reactive oxygen species (ROS) production has been proposed as a common consequence of various abiotic stresses and a positive correlation between the plants antioxidant activity to scavenge/control such highly reactive molecules and stress tolerance has already been demonstrated in several species, including *Coffea* spp. In this context, we aim at studying the acclimation mechanisms of *Coffea* spp. to single and combined stresses of drought and cold at the plant physiology and triggering of antioxidant levels.

INTRODUCTION

Abiotic stresses, particularly those related to water availability and low temperature, are major limiting factors in crops production. Under field conditions single or multiple stresses exposure can occur, with the latter being the most common situation for crops. Multiple stresses can alter plant metabolism in a novel and more complex manner that may be different from that caused by each of the different stresses applied individually, and may require a new type of response that would not have been induced by each of such individual stresses.

To cope with environmental limiting conditions, plants undergo a process of stress acclimation. This process may require changes in the flow of metabolites through different pathways, the suppression of pathways that may be involved in the production of ROS during stress, and the induction of various defense genes such as heat shock proteins (HSPs) and ROS scavenging enzymes. The complexity of signaling events associated with the sensing of stress and the activation of defense and acclimation pathways is believed to involve, among others, ROS, calcium, calcium-regulated proteins, mitogen-activated protein kinase cascades, and cross-talk between different transcription factors. Interestingly, different stress conditions such as drought and cold can result in the activation of similar stress response pathways. Thus, a high degree of overlap/complementary may exist between gene clusters activated by different stresses. This overlap may explain the phenomena of “cross tolerance” observed in some plants, in which a particular stress can induce resistance to a subsequent stressful condition different from the initial one.

The coffee crop is confined to the inter-tropical zone, from 20-25ºN in Hawaii down to 24ºS in Brazil, mainly due to ecological factors related to temperature and, within this zone, the strongest climatic limitations of coffee are low temperatures and drought. It should be remembered that in tropical areas both low and high temperatures and water deficit may occur concomitantly in field conditions. Integrated approaches dealing with responses from plant to gene could help to better understand the mechanisms involved in plant stress response. In this way, this work aims at providing new insights concerning the *Coffea* spp. cold and drought sensitive key points and the acclimation mechanisms to multiple stresses.

MATERIALS AND METHODS

Plant material and experimental design

Potted plants of Icatu (*C. canephora* x *C. arabica*), 1.5 year-old, were submitted to two irrigation regimes (with field capacity of *ca*. 70%, control, and 15%, severe drought) and, after the establishment of each regime for *ca*. 1 month, plants were exposed successively to: i) a gradual temperature decrease (0.5ºC/day) from 25/20ºC (day/night) to 13/8ºC, ii) a 3 daily chilling cycles (13/4ºC), where plants were subjected to 4ºC during the night and in the first 4 h of the next morning, followed by a rise to 13ºC applied throughout the rest of the diurnal period, iii) a 7 days rewarming period at 25/20ºC, and iv) a 7 day re-watering to 70% field capacity.

Antioxidants extraction and activity assays

α-tocopherol (vitamin E) determinations were made as described in [13]. Superoxide dismutase (SOD; EC 1.15.1.1), glutathione reductase (GR; EC 1.6.4.2) and ascorbate peroxidase (APX; EC 1.11.1.11) extractions and activity determinations were performed as in [14].

Gene expression studies

Total RNA was isolated using the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol and digested with DNAse I using on-column QIAGEN DNAse, as per manufacturer's instructions. Verification of the intactness of the extracted RNA, cDNA synthesis and quantitative real-time PCR reactions were done as described in [15]. Gene sequences were obtained via searches against the *Coffea* ESTs libraries on NCBI databases.

RESULTS AND DISCUSSION

The antioxidative system defenses include a complex network of mechanisms that, firstly try to avoid the production of highly reactive molecules, by thermal dissipating the excess of light energy, performed by photoprotective xanthophylls and the q_E mechanism that functions at the photosystems level. Yet, if such highly reactive molecules, namely those involving oxygen, are overproduced, a second line of defense is set in place, mostly relying in the ascorbate-glutathione cycle, which integrates several enzymes and non-enzyme molecules. Among the enzymes are included the SOD, APX and GR. The results clearly showed that the activity of all studied enzymes was affected both by drought and cold stresses and, in general, maximal activity values were found under the simultaneous imposition of both environmental stresses (Fig. 1).

Cold, more than drought, efficiently promoted the synthesis of α-tocopherol, contrary to what

was observed for the antioxidative enzymes, in which drought was the main triggering factor. In fact, the pre-exposure to drought increased the activity of enzymes (particularly of APX and SOD) that were further increased with the following cold imposed conditions.

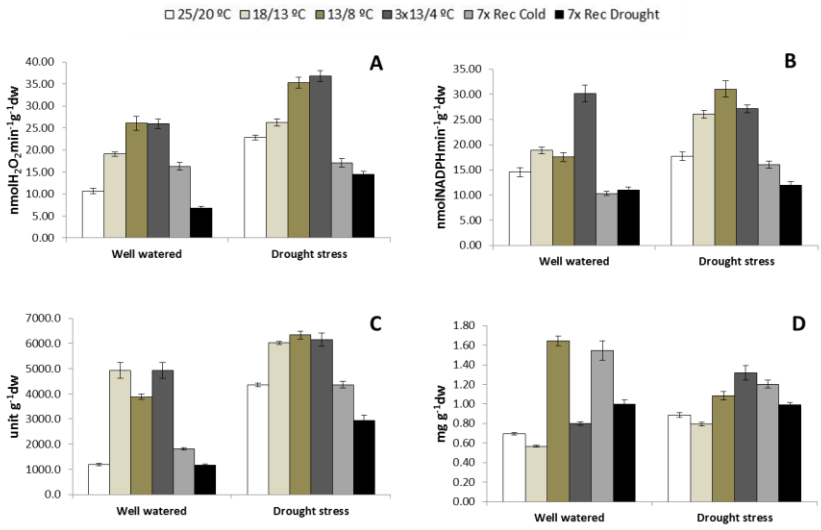

Figure 1. Changes of leaf activities of ascorbate peroxidase (APX) (A), glutathione reductase (GR) (B), superoxide dismutase (SOD) (C), as well as in α-tocopherol content (D) under water control and severe drought conditions, under temperature control conditions (25/20 ºC), by the end of the acclimation period (13/8 ºC), after 3 chilling cycles (3 x13/4 ºC), after 7 days under rewarming conditions (7x Rec 25/20 ºC) and after an extra week upon re-watering (7x Rec Drought). Each value represent the mean ± SE (n= 3).

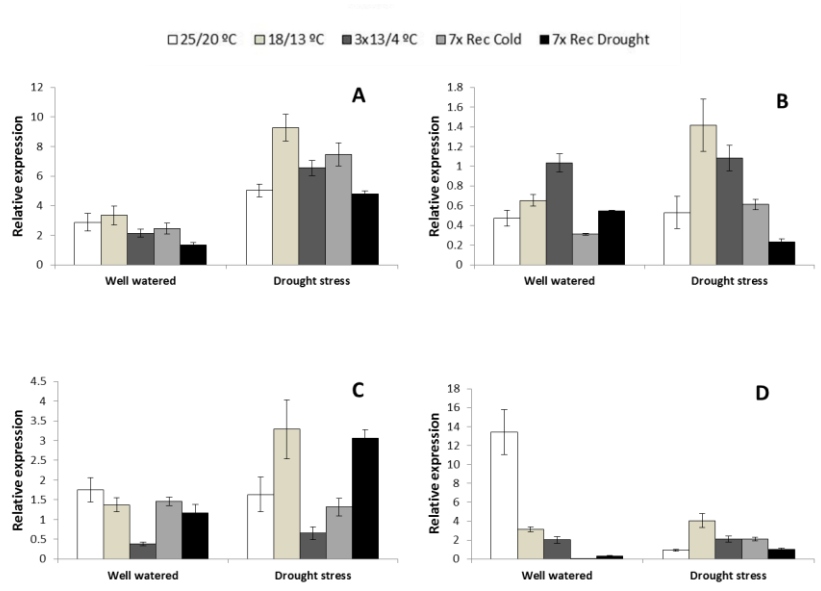

Figure 2. Relative gene expression values quantified by qRT-PCR for ascorbate peroxidase (APX) (A), glutathione reductase (GR) (B), superoxide dismutase (SOD) (C) and in tocopherol methyltransferase (D) under water control and severe drought conditions, under temperature control conditions (25/20 ºC), in the acclimation period (18/13 ºC), after 3 chilling cycles (3 x13/4 ºC), after 7 days under rewarming conditions (7x Rec 25/20 ºC) and after an extra week upon re-watering (7x Rec Drought). Each value represent the mean ± SE (n= 3).

Thus, although these enzyme and non-enzyme antioxidant molecules work in an integrate way in the ascorbate-glutathione cycle, drought seems to function as an enzyme inductor while cold induces the synthesis of both kind of molecules. That could be related to the need of a stronger antioxidative reinforcement under cold through non-enzyme components, as the enzymatic reactions are severely reduced upon low temperature. On the other hand, under drought conditions (at least under adequate temperature) a higher rate of enzyme reactions (that detoxifies the cell from ROS) would allow a faster regeneration of α-tocopherol, therefore compensating a lower pool of these molecules.

The observed increases in the maximum potential enzyme activities are in accordance with over-expression in the corresponding mRNAs, with the more pronounced effects found under multiple stress conditions (Fig. 2).

In conclusion, the presented analysis points to a clear impact of cold and drought, applied isolated, in the triggering/reinforcement of the antioxidant mechanisms related to the ascorbate-glutathione cycle. Furthermore, by pre-exposing the plants to drought conditions a stronger response was found to cold. In fact, drought have functioned as a "vaccine"/promoter by triggering tolerance mechanisms previously to cold conditions arrival, therefore, strengthening the acclimation capability of Icatu plants to the following cold exposure.

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A Decade of Contributing to a Profitable and Sustainable Coffee Industry in Tanzania: the Arabica and Robusta Improvement Programmes

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SUMMARY

Coffee berry disease (CBD) and coffee leaf rust (CLR) have threatened the sustainability of the Tanzanian Arabica coffee industry for more than half a century. Coffee wilt disease (CWD) has threatened the sustainability of the Robusta coffee industry since its first report in the country in 1997. The Tanzania coffee stakeholders unanimously agreed that the development and replanting with high yielding Arabica and Robusta varieties that combine durable resistance to these diseases with good beverage quality was the most important strategy for the economic management of these diseases and improve the sustainability of the industry. The Tanzania Coffee Research Institute (TaCRI) has, therefore, been implementing meticulous Arabica and Robusta breeding programmes with the above objectives since 2001 with strong support from the European Commission. Achievements are impressive. We have officially released 15 improved tall and four compact Arabica varieties and four Robusta varieties that meet the above objectives and several more are in the pipeline for official release. This paper gives highlights of the Tanzania Arabica and Robusta coffee improvement programmes, results and the future directions of the programmes to meet the expectations of our stakeholders.

INTRODUCTION

Comprehensive research work contributing to a profitable and sustainable coffee improvement programme in Tanzania has been documented (Teri *et al.,* 2011). Arabica coffee breeding have been implemented to develop, evaluate and propagate high yielding coffee berry disease (CBD) and coffee leaf rust (CLR) resistant varieties of *Coffea arabica* with good bean size and cup quality. Coffee Research Improvement Programme managed a 3-phased programme to develop Arabica hybrid varieties with similar properties of disease resistance, yield and quality. The first and second programmes were concentrated to develop tall Arabica hybrids, while the third focused on compact growth habit to address issues of increasing productivity. These efforts lead to official release of 15 improved tall and four (4) compact Arabica varieties. Coffee wilt disease (CWD) continue to be a threat to Robusta coffee industry, however the programme has been making good progress in identifying lines of *Coffea canephora* resistant to the disease. The selected four (4) lines were approved for official release in 2011. This is considered to be a major milestone of historic importance in Tanzania in official release of 19 Arabica coffee hybrid varieties and four Robusta clones which are disease resistant, high yielding and have bold beans and excellent beverage quality. The report highlights progress, results and future strategies to meet expectations of coffee stakeholders in Tanzania,

MATERIALS AND METHODS

Selection of Arabica elite breeding lines

Simple and complex crosses established in Field 23, 27 and Compact Variety Trial 3; were selected on basis of performance (yield, resistance to CBD and CLR, and beverage quality). The plants of the crosses originated from rooted cuttings (Fernie, 1962). Collection of *Colletotrichum kahawae* strains and screening resistance to CBD procedures have been extensively documented (Kilambo *et al.*, 1999). For CLR resistance determination, description by Eskes and Toma-bragini (1981) were followed. Breeding lines confirmed to have combined disease resistance, higher yield than N39 and KP423, and quality of higher than or equal to commercial cultivars were advanced to the multilocational and on-farm trials accordingly. Assessments of CBD and CLR, yield recording and samples for beverage have been described by Teri *et al.* (2004).

Selection for CWD resistance Robusta clones

Search for disease resistant clones was initiated in 2004, using 875 breeding lines established at TaCRI Maruku. Assessment included individual tree selection on yield, resistance to CWD and quality in terms of bean sizes and cup taste. Methods developed by Hakiza *et al.* (2004), were used in collection and preparations of *Gibberella xylarioides*, artificial inoculation of clones and assessment of the reaction. Robusta breeding lines that were found to combine genes for CWD resistance, of higher yield and excellent quality, were advanced and established in multilocational and on-farm trials of disease hot spot areas, for adaptation and adoption tests.

RESULTS AND DISCUSSION

The major characteristics of these varieties are being summarized in Table 1.

Table 1. Characteristics of 19 improved Arabica and 4 coffee wilt disease resistant Robusta varieties

Name of the variety	Yield (Kg/ha) Clean coffee	Cup taste	Bean sizes (AA + As)	Class	Description	Type
First generation Arabica tall hybrids officially released in September 2005						
N39-1	2,058	Clean cup	77	4++	Good acidity, body, flavour & aroma	Bourbon
N39-2	2,708	Clean cup	77	4++	Good acidity, body, flavour & aroma	Bourbon
N39-3	2,763	Clean cup	74	5+	Good acidity, body, flavour & aroma	Bourbon
N39-4	1,961	Clean cup	80	4+	Good acidity, body, flavour & aroma	Bourbon
N39-5	2,633	Clean cup	62	5+	Good acidity, body, flavour & aroma	Bourbon
N39-6	2,891	Clean cup	72	4+	Good acidity, body, flavour & aroma	Bourbon
N39-7	2,526	Clean cup	72	5+	Good acidity, body, flavour & aroma	Bourbon
KP423-1	2,225	Clean cup	80	4++	Good acidity, body, flavour & aroma	Kent
KP423-3	1,578	Clean cup	77	5+	Good acidity, body, flavour & aroma	Kent
First generation Arabica tall hybrids officially released in January 2011						
KP423-2	1,851	Clean cup	68	5+	Good acidity, body, flavour & aroma	Kent
Coffee Wilt Disease resistant Robusta varieties officially released in January 2011						
Maruku2	3,900	Clean cup	90	4	Nice aroma like mild Arabica	Robusta
Bukoba1	780	Clean cup	91	5	Natural Robusta coffee	Robusta
Maruku1	2,400	Neutral cup	98	5	Typical natural Robusta coffee	Robusta
Muleba1	2,400	Clean cup	94	6	Typical natural Robusta coffee	Robusta
Second generation Arabica tall hybrids officially released in January 2012						
N39-8	2,000	Clean cup	76	4+	Good acidity, body, flavour & aroma	Bourbon
N39-9	2,700	Clean cup	68	4+	Good acidity, body, flavour & aroma	Bourbon
N39-10	2,400	Clean cup	71	4	Good acidity, body, flavour & aroma	Bourbon
N39-11	2,700	Clean cup	68	4+	Good acidity, body, flavour & aroma	Bourbon
N39-12	2,400	Clean cup	79	4	Good acidity, body, flavour & aroma	Bourbon
Compact hybrids Arabica varieties officially released in December 2013						
TaCRI1F	6,000	Clean cup	69	4+	Sweet and pleasant aroma	Bourbon
TaCRI3F	5,050	Clean cup	64	4+	Sweet aroma	Bourbon
TaCRI4F	4,800	Clean cup	74	4+	Fruity aroma, dark chocolate, honey	Bourbon
TaCRI6F	6,000	Clean cup	68	5	Pleasant aroma	Bourbon
N39	1,000	Clean cup	57	4+	Pleasant aroma	Bourbon

Key: Bean sizes; >50% (AA + A) = excellent quality: Cup taste: Fine, 1=Good to Fine; 2=Good; 3=Fair to Good; 4=Fully Fair; 5=Fair Average Quality; 6=About Fair; 7=Poor to FAIR; 8=Poor.

Worldwide, there are around 40 known physiological races of *H. vastatrix* causative agent for coffee leaf rust (CLR); of which seven were recorded to exist in Tanzania (Rodrigues Jr. *et al.*, 1975). These were races I, II, III, XVII, XXIV, XI and XX. Coffee rust disease surveys carried out from 2006 to 2007 recorded new rust pathogen races XXII and XXXIV (CIFC, 2007). Two years later, five new coffee leaf rust pathogen races were recorded (TaCRI, 2009). These were races XXIII, XXIV, XXV, XXVIII and XXXI. Recently additional seven races: XLI, XLII, XV, XXX, XXXIII, XXXIV and XXXIX; were detected from all coffee growing regions in Tanzania (Kilambo *et al.*, 2013). Despite variability of 21 CLR races in Tanzania, officially released improved coffee varieties continue to maintain resistance. With regards to *C. kahawae* strains, previous studies indicated variability in pathogenicity of the pathogen in coffee growing areas (Kilambo *et al.*, 2008), but there are no reports of breaking of CBD resistance. Nature of resistance of Arabica hybrids have been confirmed (Kilambo *et al.*, 2013). TaCRI has been keen in implementing Arabica and Robusta breeding programme (van der Vossen, 2005). The institute has officially released 15 improved tall and four compact Arabica varieties that combine high yielding, durable resistance to CBD and CLR, and four CWD resistant Robusta clones combining high yields with good beverage quality. The challenge now is to make planting materials available to coffee growers.

CONCLUSION AND WAY FORWARD

TaCRI has selected top Arabica hybrid and Robusta varieties. No doubt they are amongst the best varieties in the world. To face the challenge of availability of planting materials to coffee growers, TaCRI prepared a roadmap for optimization of mass multiplication methods. They entails: documentation of protocols on horticultural methods (clonal multiplication and grafting), controlled pollination, tissue culture and male sterility to hasten availability of planting materials. In the meantime, additional coffee breeding lines are in pipeline for official release. Looking back twelve years ago, it was a decade of successful contribution to a profitable and sustainable coffee industry in Tanzania.

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Growth and productivity of Conilon coffee trees in a plantation with rubber

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SUMMARY

The study of the microclimate in two year seasons provided by trees in an agrosystem and of the behaviour of coffee trees under is important for the management and viability of coffee plantations. This study aimed to characterise the influence of rubber trees on the microclimate in a Conilon coffee plantation and the growth of the coffee trees. This study was performed in a *Coffea canephora* plantation planted in 2006 in association with *Hevea brasiliensis* trees established in 2007. The rubber trees were planted in two rows with a double spacing of 33 m x 4 m, with 2.3 m between the plants. This study consisted of five treatments according to the distance from the rubber trees: T1-3.0 m, T2-6.0 m, T3-9.0 m, T4-12 m, and T5-15 m. The along lengths of the internodes of plagiotropic and orthotropic branches, leaf area and chlorophyll. The proximity to the rubber trees, with the exposure to a somewhat lower irradiance exposure, implicated a greater elongation of the plagiotropic and orthotropic branches and an increase in leaf area. Yet, although the coffee plants closer to the rubber trees might be in more adequate environment, the yield, leaf concentrations of chlorophyll. Therefore *H. brasiliensis* use seems to have residual effects on productivity, although the plants would benefit from more adequate microclimate conditions.

INTRODUCTION

Coffee is a product of high worldwide importance being cultivated in over 80 countries, and its production is above 8 million tons since 2010, reaching ca. 8.6 million tons in 2012, mostly arising from South America and Asia countries, with 35% accountable to the *Coffea canephora* Pierre ex A. Froehner, or Robusta coffee type.

In turn, rubber trees (*Hevea brasiliensis* Müll. Arg.) are also important as the main source of natural rubber, which is used in the manufacturing of a large number of products, especially in the tire industry. Rubber trees are cultivated in many regions worldwide, with a production of over 11 million tons.

Coffee evolved in African forest understories and has therefore traditionally been considered a shade-requiring species. Even so, appropriate management of the plantation may allow their cultivation under full sun, frequently achieving higher yields than shaded coffee plantations. It could be assumed, therefore, that adult coffee trees should have sufficient phenotypic

plasticity to acclimate themselves to contrasting light environments. Under good hydric and mineral nutrition conditions, the sun is the decisive factor for the production of coffee seeds; still, it has been reported that some amount of shading may result in greater plant health and longevity, and moderate shading provided by a greater density of coffee trees or by their association with other species has been described as positive for the plantations.

The regions where coffee plantations are located are often subjected to summers and high temperatures, which may sometimes exceed 38ºC during the critical grain-filling phase. These conditions, combined with the occurrence of strong winds and high evapotranspiration rates, cause some environmental stress for the crop, requiring different techniques to mitigate these problems.

The study of the microclimate provided by trees in an agrosystem and its impact on the coffee trees is important for the management and viability of coffee plantations. Taking that into account and that this work is work in progress, we aim at study the influence of rubber trees intercropping on the microclimate in a Conilon coffee plantation and its nutrition, growth, and yield.

MATERIALS AND METHODS

Plant material and experimental design

This research was conducted between January 2012 and May 2013 on a rural property in the Jaguaré municipality, Espírito Santo, Brazil (18º56’ S; 39º58’ W, altitude 70 m). The climate is warm tropical, the location was plane, with an average annual temperature of 23.3ºC. Average annual precipitation is 1,200–1,300 mm, with rains predominantly in the months of October to January. The soil is classified as dystrophic red-yellow Oxisol with a loamy sand texture.

The experiment involved establishing a Conilon coffee (*Coffea canephora* Pierre ex A. Froehner cv. Conilon-Verdebras G35) plantation with a spacing of 3 x 1 m (3.333 plants ha^{-1}) associated with rubber trees (*Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg.) planted along two rows double-spaced (33 x 4 m), with 2.3 m between the plants (135 plants ha^{-1}). The coffee trees were planted at the end of 2006 and the rubber trees at the end of 2007. Both rubber tree rows were oriented at a direction of 65º northwest. By the time of the present experiment (2012), the rubber trees had a diameter at breast height (DBH) of 45 cm, a height of 10 m, and a canopy diameter of 7 m. The rows were 100 m long and had windbreaks composed of 35 year old rubber trees with a height of 17 m, a DBH of 105 cm, a canopy diameter of 14 m, and a spacing of 3 x 7 m at the front and rear ends.

Growth characterization and yield

The growth characteristics of the coffee trees in the plantations were assessed simultaneously with the microclimate measurements. For this, the number of nodes was quantified, and the lengths of orthotropic branches, of plagiotropic branches with fruits, and of young plagiotropic branches without fruits were measured. A total of 20 plants were assessed for each treatment (10 plants on the north side and 10 on the south side). The average internode length (etiolation) was obtained by dividing branch length by the number of nodes. For the branches with fruits (old branches), the distance between the nodes with coffee fruits and the branch type without fruits was also measured. The measurement of the young branches (without fruits) consisted of the entire branch, and the orthotropic branch was measured from the youngest branch without coffee fruits to the tip of the orthotropic branch.

The yield of two years (2012 and 2013) was evaluated in order to overcame the effect of yield variation between consecutive years. Coffee seed were collected manually in May 2012 and May 2013, when approximately 80% of the fruits were ripe. Three plants were sampled in each of the four plots, with a total of four replicates per treatment. The average coffee seed yield was quantified in liters per plant and extrapolated to kg per hectare. For this, the average ratio obtained for this plantation, of 5.33 liters of mature fruit per kg of processed seed coffee, was used.

Chlorophyll estimate

The sampling also included, for both seasons, an estimation of the amounts of chlorophyll *a* and *b* as well as the total chlorophyll concentration of the leaves located in the plant's mid-upper third, with 20 leaves per treatment. The third and fourth pairs of leaves were sampled with an electronic chlorophyll meter (Flaquer clorofila CFL 1030). Sixty leaves per treatment (30 on each side) were collected using the same sampling pattern for the analysis of nutrient concentration and the measurement of leaf area. Leaf area was estimated according to Partelli et al. [7].

Statistical analysis

The hypothesis of equality of the sampled variables between the treatments was tested using analysis of variance. Tukey's test was used for multiple comparisons between the means at a 95% confidence level. Descriptive statistics were also used, especially for microclimate data.

RESULTS AND DISCUSSION

Plant growth

All of the branch types during both seasons (Table 1) increased in average internode length in response to shading, as it was also observed by Ricci et al., also for coffee plants. This process is known as etiolation in plants subjected to severe shading but may also be observed in plants growing under tree canopies. Because of shading, solar radiation, after passing through the tree canopies, has an altered spectral distribution, and the radiation received by plants under the tree canopies has a greater amount of far red relative to red wavelengths. This promotes the transformation of far-red phytochrome (Pfr) into red phytochrome (Pr), reducing the ratio between (Pfr) and total phytochrome (Ptotal) (Pfr/Ptotal).

Simultaneously, the shading caused by the trees led to a strong increase in average leaf area for all treatments and during both seasons (Table 1). Leaf area increased gradually with decreasing distance from the tree rows, with the maximum difference observed between treatments T1 and T5, namely, 105% in summer and 216% in winter. These results corroborate the observations made by other authors in studies with coffee plants, who report decreasing leaf area in plants further from the shading trees in *C. arabica* and *C. canephora* cultivated in a shaded system compared to the same species under full sun, or when increases in the shading of *C. arabica* saplings grown under different radiation levels. Additionally, it was observed that shaded coffee has higher transpiration and photosynthesis rates, greater height growth, a smaller number of plagiotropic branches, and larger leaves. However, an interception of up to 50% of the incoming radiation may have no effects on the growth, maturation, production, or size of coffee beans.

Table 1. Lengths of the internodes of the plagiotropic and orthotropic branches, area per leaf, average amount of chlorophyll in the leaves, and average yield (2012 and 2013) of Conilon coffee cultivated in association with rubber trees in summer (S) and winter (W) of 2012.

	Growth of plagiotropic branches (cm)						**Leaf area (cm^2)**	
Tr.	**PBWF(S)**	**TPPB(S)**	**YPB(S)**	**OB(S)**	**YPB(W)**	**OB**	**S**	**W**
T1	5.1ab	3.6a	5.7a	4.1a	4.1a	2.5a	93.2a	109.1a
T2	5.2a	3.6a	5.1b	3.7a	4.1a	2.4a	84.6a	71.0b
T3	4.4ab	3.1ab	4.7bc	3.2b	3.8ab	2.3ab	72.1b	62.0c
T4	4.8ab	3.0b	4.5c	2.8b	3.5b	2.1b	56.5c	50.8d
T5	4.3b	2.9b	4.4c	2.9b	3.5b	2.0b	45.4d	34.6e
CV%	32.2	27.9	17.7	23.6	19.2	22.8	22.4	23.5

	Chlorophyll *a*		**Chlorophyll *b***		**Total Chlorophyll**		**Yield** Kg ha^{-1}
Tr.	**S**	**W**	**S**	**W**	**S**	**W**	
T1	41.1a	39.6a	18.3a	17.0c	59.1a	56.5c	71.3a
T2	42.6a	41.4a	22.9a	25.5ab	65.5a	66.9ab	75.2a
T3	43.0a	38.6a	20.7a	19.9bc	63.7a	66.9ab	73.6a
T4	42.6a	41.2a	18.1a	23.7abc	60.7a	58.5bc	68.3a
T5	42.0a	40.6a	19.8a	27.5a	61.7a	68.1a	71.8a
CV%	5.5	9.4	52.7	36.1	20.0	17.3	20.2

Means followed by the same letter in the same column are not significantly different from one another according to Tukey's test (P<0.05). T1: coffee row at 3 meters, T2: coffee row at 6 meters, T3: coffee row at 9 meters, T4: coffee row at 12 meters, and T5: coffee row at 15 meters from the rubber trees. Plagiotropic branches with fruits (PBWF), tip of the productive plagiotropic branches (TPPB), young plagiotropic branches (YPB), orthotropic branches (OB).

It must be highlighted that the productivity of the rubber trees may provide a supplementary source of economic income, reducing the implementation costs of the rubber trees as the cultural practices performed on the coffee plants were also used on the rubber trees and vice-versa, making this activity economical and sustainable.

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Phosphate solubilization by phosphate-solubilizing fungi isolated from Colombian Coffee Zone soils

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SUMMARY

Phosphorus (P) is necessary in coffee plants during the nursery stage and for vegetative growth. In order to supply this requirements, Colombian coffee growers use nearly 200 kg of $P_2O_5.ha^{-1}$, but this practice is non cost effective since that the industry of P fertilizers depends on international oil prices, and under some conditions, like the volcanic ash soils, high P fixation is a constant, causing low availability of P in the rhizosphere. These problems can be alleviated through several strategies such as the application of large quantities of P fertilizers, the arbuscular mycorrizal inoculation and the use of low-solubility sources like phosphoric rock (PR) alone or inoculated with native P solubilizing microorganisms. With the aim of contributing to the newly found options to improve the efficiency in the use of this nutrient in coffee crops, fungi with capability to P solubilization (FSP) were isolated from Chinchiná (*Pachic Fulvudand*) y Timbío (*Typic Melanudand*) soils, due to their high P fixation potential, from 100 to 52%. All of the isolated fungi showed solubilization capability, according to the fungi + PR *in vitro* test, with solubilization rates ranging from 15 to 107 $mg.L^{-1}$ P in solution respect to PR alone (4 $mg.L^{-1}$ P). No correlation between pH and P in the solution was observed, indicating that other variables must be involved in the solubilization processes, like secondary metabolites or specific enzymatic activity.

INTRODUCTION

Volcanic ash soils exhibit high capacity to P fixation onto their surface. This condition produces low availability of this nutrient in the soil solution, where the plant roots must uptake it. To overcome this problem, farmers apply high soluble P-fertilizers levels such as DAP, MAP, and others, although this practice may be too expensive. In this case, options as liming and the use of alternative P sources like PR, alone or with partial acidulation are recommended. Nevertheless their effectiveness depends on soil acidity conditions. Recently, there is increased interest in microorganisms capable of increasing PR agronomic effectiveness as crop fertilizer, which through mechanisms such as the release of organic acids and discharge of protons originated by NH_4^+ assimilation, may transform insoluble P forms to phosphates available to plants. Many soil microorganisms are recognized by their ability to solubilize inorganic P compounds. Accordingly, benefits are reported with *Brevibacillus*, *Aspergillus*, *Penicillium expansum, Mucor ramossimus, Candida krisii, Trichoderma viride* and *Aspergillus níger, Aspergillus aculeatus, Trichoderma sp, Aspergillus oryzae, Paecilomyces sp, Gongronella butlieri, Fusarium redondels, Pseudomonas fluorescens, Serratia, Pseudomonas, Bacillus* and *Enterobacter*, *Mortierrella* spp., etc. Whereas Colombia's coffee growing zone has a large diversity of soil types, volcanic ash soils near 40%, where P fixation may be from 8 to 99%. In addition, the whole consumption of P fertilizers is this country is imported, therefore search for sustainability strategies is receiving greater attention. For this reason the aim of our study was to look for new options in

order to improve the P fertilization efficiency under the Colombian coffee growing conditions, through FSP use.

MATERIALS AND METHODS

This study was carried out under laboratory conditions at the National Coffee Research Center (Cenicafé). Rhizospheric soil samples from coffee fields in production stage were collected from the A horizon (0-20cm of depth) in a zig-zag pattern using a hole digger tool. from both *Pachic Fulvudand* (Chinchiná - Caldas) and *Typic Melanudand* (Timbío - Cauca) soils. In each soil, samples were combined into a single 2kg sample.

FSP isolation

Ten grams of each soil in dry basis, were shaken at 120r.p.m. in 90mL of NaCl (0,85%) during 3h. Serial dilutions from 10^{-1} to 10^{-6} in saline solution + Tween 20, were aseptically transferred to PDA medium with Chloramphenicol (0,15%) and after 7 days of incubation at 28°C, individual colonies were isolated and purified in a specific medium to P solubilization, which further contains Chloramphenicol 1,5g.L^{-1}, bromocresol purple (0,05%) and FR 3,5g.L^{-1} as the only P source.

P solubilization test

After 10 days, when color change developed around the colonies, they were inoculated by triplicate in a liquid P solubilization medium (same as above but without Agar), then the culture medium was centrifuged at 4000 r.p.m. for 15 min and filtered; the supernatant was recollected and the P and pH in the medium were evaluated by using both the phosphomolybdate (UV-Vis 420nm) and the potentiometric methods, respectively.

The results were analyzed by descriptive statistics (average, maximum, minimum and confidence intervals at 0,05%), in order to stablish differences between strains with capability to P solubilization from PR.

RESULTS

The strains isolated from the two soils studied were capable of solubilizing P from PR. A total of 28 strains were obtained from Chinchiná and 19 from Timbío soils. The available P levels varied according to the soil type, indicating for the *Pachic Fulvudand* soil a minimum content of 15,5 mg.L^{-1} and a maximum level of 58 mg.L^{-1} (Figure 1.); in comparison with PR alone (control), where soluble P was less than 4,2 mg.L^{-1}.

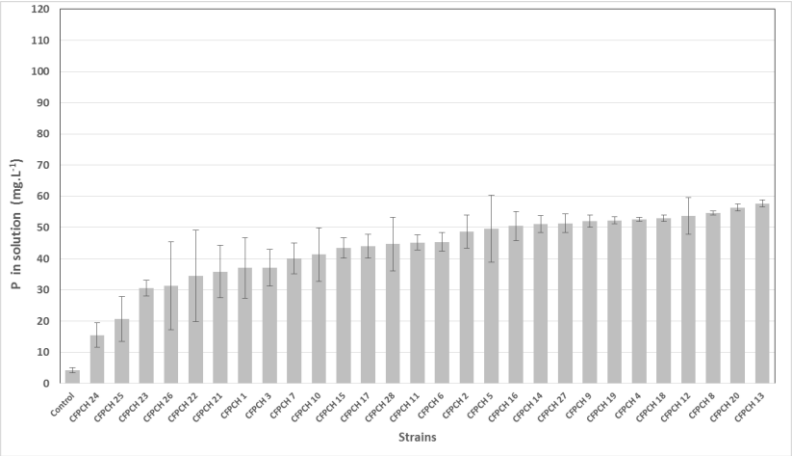

Figure 1. P solubilization from FSP strains isolated from Chinchiná soil.

The maximum P solubilization was detected with strains isolates under conditions of the *Typic melanudand* soil, where P levels in solution ranged from 52 and 107mg.L^{1} (Fig. 2.).

The differences mentioned seem to correspond to possible associations with the soil P fixation potential. In fact, despite considering the two soils in the Andisol order, in Chinchiná the P fixation was near of 99%, while in Timbío the magnitude of this phenomenon was 52%.

The P levels founded are in accordance with previously reported studies, where strains of *Penicillium*, *Mucor* and *Candida* isolated from phosphate mines, showed P in solution around of 105mg.kg^{-1}, and more effectiveness than *Mortierella* spp. reported by Osorio and Habte.

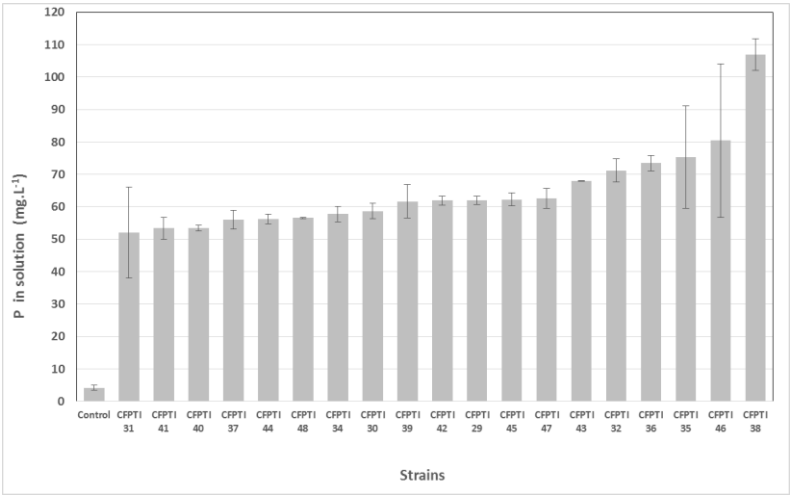

Figure 2. P solubilization from FSP strains isolated from Timbío soil.

Figure 3 shows that PR solubilization by isolated strains was not concomitant with medium acidification, indicating that other variables must be involved in the solubilization processes, like secondary metabolites and/or specific enzymatic activity. Yadav *et al*. optimized culture medium for P solubilization, when the temperature, pH, and carbon, nitrogen and potassium sources are specific.

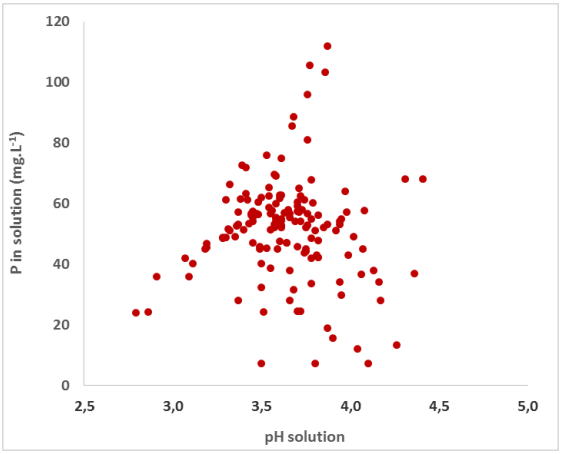

Figure 3. Relationship between pH and available P in liquid medium with PR, inoculated with FSP.

CONCLUSION

These results indicate that FSP isolates from the Colombian Coffee Zone, have a significant potential to be used in effective ways to solve the P availability limitations in coffee soils.

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Transforming Coffee Farmers in Rural Entrepreneurs through the Method of "Identification of Management Degree" (MIGG)

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SUMMARY

The aim of this study is to identify the major bottlenecks in the management of the agricultural coffee production, to subsidize the corrections that raise the level of quality management of those companies, increasing its competitiveness and enabling their survival or long-term growth. This method classifies firms into management levels from 1 (lowest) to 9 (highest level). The results presented refer to the survey from May 2013 to July 2014.

INTRODUCTION

The concern with respect to quality management has continually grows between companies from different economic sectors. The development of internal management systems has become essential to maintain or increase the competitiveness of businesses around the world, for a continuous increase in the satisfaction and trust of customers, reducing internal costs, increased productivity, improve the image and processes, and access to new markets.

For companies in the coffee sector the reality is similar: the establishment of internal management mechanisms is very important, since the improvement of agricultural processes until the product placement in the target market. The coffee producer or rural businessmen as in their work process dispenses much of his time and energy to technical issues and conducting routine tasks, relegating to the background the administrative aspects of the activity.

Despite the broad technical experience in cultivation, resulting from years of work, the management of the business is still, in most cases, primitive and intuitive. The use of information is done empirically, and grounded in feelings. Decision-making are not very rational, since they are not guided by methods that enable a systematic reproduction of processes. A challenge for the industry is the training of rural businessmen, to assimilate it and apply the concepts of competitiveness, quality and management, replacing the simple idea of making a profit.

The identification of the degree of management of coffee companies, in a simple and fast way, by applying the Model Identification of Management Degree - MIGG, contributes to the structuring of their business in an organized manner and to obtain superior finished products. Then, this study aimed to identify the major bottlenecks in the management of the agricultural coffee production, to subsidize the corrections that raise the level of quality management of those companies, increasing its competitiveness and enabling their survival or long-term growth.

MATERIALS AND METHODS

The MIGG (Method of Identification of Management Degree) [1-3] was developed with base on the criteria recommended by the National Quality Foundation (FNQ) and used in your system of evaluation of management companies, the Model of Excellence in Management® (MEG) [4-6], for flowers, greenery, fruit and coffee sector. MIGG - Coffee questionnaire incorporates a itinerary to assess the management of rural enterprises (coffee farms).

The questionnaire is evaluated by values and weights. The questions that comprise the evaluation questionnaire are straightforward, direct and admit only two answers: yes or no. Therefore subjectivity that often accompanies the descriptive or qualitative methods is minimized. The itinerary aims to continuously raising the standards of quality in all stages of the agribusiness system. This method classifies firms into management levels from 1 (lowest) to 9 (highest level).

RESULTS AND DISCUSSION

Tables 1 and 2 show the results of the questionnaires Migg, answered by rural entrepreneurs (coffee farmers), who were interviewed between May 2013 and July 2014 Table 1 shows the resul ts for the state of Bahia, and Table 2 the results for the states of Minas Gerais, São Paulo and Espírito Santo. In these tables we present the scores obtained by the respondents individually, for each of the evaluated criteria (strategies, leadership, customers, society, knowledge and information, people, processes and results), the total score and their classification according to degree of management. Follow the main comments on the results:

- West of Bahia state: Climate and technological homogeinedade the region and entrepreneurs, based on a previous concept of agribusiness results, resulted in a high average degree of management of the assessed organizations, reflecting the good results in all concepts. we should highlight in the evaluated organizations, rigor in the conduct of processes, consequently giving positive results for the sustainability of the activity.
- Southeast of Bahia Plateau, Bahia state: Despite the small number of organizations evaluated to date, the results indicate a trend of lower levels of management. The indices of planning, strategies and information are in general very low. The exception is a producer working on a vertical integration model (production, processing, roasting and grinding), aiming at export market niche - organic coffee.
- In the Plateau actual the average levels of management are a little higher. But still heterogeneous when the results are analyzed individually.
- The southwestern Bahia Plateau reflects low levels of management, with few exceptions, probably because of the predominance of family production, with less access to information technology and the low degree of organization of cooperatives and regional associations.
- Minas Gerais state: Despite the small sample, there is a trend of higher levels of management, probably resulting from an effort to produce quality coffee, and the history of regional cooperatives, disseminating information and technical assistance to the grower.
- São Paulo: Shows great variation in levels of management. The profile of respondents showed that smaller organizations or families had lower levels of management.
- Concerning the Espírito Santo, only one organization was analyzed yet, therefore it was not possible to assess the trend for coffee regions.

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Opportunities and Hazards of Nanotechnology Innovations in the Sectors of Production and Industrialization of Coffee in Brazil

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SUMMARY

The aim of this study is to identify opportunities for innovation in the nanotechnology area, and actual and potential hazards arising from them in social, environmental and economic dimensions, for the production and industrialization of coffee in Brazil. We use the Delphi technique to identify key opportunities for the application of nanotechnologies in different segments of the coffee industry, identify and rank the main impacts of its use and describe the most outstanding contributions to this branch of science can potentially arise. The results indicate that, in general, representatives of the coffee sector are not familiar with that subject and they have no opinion about the merits and risks arising from their use. Some companies reported the use of nanotechnology in some of their products, but actually it develops and markets products with very small particles, but not with nano dimensions. Other companies reported that their technical departments work in order to develop and commercialize products based on nanotechnology in the next five to ten years.

INTRODUCTION

Nanotechnology involves manipulating of matter at the atomic and molecular scales. It is often cited as the basis of a new scientific revolution. The global market for products and processes that include nanotechnology components will reach US$ 1 trillion over the next 10 years. In 2010 the Brazilian market for national nanotechnology based products accounted for R$115 million in business. On the other hand, there are also great concerns about the risks of these products - and its residues - on human health and the environment. While the results of nanotechnology stand out in many sectors of the world economy, its use in agriculture and the food industry is still modest, although there is immense potential.

This study aimed to identify opportunities for innovation in the nanotechnology area and the actual and potential risks arising from them in environmental, social and economic dimensions, in the production and processing of coffee chain in Brazil.

It is expected that the information obtained contribute in the diagnosis updated, of the development of nanotechnology in the coffee chain products in Brazil, and to evaluate the environmental, social and economic impacts arising from them. It is also expected that the results subsidize the production sector, enabling or facilitating the implementation or (re) formulation of public policies related to information and regulation of nanotechnologies.

MATERIALS AND METHODS

To assess the potential impacts of nanotechnology (ex-ante) we used the Delphi technique. This technique allows a qualitative approach, based on expert opinion obtained by applying a

structured questionnaire. It allows predictions in situations where there is no historical data on the performance parameter or are expected to have structural changes in the business environment, as expected in the case of nanotechnology. This technique consists of repeated rounds of questionnaires, among a set of experts, anonymous to each other, used to identify the main opportunities for application of nanotechnologies in different segments of the coffee industry, identify and rank the main impacts of its use and describe the most prominent contributions, which can potentially result from this branch of science.

From the Lattes system / CNPq, we identified informants related to agribusiness and industries associated with it, such as pharmacy, chemistry and the environment. Through exploratory interviews, we also identify key informants belonging to the agro-industrial chain of coffee. In the first phase of this study, we developed a questionnaire to assess the impact of nanotechnologies according to the Delphi Technique. The questionnaire was initiated by a pre-test, applied to the members of Brazil Nano Network, via e-mail. Next, we apply the questionnaires in sector scientific events, and then we visited the key informants identified, for application of the questionnaire *in loco*. In total, we distribute 75 questionnaires to evaluate the impacts.

RESULTS AND DISCUSSION

We received 58 completed questionnaires. Of this total, 38 (65.5%) have heard of nanotechnology and 20 (34.5%) know any possible agricultural use. From those 58 respondents, eight (13.8%) provided examples of use of nanotechnology different from those we used in the Delphi questionnaire.

The greatest difficulty of the study relates to companies and researchers who effectively work with nanotechnology products and better master this subject, most of them related to the food industry. These companies and individuals have refused to answer the questionnaire because they fear that the results of this study arouse the interest of competing companies or researchers, so your technicians are subject to agreements that require them to confidentiality about their activities. This fact may generate results with significant bias, especially on the use of coffee in the industrial processing sector.

Some companies, related to the inputs for agricultural production sector and the food industry, have reported that work with nanotechnology products, but visits to some industries revealed that these companies actually develop and market products with very small particles, some with dimensions "micro" but not with nano dimensions. Other companies know that its products are not at the nanoscale and claim that have worked in order to develop and commercialize products based on nanotechnology, the next five to ten years.

Table 1 shows the opinions of thirteen respondents who assessed the scale of impacts of potential risks of nanotechnologies in the coffee business. Table 2 presents the opinions of the nine respondents who assessed the scale of importance of the regulation of the production and generation of nanotechnology products and processes in the coffee business. Since we intend to expand the sample, the results are not definitive.

The representatives of the coffee industry are not familiar with the theme "nanotechnology". At some point they have heard or read any comments or article on this subject, but they do not know exactly what is a nanoproduto and confuse them with very individualized products, but larger than nano. Generally, they have a lot of difficulty to provide examples of nanoproducts. Moreover, they do not know the existing laws on the subject and they have no opinion about the merits and risks arising from the gradual introduction of nanotechnologies in the coffee

production chain. Therefore it is important that scientists and companies, public or private, which effectively utilize or research Nanotechnology-based products, implement actions for dissemination and explanations regarding the benefits and risks of these products within the sector itself, preparing it for discussions with the society.

Table 1. Opinions of the thirteen respondents who assessed the scale of impacts of real or potential risks of nanotechnologies in the coffee business.

Real or potential risks of nanotechnologies in the coffee business		Scale of impacts - frequency of responses					
		Very high	High	Medium	Low	Very low	No opinion about it
1	Toxicity in plants, animals and humans (inhalation, ingestion, skin penetration).		3	1	4	4	4
2	Induction of mutagenic processes or changes in the duplication of DNA in humans and animals (inhalation, ingestion, skin penetration) process.		2	2	5	2	3
3	Risks to the safety and health of workers in the handling of products (mixtures of defensive grout, nanoencapsulation).		3	4	3	3	1
4	Risks of contamination of the environment.		2	4	5	3	
5	Tools and laboratory methods available for assessment of intrinsic risks of nanoparticles are enough to measures them.	1	5	2		1	5
6	Transparency in research and development of nanotechnology innovations (military weaponry, lettering, reliable public disclosure about its safety).	1	3		2	2	6
7	Rrisk of nanotechnologies promote disintegration of relevant industries in the productive structure of the country (bottom-up trajectory, decreasing wealth, unemployment, increasing external dependence).		2	3	3	3	3
8	The knowledge of nanoscience techniques (public or private) can extend the socioeconomic inequality between nations.	1	2	2	3	5	1
9	Other examples: were not cited other examples of potential risks.						

Source: Results of the study.

Table 2 Opinions of the nine respondents on the importance of the regulation of production and generation of nanotechnology products and processes in the coffee business.

Regulation of production and generation of nanotechnology products and processes in the coffee business.		Scale of impacts - frequency of responses				
		Fully agree	Partially agree	Somewhat disagree	Strongly disagree	Unaware legislation
1	The current legislation is sufficient to control or monitor initiatives in nanoscience.	1	2	2	3	6
2	The legislation relating to intellectual property is suitable to the requirements of the development of nanotechnologies.	2	4	4	2	2
3	International standardization proposed by ISO / TC 229 Technical Committee - Nanotechnology is necessary and sufficient to	2	4	1		7
4	A framework for regulation of nano innovations base is sufficient.		2	1	4	7
5	The precautionary principle should be understood as: adopting proactive actions to protect the health of individuals and ecosystems; and absence of evidence can not be taken as evidence of absence.	5	5	1	1	2
6	Financing structures and funding to public research in nano area should be managed by agencies with civil society representatives.	6	4	3		1
7	The accounting of liabilities of the processes of nano based products (ecotoxicity, negative energy balance, costs for collection and treatment of waste, damage to human health) is inadequate or partial.	2	5	3	1	3
8	Other examples: no other examples of regulatory possibilities were mentioned.					

Source: Results of the study.

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Separation in Clusters with Relation to the Good Agricultural Practices in Coffee Properties in Municipality of Franca-SP, Brazil

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SUMMARY

This study aimed at separating of groups of coffee producers according to their performance in relation to Good Agricultural Practices (GAPs) in the coffee production through cluster multivariate analysis. This technique allows the identification of similar groups inside a large heterogeneous group, which may facilitate the implementations of differentiated technical support and rural extension policies. As a conclusion, the producers in the Group 1 showed better performance in relation to GAPs when compared with Group 2. The applied methodology was able to categorize groups of coffee properties in accordance to their performance in relation to BPAs.

INTRODUCTION

The global Market for coffee producers in a sustainable manner has grown in recent years what makes necessary an implementation of public policies and other mechanisms that facilitate the access of new coffee producers to the market of differentiated coffee. With this new market reality, it was able to verify a growth in the demand for "sustainable coffees", which are produced following the rules and conduct codes done by independent entities that seeks sustainable activities in their production and emphasize environmental, social and economic extensions (GIOVANNUCCI; PONTE, 2005). The production of agro-food considering Good Agricultural Practices (GAPs) principles became essential to attend the demand of this market, which consists of following the sustainability principle and food safety in food production. The Good Agricultural Practices, according to FAO (2007) proposed by Izquierdo et al., (2007) is "Do things well and give warranty of them". This study aimend at separating of groups of coffee producers according to their performance in relation to Good Agricultural Practices (GAPs) in the coffee production through cluster multivariate analysis. This technique allows the identification of similar groups inside a large heterogeneous group, which may facilitate the implementations of differentiated technical support and rural extension policies.

METHODOLOGY

This study was performed in 2008 with 42 coffee producers of the municipality of Franca, São Paulo, Brazil, through a survey type structured questionnaire. According to Oppenheim (1992), Survey is conducted as a representative sample of a population that is applied in general way a structured questionnaire, where the obtained data are studied using statistic techniques to measure relations among variables. The questionnaire was elaborated by Agronomists and Technicians with wide experience in Coffee Culture in COCAPEC – Cooperative of Coffee Planters and Agro-Cattle Breeders, settled in Franca, SP, based on the

main norms, conduct codes of certification programs and existing laws in the country that deal with agricultural issue. The questionnaire application was performed by the COCAPEC technicians supported by SEBRAE-SP and was divided into two parts: the first with 35 questions regarding the description of the producer and his property. In the second part, with 158 questions, it was used a three point scale that could have the answers: yes, partially and no.

The statistical analyses were done by SPSS statistical software. Initially it was done Cluster multivariate analysis. According to Hair Junior et al. (1995) and Malhotra (2006), Cluster is a technique that does not have dependence among variables and, this way, classify the individuals in homogeneous groups or conglomerates called Clusters. It is known that groups created by analysis are similar among them (inside a minimum variance) and different of other Clusters (among Clusters the variance is maximum). After the separation by Cluster analysis, a discriminating analysis was made, which presented the identified variables by SPSS that caused the biggest divergence or differentiated both groups of producers. Malhotra (2006) defines that discriminating analysis or linear combinations divides the variables that better discriminate the categories of the dependent variable (groups). It is important to highlight that, in this study, there is no hypothesis to be confirmed but it is intended to gather elements with given similarity.

RESULTS AND DISCUSSION

The Cluster analysis divided the coffee producers in two groups according to their performance in relation to GAPs. The Group 1 consisting of 25 producers and the Group 2 by 17 producers. In Figure 1 is observed the division of 2 groups of rural producers done by Cluster multivariate analysis, dividing the producers according to their similar agricultural practices.

Through the discriminating analyses that were done after the separation of the groups, it was possible identify variables that are more different inside of two groups and that considered by Cluster analysis to divide the groups. It was identified 12 variables that more discriminated the two groups of producers.

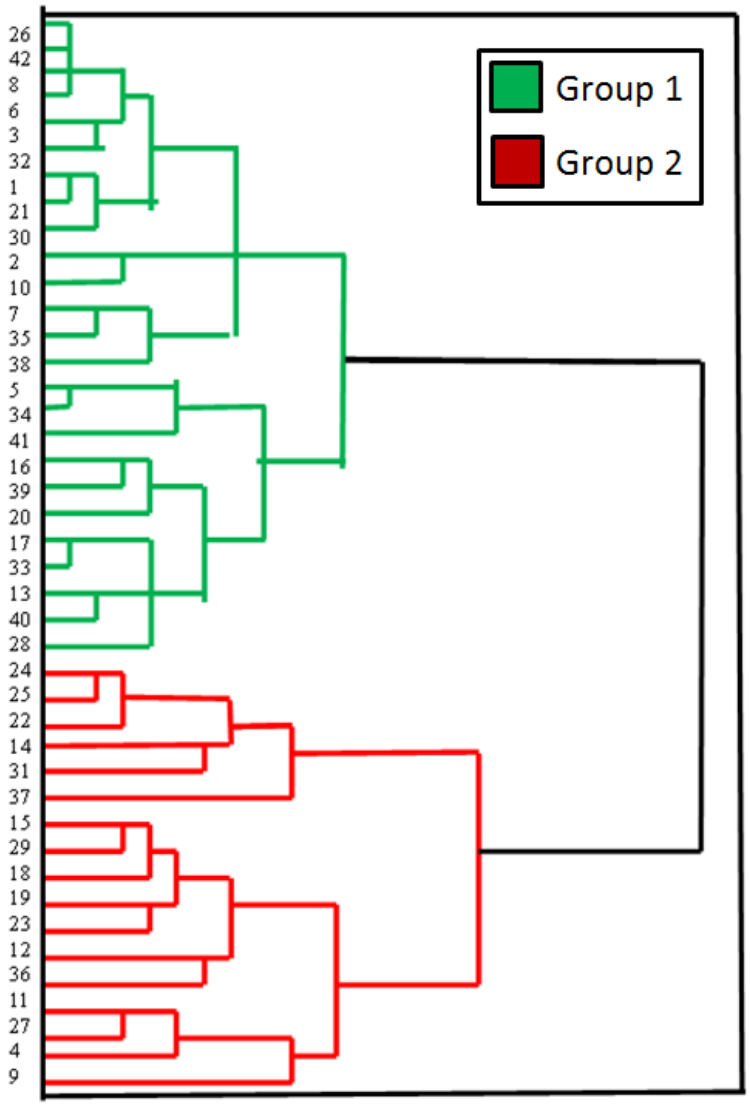

Figure 1. Cluster Dendrogram with the division of the groups.

Table 1. Variable that differed both groups of producers in accordance to their performance of Good Agricultural Practices.

DISCRIMINATING ANALYSIS		No	Partial	Yes	N.A.
1. Do you have appropriate control and registration of all costs of activities?	*Group1*		32%	68%	
	Group2	16%	64.7%	17.6 %	
2. Do you require the appropriate maintenance of IPE after all the applications?	*Group1*		8.0%	92%	
	Group2	23.5%	23.5%	52.9%	
3. Do you have knowledge about the options in the market?	*Group1*	8.0%	8.0%	84.1%	
	Group2	52.9%	11.8%	35.6%	
4. Do you record and file the course certificates of employees?	*Group1*		8.0%	56%	36%
	Group2	17.3%		11.8%	7%
5. Do you file the return receipts of empty packages?	*Group1*	4.0%	4.0%	92.0%	
	Group2	41.2%		52.9%	5.9%
6. Do you respect the windrow thickness in the yard of drying?(Max. 10 cm)	*Group1*			100%	
	Group2		5.9%	94.1%	
7. Do you look in the COCAPEG site to have more market information?	*Group1*	32.0%	4.0%	64.0%	
	Group2	70.6%		29.4%	
8. Do you wash the IPEs separated of other clothes?	*Group1*			100%	
	Group2	5.9%	11.8%	82.4%	
9. Do you demand medical checkup of your employees?	*Group1*	16.0%	4.0%	80.0%	
	Group2	64.7%	5.9%	29.4%	
10. Are the phytosanitary products stored properly?	*Group1*	64.0%		32.0%	4.0%
	Group2	82.4%		5.9%	11.8%
11. Are the employees trained to their job?	*Group1*		28%	72%	
	Group2		29.4%	70.6%	
12. Do you do foliar analysis every year?	*Grupo1*	12.0%		88.0%	
	Group2	11.8%	17.3%	70.6%	

The variables found by discriminating analysis show that Group 1 has better performance in relation to fulfillment of GAPs when compared to Group 2, in all 12 variables presented in Table 1. The applied methodology was able to categorize groups of coffee properties in accordance to their performance in relation to GAPs.

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Agricultural Profile and Practices of Coffee Producers in Municipality of Jeriquara – SP, Brazil

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SUMMARY

This study aimed at describing the profile of 67 coffee producers in municipality of Jeriquara, São Paulo – Brazil, linked to cooperative COCAPEC (Cooperative of Coffee Planters and Agro-Cattle Breeders, settled in Franca – SP) dividing them in groups according to their similarity to Good Agricultural Practices in the coffee production. According to Cluster analysis, the producers were divided in two groups considering the similarity of their agricultural practices. The Group 1 was formed by 28 producers and the Group 2 by 39 producers. After the division of the two Clusters, aiming show the variables with the biggest difference between the two groups, was done a statistical test Qui-Square of Pearson, considering the variables with differences of $p< 0.05$. The present study proposes a way of divide groups of coffee producers according to their performance in Good Agricultural Practices through multivariate analysis, instead of working the variables separately, and this way, propose differentiated technical assistance.

INTRODUCTION

Consumer concerns with the environment and food safety has led to the creation of Market niches, which seek to differentiate the quality of agricultural products considering social-environmental aspects in the production. In this context, it became indispensable to observe the Good Agricultural Practices in food production in order to meet the market demands for more sustainable products. According to Santos et al. (2007), the Good Agricultural Practices result in increasing of the efficiency of services and decreasing in social and environmental impacts. According to Pereira (2013), the Good Agricultural Practices (GAPs) are based in the food safety principles, environmental conservation and social conditions that respect the individuals involved in the production process, gathering under just one concept the agricultural and market requirements. The GAPs are a competitiveness component that allows the rural producer, in this case the coffee producer, differentiate his product of other, increasing its quality, broadening the access to new market, consolidating the presents and decreasing the costs. They understand the knowledge, planning, measurement, registration and management of social, environmental and specific productive objectives, using tools that show by adequate processes to evidence that they are doing the things in a correct way through agro-food chain. The objective of this study is to describe the profile of producers from the municipality of Jeriquara, São Paulo – Brazil, linked to the COCAPEC cooperative (Cooperative of Coffee Planters and Agro-Cattle Breeders, settled in Franca – SP) dividing them in groups according to their similarity to Good Agricultural Practices in the coffee production.

METHODOLOGY

The study was performed on the properties of the associates in Jariquara, in 2008, with 67 coffee producers, through a survey type structured questionnaire. The questionnaire was organized in a way to detect the reality of the practices adopted by the producers and was developed and applied by a group of Agronomists, collaborators and technicians from COCAPEC specialized in coffee culture and supported by SEBRAE SP. The questionnaire is based in the main norms, conduct codes of certification programs and laws that deal with agricultural issues, specially the coffee culture. The questionnaire comprises a survey of the GAPs through a three point scale that could have the answers: yes, partially and no, and also "does not apply". After the questionnaire application, the date were tabulated and analyzed by the SPSS software (*Statistical Package for the Social Science)*

It was done frequency analysis to view each category of date. According to Levin (1985), the distribution of frequencies helps the researcher to transform the gross date in a set of measurements, organized and endowed with sense. Next, it was done the Cluster multivariate analysis, which was the main methodology of this study. In accordance with Everitt (1993) and Manly (1986), the Cluster analysis is a technique that aims gather individuals (cases) that have similar characteristics in function of a set of selected variables. After the grouping of the variables by Cluster analysis, it was applied the nonparametric statistical test of Qui-Square of Pearson aiming identify the variables with statistical difference between two groups.

RESULTS AND DISCUSSION

The Cluster multivariate analysis divided the producers that participated in the survey in groups according to their similarity in the agricultural practices. The producers were divided in two groups. The Group 1 consisting of 28 producers (41.8%) and Group 2 by 39 producers (58.2%), totaling of 67 (100%) producers that made part in the survey.

In the Table is possible observe the group division according to Cluster analysis:

Table 1. Division of the Clusters

Cluster		Percentage	Frequency
	1	41.8%	28
	2	58.2%	39
	Total	100.0%	67

The study presented a way to separate groups of coffee producers according to their performance in Good Agricultural Practices through multivariate analysis, instead of working the variables separately, allowing propose differentiated technical assistance.

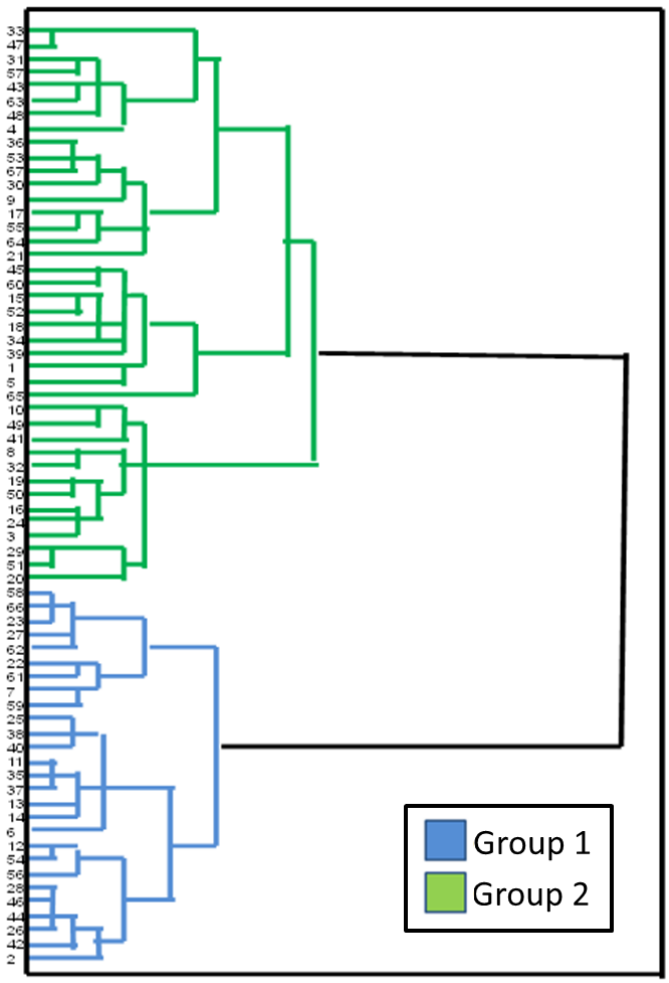

Figure 1. Cluster Dendrogram with the division of two groups.

After the division of the two Clusters, aiming at presenting the variables with the biggest differences between the two groups, it was done a statistical test of Qui-Square of Pearson, considering the variables with differences of $p<0.05$.

Table 1. Variables that differentiated the two groups of producers according to their performance in Good Agricultural Practices.

		No	Partial	Yes	N.A.
1. Do you have control of the expenses of firewood of the dryer?	*Group1*	**53.6%**	**28.4%**	**17.9%**	
	Group2	**12.8%**	**51.3 %**	**35.9%**	
2. Do you submit the employees that work with the pesticide application a medical checkup every year?	*Group1*	**75%**	**3.6%**	**21.4%**	
	Group2	**25.6%**	**0%**	**74.4%**	
3. Are all your temporary employees registered?	*Group1*	**21.4%**	**39.3%**	**39.3%**	
	Group2	**2.6%**	**30.8%**	**66.7%**	
4. Do you have all the legal reserve areas demarcated and annotated?	*Group1*	**82.1%**	**3.6%**	**14.3%**	
	Group2	**53.8%**	**25.6%**	**20.5%**	
5. Do you do organic fertilization in coffee?	*Group1*	**35.7%**	**17.9%**	**46.4%**	
	Group2	**10.3%**	**12.8%**	**76.9%**	
6. Do you do foliar analysis every year?	*Group1*	**50%**	**15.3%**	**35.7%**	
	Group2	**5.1%**	**5.11%**	**89.2%**	
7. Do you have a traceability system appropriate?	*Group1*	**3.6%**	**37.1%**	**39.3%**	
	Group2	**0%**	**25.6%**	**74.4%**	
8. Do you keep registers and controllers of field operations?	*Group1*	**92.9%**	**0%**	**7.1%**	
	Group2	**15.4%**	**5.1%**	**79.5%**	
9. Do you keep registers and controllers of field operations?	*Group1*	**35.7%**	**25%**	**39.3%**	
	Group2	**7.7%**	**15.4%**	**76.9%**	
10. Do you keep registers and controllers of all cost of production?	*Group1*	**35.7 %**	**42.9%**	**21.4%**	
	Group2	**5.1 %**	**41.0 %**	**53.9%**	
11. Do you use computers to control the activities?	*Group1*	**50%**	**17.9%**	**32.1%**	
	Group2	**23.1%**	**5.1%**	**69.2%**	**2.6%**
12. Do you follow and register the bombs number used in each pulverization?	*Group1*	**10.7%**	**25.0%**	**64.3%**	
	Group2			**100%**	
13. Do you have minimum knowledge to determine the kind of drink?	*Group1*	**10.7%**	**71.4%**	**17.9%**	
	Group2		**53.8%**	**46.2%**	
14. Do you do the monitoring and control of the drill?	*Grupo 1*	**10.7%**		**89.3%**	
	Group2			**100%**	

In relation to the variables found by the Qui-Square of Pearson test, it was possible observe that Group 2 has better performance in relation to GAPs fulfillment when compared to Group 1, in all 14 variables represented in the table that has significance $p<0.05$.

As a conclusion, the suggested methodology showed be able to categorize groups of coffee properties according to their performance in relation to Good Agricultural Practices. The Group 2 presented better performance in relation to Good Agricultural Practices when compared with Group 1.

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Infestation Level and Severity Caused by *Dirphya nigricornis* Olivier in Lushoto District, Tanzania

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SUMMARY

A new pest of coffee was reported by farmers in Lushoto district, Tanga region Tanzania. We carried out a cross-sectional survey in January, 2013 to identify the pest and establish its spread, incidence and severity. Multistage sampling was employed whereby divisions and wards were selected based on the status of coffee cultivations. In each selected ward, a random selection of villages and farms were done. The pest was identified as the yellow headed borer, *Dirphya nigricornis* (Olivier). The pest was found in almost all the study divisions attacking coffee plants. The infestation was 67.8% of 84 Arabica coffee farms surveyed. The farms infestation level was high in Soni (94.9%), medium in Mtae (85.2%) and Lushoto (84.9%) and low in Bumbuli (39.5%) and Mlalo (34.5%) divisions. The average incidence in five divisions was 31.6% with 24.6% severity of their branches bored with yellow headed borer larvae. Mtae division had much higher incidence (8.6%) and severity (44.57%) as compared to rest of divisions. *Leucaena leucocephala* was the only identified host plant infested by the pest around coffee farms. The majority (94.4%) of farmers are ignorant about the pest. A total of 12 extension workers were trained on best ways to manage the pest during the survey, and up scaling of the same is recommended with focus on removal and burning/burying of infested branches and other best farm practices.

INTRODUCTION

Yellow headed borer (YHB), *Dirphya nigricornis* Olivier (Cleoptera: Ceramycidae), is reported as a minor pest of Arabica coffee (Waller *et al.*, 2007). The pest is found in tropical countries including Cameroon, Congo, Uganda, Kenya, Malawi and Tanzania (Bohlen, 1978; Rutherford and Phiri 2006). In Tanzania it is generally considered as a minor pest though severity localized outbreaks have been reported to occur. The larvae of the pest bore through the wood of the stem pith which disrupts continuous translocation of minerals thereby interfering with the normal physiological functioning of the plant that finally leads to direct loss of berry yields. Symptoms of its severity causes wilted tips of primaries on the coffee farms infested by the pest. Also causes a series of holes, flute-holing, on one side of a branch or main stem, and frass (wooden shavings) injected outside the branches and stems. When there is a serious infestation, broken branches commonly observed in coffee farms bearing a heavy crop (Minai, 2009). Cultural control measures involve cutting and burying or burning for charcoal the infested branches and stems. Use of Deltamethrin 25g/l, Chlorpyrifos 48% and Ethion 50% have been used in Tanzania and Kenya for controlling the pest (Paul *et al.*, 2001; Magina, 2011). Botanicals like Neem, *Azadirachta indica*, Fish bean, *Tephrosia vogelii* and Jimson weed/Thorn apple, *Datura stramonium* are reported to give a good control as well (Paul *et al.*, 2001). The pest had previously been reported to occur in Mbinga district, Ruvuma region (2000), Lushoto district, Tanga region (2080) as a minor pest, but its severity somewhat diminished until recently (in 2012) when an outbreak of the pest was reported as an "unknown" pest. As a follow-up action, TaCRI organized a survey in January, 2013 with the

objective of establishing the identity of the pest, its incidence, symptoms, severity and economic importance. This paper summarizes the findings of the survey and future plans.

METHODOLOGY

The survey involved a multistage sampling whereby divisions and wards were selected based on the status of coffee cultivations. Five divisions were involved in the survey which includes: Mtae, Lushoto, Mlalo, Soni and Bumbuli. In each of the 5 divisions 3 wards were selected and in each ward, 2 villages were selected and in each village 3 farms were randomly selected to make a total of 84 farms. Global Positioning System (GPS) was used to mark the geo reference of each surveyed farm in the study area. Each study farm or block was subdivided into 4 roughly equal quadrants regardless of the size of the farm. Three coffee trees were randomly sampled along the diagonals of each quadrant, making twelve trees per farm. In total 1008 coffee trees were sampled and examined in detail to observe the presence of the pest. Structured questionnaire was also used to collect data from farmers and extension workers on background and knowledge of yellow headed borer problem in their respective areas. Data on the percentage of infested farms (infestation), yellow headed borer infested trees (incidence) and bored branches/twigs (severity) and farmers knowledge about the pest were analysed using Statistical Package for Social Science (SPSS) version 16.0.

RESULTS AND DISCUSSION

Careful examination of the infested plants for diagnostic symptoms (Plate 1) enabled the identification of the "unknown" pest as yellow headed borer (YHB), *Dirphya nigricornis* (Olivier). The pest was found in all the study divisions at varying severity levels. The overall infestation was 67.8% of 84 Arabica coffee farms surveyed. The farms infestation level was high in Soni (94.9%), medium in Mtae (85.2%) and Lushoto (84.9%) and low in Bumbuli (39.5%) and Mlalo (34.5%) divisions. The average incidence in five divisions was 31.6% with 24.6% severity of their branches bored with yellow headed borer larvae. Mtae division had much higher incidence (8.6%) and severity (44.57%) as compared to the other divisions. The most serious severity was recorded in Masereka ward (80.7%) at Mtae division followed by Soni ward (63.5%) at Soni division, Sunga ward (32.0%) at Mtae division and the rest of the surveyed wards showed substantially less severity (Table 1). *Leucaena leucocephala* was the only identified host plant infested by the pest in the field. High level of infestation of the pest at Soni division may be due to the fact that coffee was first introduced and has been growing there for a long time, hence the pest has had time to reproduce and build up to high populations. Similar observation have been reported by Oerke *et al.,* 2010 that in perennial crops pests can reproduce and potentially accumulate over time if there is availability of food. Masereka is a special case with very few recently established coffee trees (new varieties) and the high infestation may be due to the few farms present and/or the attractive characteristics of the new improved varieties. On-spot training of 12 extension workers on pest management practices was conducted as a follow up strategy to minimize further spread.

Table 1. Infestation, incidence and severity due to yellow headed borer in Arabica coffee at Lushoto district, Tanga region

Divisions	Wards	% of infested farms (infestation)	% of infested trees (incidence)	% branches infested (severity)
Lushoto	Gale	100.0	33.3	21.5
	Lushoto	80.9	35.4	6.0
	Kwemshai	73.7	29.2	14.0
	Average	**84.9**	**32.6**	**13.8**
Mlalo	Kwemshasha	38.3	27.8	12.7
	Dule M	26.0	21.7	7.6
	Mwangoi	39.3	22.2	15.0
	Average	**34.5**	**23.9**	**11.8**
Mtae	Sunga	66.7	25.0	30.0
	Masereka	100.0	83.3	80.7
	Mtae	88.9	37.5	32.0
	Average	**85.2**	**48.6**	**47.6**
Bumbuli	Funta	58.6	21.4	23.3
	Dule B	60.0	12.5	11.0
	Dule "M"	0.0	0.0	0.0
	Average	**39.5**	**11.3**	**11.4**
Soni	Mamba	93.8	28.3	13.8
	Soni	95.9	54.8	63.5
	Average	**94.9**	**41.6**	**38.6**
Overall Average		**67.8**	**31.6**	**24.6**

Source: Survey 2013

Farmer's comments regarding the yellow headed borer infestation

The comments made by farmers interviewed regarding the yellow headed borer (YHB) infestations are summarized in Figure 1 and Table 2. The questionnaire data revealed that 94.4% of farmers are not aware of the pest and were simply confused with white coffee stem borer (WCSB), assumed to attack branches at early stages of its development and stems at the late stages when they are more mature. The pest of economic importance in the area includes: WCSB (41.3%), Antestia bugs (16.5%), yellow headed borer (19.1%) and rodents (11.7%), while minor pests are: berry moth (3.5%), stinging caterpillar (3.5%), coffee berry borer (3.5%), leaf miner (0.9%) and aphids (0.9%) (Figure 1). Climate change and farms with poor agricultural practices was the factors mentioned by farmers as causes for outbreak of yellow headed coffee borer in the study area.

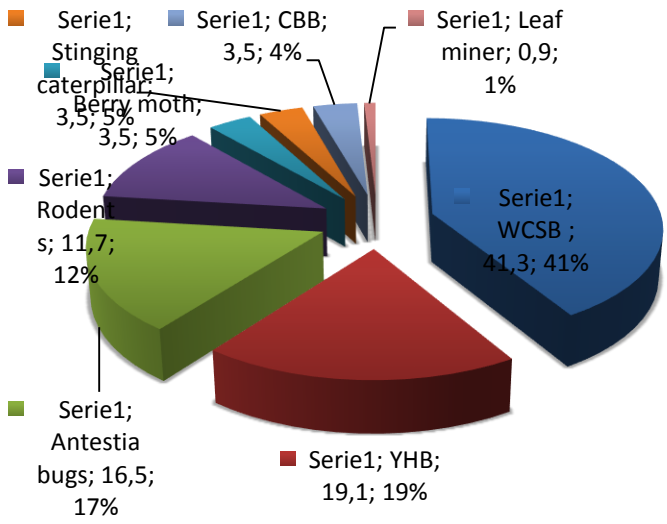

Figure 1. Coffee economic pests mentioned by farmers in Lushoto district

Table 2. Percentage of farmers who made comments during the survey of yellow headed borer at Lushoto district.

Farmers and extension comments	Percentage
Farmers with no idea about yellow headed borer (YHB) insect pest	94.4
Farmers mentioned that YHB outbreak is caused by climate change	11.3
Farmers mentioned that YHB outbreak is caused by poor agricultural practices	5.6
Farmers with an idea on the management of YHB in the field	0.5

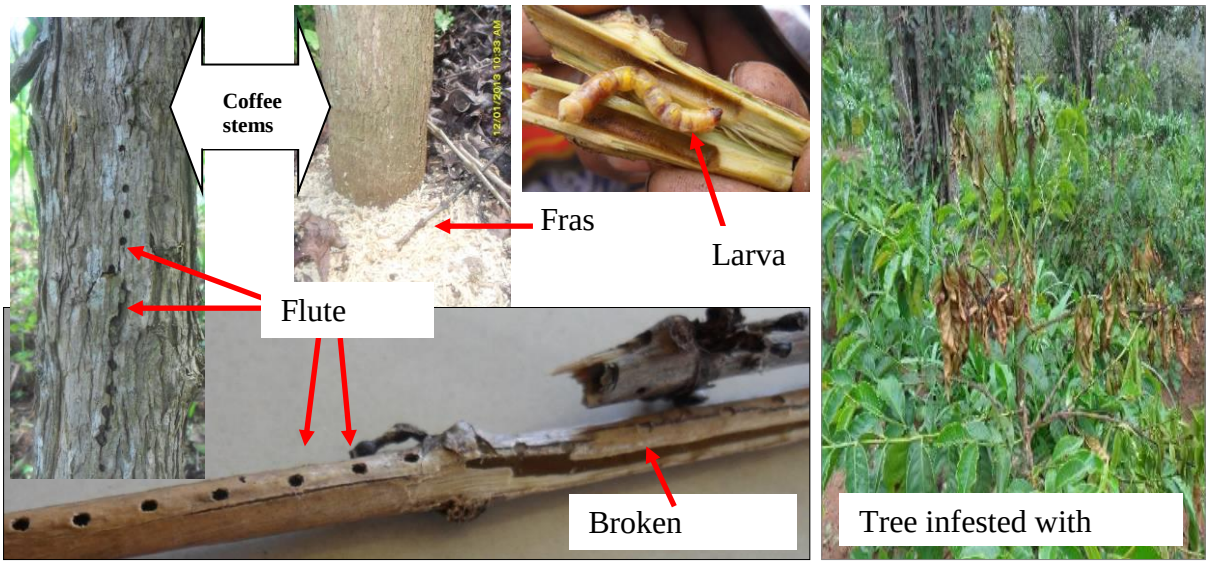

Figure 1. Larva of YHB, flute holing, broken branch and frass (left) and coffee tree with branches and growing tips (right) killed with YHB (Photo: Magina F).

CONCLUSION AND RECOMMENDATIONS

Based on symptoms of severity, the pest was identified as yellow headed borer, *Dirphya nigricornis (Olivier*) and it was found in all coffee growing divisions. The pest had so far

infested 67.8% of the farms and 24.6% of the primary branches. Since the pest is still unknown to most farmers, it is recommended to upscale by training farmers focusing on removal/burning or burying of infested branches and avoidance of the alternate host plants near coffee farms.

ACKNOWLEDGEMENTS

We are grateful to coffee growers in Tanzania, the European Commission (EC) in Tanzania and the government of Tanzania for supporting this work.

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Progress in the Management of the Coffee Berry Borer, *Hypothenemus hampei* Ferrari in Tanzania

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SUMMARY

A study was conducted in Lushoto district, Tanga region, Burka coffee estate, Arusha region and TaCRI Maruku, Kagera region in Arabica and Robusta coffee farms from September 2013 to March, 2014. The objective was to evaluate the effectiveness of pheromone lure and different alcohol brands in trapping adult coffee berry borer (CBB). A pheromone (ethanol + methanol in a ratio of 1:1) from India was compared with three local brews ("mbege" "rubisi" and "dengelua"), banana juice, methylated spirit (standard) and water (control). The split-plot design was used in a completely randomised block design with three replications. The main factor was colour traps painted with red, blue and white; sub factor was the attractants. Results show significant differences ($P \leq 0.05$) between mean CBB trapped per location. In Burka high numbers of the pest was attracted by methylated spirit followed by banana juice, "mbege", pheromone and water (control) was the least. In Maruku higher ($P \leq 0.05$) significant catch was observed for Methylated spirit as compared to "mbege", banana juice, "rubisi", pheromone and water was the least. At Lushoto, high significant differences ($P \leq 0.05$) were observed for Methylated spirit as compared to banana juice, pheromone, "dengelua" while water attracted less of the pest. However no significant differences ($P > 0.05$) was found between pheromone, banana juice and local brews ("rubisi", "dengelua" and "mbege") in all locations. Also the use of traps painted with red colour improved the efficiency of the traps. This study has found out that banana juice and locally made alcohols are as effective as the pheromone and are therefore recommended to farmers, in the respective areas where they are commonly made. On the other hand, methylated spirit had best performance, so it can be adopted by coffee estates which are better positioned in terms of resources. Pheromones could not compete with the standard methylated spirit. As it is being tested in Tanzania for the first time, the one-year data may not be conclusive, hence evaluation will continue for two more years.

INTRODUCTION

Coffee berry borer (CBB), *Hypothenemus hampei* (Ferrari)(Coleoptera: Curculionidae) is a serious coffee pest worldwide (Mugo and Kimemia, 2009). CBB is one of the major coffee pests in Tanzania for Robusta and low and medium Arabica coffee (Le Pelley, 1968; Magina, 2011). Both adult and larval stages cause damage by feeding inside mature and immature berries, resulting in crop loss of about 50% and more as well as significant reduction in quality of the crop (Le Pelley, 1968). In various countries in the past CBB was managed by

use of Endosulfan chemical (Mugo and Kimemia, 2009; Magina, 2011). The chemical is normally surface-active, and thereby unable to control the pest that has entered the berry. Frequent uses of the chemical have led to the development of pest resistance and are expensive and not environmentally friendly. The chemical have been black-listed by the Stockholm Convention as a Persistent Organic Pollutant (POP), and is aimed to be eliminated worldwide.

Mass trapping is a technique currently being considered for control or suppression option of CBB in the field (Durfour and Frerot, 2008). Various authors have reported to establish the control of adult CBB by use of traps baited with lures; for example methanol and ethanol (1:1) (Prakasan *et al*., 2001), methylated spirit and water (1:1) (Magina, *et al.,* 2006); and local brews ("Mbege") (Maro *et al*., 2008) respectively. On the other hand female sex pheromones have been used in monitoring and control or suppression option of insect populations which results either trapping and killing the pest or confusing their mating patterns (Cork, 2004). The IPM CRSP IL project in East Africa in which TaCRI is a collaborator, has initiated a study of evaluating different alcohols (local brews) available in different coffee growing regions in Tanzania (Tanga, Kilimanjaro and Kagera regions) since 2012. The objective of this study was to evaluate the effectiveness of pheromone lure and other alcohol brands in trapping adult CBB for use in the integrated management.

METHODOLOGY

The study was conducted at Burka coffee estate, Arusha region (at 1384 masl), a smallholder coffee farm at Shashui Village, Lushoto district, Tanga region (at 1315 masl) representing Arabica coffee and TaCRI-Maruku, Kagera region (at 1366 masl) representing Robusta coffee from September 2013 to March, 2014. We used split-plots in a completely randomised block design, with three replications. The main factor was colour traps painted with red, blue and white and sub factor was the attractants which includes: pheromone lure (ethanol + methanol in ratio of 1:1), methylated spirit and water (1:1), banana juice, local brews ("mbege", "rubisi", and "dengelua") and water (control). The attractants were put in a modified inverted plastic bottle (two litre) painted with red, blue and white colour at the upper part, with two holes on sides (7 cm x 5 cm) and added with water at the lower part and lures/attractants (20 to 30mls) were put in a small bottle (50 mls) and hanged with nylon or wire at the upper part (Figure 3). The bottles were then suspended on branches of coffee trees at a height of 1.5 m from the ground. The installation of traps in the field was done after every two lines of coffee at an interval of 10 metres from one trap to the other (Dufour and Frerot, 2008). The traps were serviced on one week basis, by replenishment of trapping lures depleted by evaporation and counting the number of adult CBB trapped. The number of trapped adult CBB was subjected to analysis of variance at 95 % significance level, using GENSTAT statistical package.

Table 1. Treatments (attractant) established in trial in different locations

Treatments	Lushoto (Tanga region)	Maruku (Kagera region)	Burka (Arusha region)
T1	Pheromone lure	Pheromone lure	Pheromone lure
T2	Banana juice	Banana juice	Banana juice
T3	"Dengelua"	"Rubisi"	"Mbege"
T4	Water (control)	Water (control)	Water (control)
T5	Methylated spirit and water (1:1) (standard)	Methylated spirit and water (1:1) (standard)	Methylated spirit and water (1:1) (standard)

"Dengelua", "Rubisi" and "Mbege' are local brews commonly made from locations as indicated above. "Dengelua" is made from fermented sugarcane (Saccharum officinarum), "mbege" from fermented banana (Musa spp) and finger millet (Eleusine coracana) and "Rubisi" from fermented banana and sorghum (Sorghum bicolor).

RESULTS AND DISCUSSION

The results show significant differences ($P \leq 0.05$) between CBB trapped in different treatments and between treatments per location (Figure 1). In Burka higher significant ($P \leq 0.05$) catches were observed for Methylated spirit (607.9) as compared to banana juice (146.57), "Mbege" (146), pheromone (116) and water (74.22). In Maruku higher significant ($P \leq 0.05$) catch was observed for Methylated spirit (310.4) as compared to "mbege" (69.2), banana juice "rubisi" (69.0), pheromone (64.0) and water (20.5) was the least. At Shashui high significant ($P \leq 0.05$) differences was observed for Methylated spirit (168.4) as compared to banana juice (45.0), pheromone (36.0), "Dengelua" (20.0) while water (9) was the least. However no significant different ($P > 0.05$) was observed between pheromone, banana juice and the local brews ("mbege", "dengelua" and "rubisi") in different locations. As regards colours of traps, red (401.1) showed significant ($P \leq 0.05$) difference as compared to blue (214.02) and white (199.74) trap colours, the last two not showing significant difference. Comparing locations, more pests were captured in Burka as compared to Maruku and Shashui, Lushoto. This may be due to different population levels of the pest in each location, which is also a function of the size of the farm and therefore the number of coffee trees available for infestation. The observation that methylated spirit and pheromone differed so much in trapping the pest is difficult to explain since they both contain methanol and ethanol which were evaluated by Prakasan *et al., (*2001); Magina *et al., (*2006); Dufour and Frerot (2008) and indicated to perform better in attracting the pest. The other lures "mbege", "dengelua", "rubisi" and banana juice performed better than water, presumably due to attractive smell or alcohol contents associated with fermentation process. High performance of "mbege" in attracting the pest agreed with the findings by Maro *et al.,* (2008). More catch of CBB in red colour agreed with the findings by Dufour and Frerot, (2008) who found that red colour of the trap substantially increased CBB attraction.

CONCLUSION AND RECOMMENDATIONS

The banana juice and locally made alcohols traps ("mbege", "rubisi" and "dengelua") have shown to be as effective as the pheromone for mass trapping of CBB. In addition they are safe, simple and cheap to make and are therefore recommended to be used by coffee farmers in the respective areas, where the local brews are commonly made. On the other hand, methylated spirit had best performance, so it can be adopted by coffee estates which are better positioned in terms of resources. Use of colour traps, especially the ones painted red, has shown to improve the efficiency of the traps. Pheromones could not compete with the

standard methylated spirit. As it is being tested in Tanzania for the first time, the one-year data may not be conclusive, hence evaluation will continue for two more years.

ACKNOWLEDGEMENTS

We are grateful to European Commission (EC, Tanzania), IPM CRSP IL in East Africa and Tanzania coffee growers for their financial support for this study.

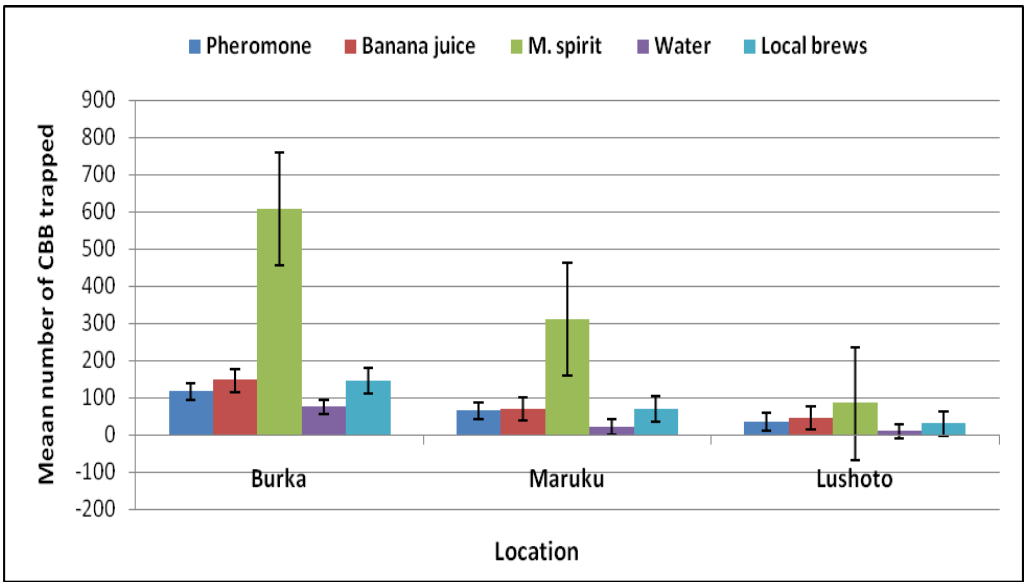

Figure 1. Mean population level of adult CBB at Shashui (Lushoto), Maruku and Burka coffee estate

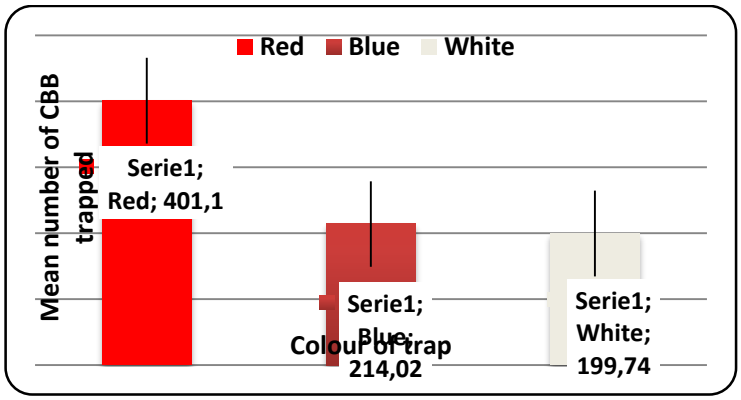

Figure 2. Mean number of CBB trapped in different colour traps for a period of 7 months.

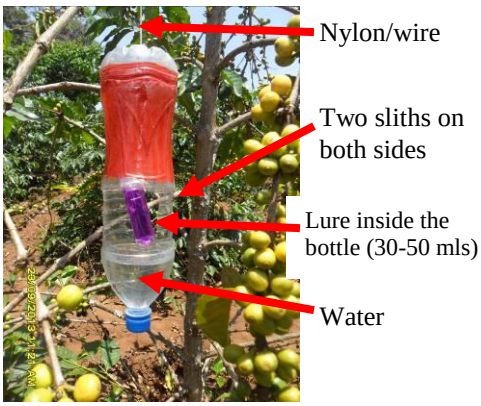

Figure 3. Modified inverted plastic bottle for CBB trap.

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Exploring the Agronomic Attributes of Some Coffee Varieties Grafted with Scions of Improved Arabica Hybrids in Tanzania

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SUMMARY

A study was conducted to explore the potential of different coffee varieties used as rootstock against new variety scions, thus taking advantage of variable rooting habits. Common improved Arabica coffee hybrid N39-3 (renowned for its high yields) was used as a scion and top worked on rootstock of several coffee varieties; KP 423, K-7, Robusta SM, POP (FC 6), and PNI 088 with N39 rootstock used as a check. Grafting was done when both rootstocks and the scion were pencil-thick. The trial was set according to the Randomized Complete Block Design with four replications and six treatments which were the selected rootstocks. The grafted coffee seedlings were planted at a space of 2.5m x 2m with six trees per plot. Parameters assessed were plant characteristics (plant height, plant canopy, number of primary branches and plant girth) observed during the second year and coffee yield recorded for three years. The data collected was subjected to analysis of variance using Gen Stat software. All the observed agronomic attributes differed among themselves but not significantly ($p > 0.05$). Plant height followed the order PNI088> N39> Robusta> K7> KP423> POP. The same trend was observed with stem girth, except that N39 and Robusta exchanged positions. The number of primary branches followed the order PNI088> Robusta> K7> N39 > POP > KP423. The order was altered with canopy size whereby N39 recorded largest size, followed by PNI088, K7, Robusta, KP423 and POP. Variety PNI088 excelled in three of the four selected agronomic attributes, being second in canopy size. On the other hand, Robusta, which is the only non-Arabica species tested, was ranked second in stem girth and number of primary branches, third in plant height and fourth in canopy size. Mean yield of three seasons ranged from 870 to 1024 kg clean coffee ha^{-1} in the decreasing order PNI088> K7> POP> N39> Robusta> KP423. The first three are also known to exhibit some degree of drought tolerance. Because the difference in yield was not significant, farmers are free to use any of the tested varieties as rootstocks for accelerating multiplication of improved Arabica varieties.

INTRODUCTION

Coffee can be propagated conventionally by seeds or vegetatively using cuttings, with the propagation by seeds being associated with inborn uncontrolled genetic stability due to segregation resulting into variable progenies (Rehm and Espig, 1991). On the other hand vegetative propagation aim at producing plants that are genetically the same with a single parent and so guarantee the uniformity with their mother parents (Wrigley, 1982). More recently techniques have been developed, which transfer the multiplication from the field to the laboratory, such methods include somatic embryogenesis or regeneration from callus (Wrigley, 1982; TaCRI, 2008).

Tanzania coffee research institute (TaCRI) has already perfected two methods for hybrid seedlings multiplication, namely clonal propagation and grafting. The progenies arising from plants propagated by clonal materials are exactly true to type Nzallawahe *et al*. (2004) carrying with it the full attributes of the mother plant. On the other hand, grafting method

involves two plants with different agronomic attributes, which are shared in the resulting plant. For TaCRI, grafting usually involves the scion of improved hybrid varieties (which are high yielding) and the rootstocks of traditional coffee varieties (Van der Vossen *et al*., 1977; TaCRI, 2008 and 2009). Grafting has shown, through experience, to be very successful and has been adopted as an easy way of multiplying new varieties by a majority of coffee farmers. According to Jose Luis *et al.* (2007), Mshihiri *et al. (2012),* some rootstocks are known to be better than others with respect to use water resources and intensity of adaptation to different condition. However there is still a knowledge gap on how rootstocks from coffee varieties with variable rooting habits influences agronomic attributes of scions from improved coffee varieties and how this can be effectively manipulated. The objective of this work was therefore to explore how coffee rootstocks with different rooting habit combine with the scion of improved coffee variety and the effects of the combination on plant vigour and yield.

MATERIALS AND METHODS

The experiment was conducted at TaCRI Lyamungu station from 2006 to 2010 using a randomized complete block design with three replication comparing six treatments as shown in Table 1. Different rootstocks were raised in the nursery through the use of seeds, and the scion of new variety was collected from mother garden at TaCRI Lyamungu. Grafting was done when both the rootstock and the scion had reached at pencil thickness. Coffee seedlings were planted in the field at a spacing of 2.5m x2m with six trees per plot. Six different rootstocks from traditional coffee verities and one scion from improved variety were used for the investigation. The experiment was managed as recommended for coffee. Data collected were on; plant characteristics which were taken during the second year of the growth (plant height, plant canopy, number of primary branches and plant girth) and coffee yield. The collected data were subjected to analysis of variance (ANOVA) test, using GenStat software and means were separated by Duncan's multiple range tests at 0.05 level of significance.

Table 1: The combinations of scions and rootstocks tested in this work

	Rootstock	**Scion**
TR 1	KP423	N39-3
TR2	K-7	N39-3
TR3	N-39 (CONTROL)	N39-3
TR4	Robusta MS	N39-3
TR5	POP(FC6)	N39-3
TR6	PNI (088)	N39-3

RESULTS AND DISCUSSION

Effects of scion rootstock compatibility on coffee yield

Mean yield of three seasons ranged from 870 to 1024 kg clean coffee ha^{-1} in the decreasing order of PNI088> K7> POP> N39> Robusta> KP423. There was an increase in yield from year one to year three (Figure 1) which is normal for Arabica coffee to reach its production potential from Year 3 onwards (Wrigley, 1988). The annual increase in yield across treatments indicates good compatibility between the grafted rootstocks from old coffee varieties and the scion from improved variety; and the yield difference across treatments was not significant at $p>0.05$ level. The mean yield obtained per ha at Year 3, which ranges from 1706-2075 kg appears to represent the potential of the common scion material N39-3, which is about twice the amount usually obtained in the old coffee varieties (1283 Kg/ha), thus

explaining the importance of grafting technique in converting old coffee varieties into improved coffee varieties. The contribution of rootstock varieties to yield differed but not significantly. Varieties PNI 088, K7 and POP/FC6 showed higher yields in excess of 2 tons per ha, which can be attributed to their prolific rooting system which implies better water and nutrient use efficiency.

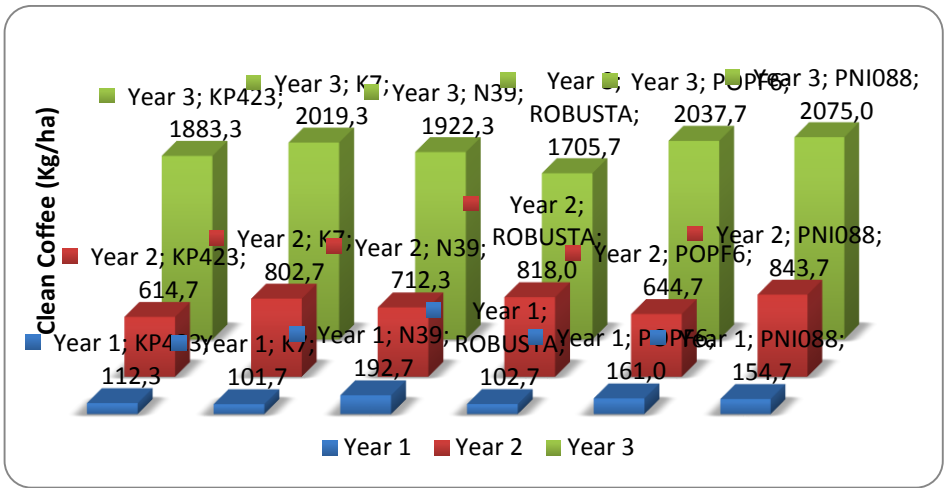

Figure 1. Yield (Kg/ha) for different rootstocks grafted on the same scion.

Effects of scion rootstock compatibility on the plant agronomic characteristics

Comparing the agronomic attributes (Table 2), no significant difference was noted among them ($p > 0.05$). Plant height followed the order PNI088> N39> Robusta> K7> KP423> POP. The same trend was observed with stem girth, except that N39 and Robusta exchanged positions. The number of primary branches followed the order PNI088> Robusta> K7> N39 > POP > KP423. The order was altered with canopy size whereby N39 recorded largest size, followed by PNI088, K7, Robusta, KP423 and POP. Variety PNI088 excelled in three of the four selected agronomic attributes, being second in canopy size. On the other hand, Robusta, which is the only non-Arabica species tested, was ranked second in stem girth and number of primary branches, third in plant height and fourth in canopy size. The implication here is that at the location where the experiment was set, any of the tested rootstocks is equally good; but this may change if the coffee is exposed to more harsh conditions such as extended drought.

Table 2. Plant characteristics

Treatments	No of primary branches	Plant canopy (cm)	Plant height (cm)	Plant girth (cm)
KP423 +N39-3	27	144.8	131.0	2.4
K-7+ N39-3	28	149.8	132.0	2.4
N-39 +N39-3(control)	28	152.5	133.5	2.7
Robusta SM+N39-3	29	148.5	133.0	2.6.
POP(FC6) +N39-3	27	136.5	127.0	2.6
PNI (088) +N39-3	30	152.3	133.8	2.6

CONCLUSION

This study has proved that grafting is a viable and successful method for rapid conversion of old coffee varieties into new coffee varieties. It also shows that all the selected rootstocks are compatible with the selected scion of new coffee variety as their difference did not cause any

significant effect on plant characteristics or yield. The observed difference was attributed to the rooting habits of different rootstock varieties with PNI 088 and K7 excelling in most of the observed attributes. The two, together with Robusta, are known to have prolific rooting system and hence an added attribute of drought tolerance. Our message to the farmers is that they can use any of the tested varieties as rootstock for accelerating the production and distribution of seedlings of the improved Arabica varieties. A follow-up series of experiments is underway to assess the behavior of the tested rootstocks in different drought-prone areas in Tanzania.

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Loss Caused by Meloidogyne exigua on the Caturra Variety Under Controlled Conditions in Costa Rica

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SUMMARY

Meloidogyne exigua is the root-knot nematode most commonly found in the Costa Rican coffee plantations, although its effect on the coffee yield is not clearly determined yet. This study aims at determining the loss caused by *Meloidogyne exigua* on adult coffee plants of Caturra variety under semi-controlled field conditions.

The trial was conducted at the Center for Coffee Research (CICAFE), Heredia, Costa Rica, at 1180 m.a.s.l., with a mean annual temperature of 21.5°C and a total annual rainfall of 2650 mm. Coffee plants formed in two axis, with a 2.0 x 1.0 m. planting distance, were planted in August 2009 in 100 L pots with disinfected soil. A randomized complete block design with 10 replicates was used. The treatments were defined by the initial inoculum (Pi) of 0, 125, 250, 500, 1000 and 2000 eggs $+J_2$ *M. exigua*/100 cm^3 soil, applied two months after planting. Adequate fertilization has been provided. The plant development was evaluated in 2010 and 2011. Foliar samples were collected twice a year for chemical analysis of each plant. Root samples were collected in September 2011 and 2013, for the analysis of the *M. exigua* density. Has been evaluated three year production.

The evaluations of development showed no response to the initial inoculum of nematodes. The foliar analysis indicated that the levels of elements were within the normal ranges and showed no response to initial inoculum. In 2011 the density of *M. exigua* in root reported a strong relationship with the initial inoculum and regression clearly separated the highest Pi ($y = 0.0473x^2-36.776x+78087$; $R^2 = 0.9766$). Analysis of density in 2013 showed no significant response to initial inoculum. Regression analysis of the 2013/2014 harvest depending on the initial inoculum showed a significant trend ($p < 0.01$) at the expense of production due to the pathogen, but low relationship ($r = -0.12$) with the density of nematode in roots in 2013. In the latest crop, production declined from 8% to 16% compared to treatment with lower density of nematodes.

INTRODUCTION

Nematodes have been a significant obstacle to the production of coffee in Costa Rica. They are located in all growing regions and its spread has been mainly through the transfer of nursery plants to nematode unpopulated areas. The genera *Meloidogyne* and *Pratylenchus* are more spread out in the country (ICAFE, 1998).

The predominant species is *Meloidogyne exigua* in coffee plantations in Costa Rica (Villain et al. 1999; Alpízar & Alvarado 1999; Flores & López 1989 cited by Bertrand et al. 2000; Rojas 2008) and according Bertrand et al. (1998) its attack can lead to a decrease of 15% of the crop.

Barbosa et al. (2004) conducted a study in 125 coffee plantations with and without infestation of *M. exigua*, categorizing two ages of cultivation (less than 5 years and more than 5 years) and three levels of technology management according to the application of fertilizer and pest control. The field study indicated that *M. exigua* was not the major cause of decline in production in plantation with management level medium or low. In contrast, well-managed farms were intolerant populations as low as 3 J_2/100 cc of soil, with yield losses that reached 45%.

This study aims at determining the loss caused by *Meloidogyne exigua* on adult coffee plants of Caturra variety under semi-controlled field conditions.

MATERIALS AND METHODS

The trial is set to the Centro de Investigaciones en Café (CICAFE), in Barva, Heredia, Costa Rica. The site is located at 1180 masl in an Andisoil, with average annual temperature of 21.5 °C and 2650 mm of total rainfall per year. The variety used is Caturra, with plants formed two axes, set to 2.0 x 1.0 m and full sunlight. It uses a design randomized complete block with 10 replications. The experimental unit comprises a potted plant. Treatments consisted of applying initial inoculum of 0, 125, 250, 500, 1000 and 2000 eggs+J_2 of *M. exigua*/100 cm^3 soil. The plants were established on August 25, 2009 in plastic barrels with approximate volume of 95 L and disinfected soil with Dazomet. The pots were inoculated two months after transplantation. Adequate fertilization has been provided.

The plant development was evaluated in 2010 and 2011. Foliar samples were collected twice a year for chemical analysis of each plant. Root samples were collected in September 2011 and 2013, for the analysis of the *M. exigua* density. During the years 2012 to 2014, after finding contamination with *M. exigua* in treatment that should be free of nematodes, nematicide was applied in May and August, to keep down the infestation. Has been evaluated three year production.

RESULTS AND DISCUSSION

The evaluations of development showed no response to the initial inoculum of nematodes. The foliar analysis indicated that the levels of elements were within the normal ranges and showed no response to initial inoculum (data not shown). In 2011 the density of *M. exigua* in root reported a strong relationship with the initial inoculum and regression clearly separated the highest Pi (Figure 1A). Analysis of density in 2013 showed no significant response to initial inoculum (Figure 1B).

Regression analysis of the 2013/2014 harvest depending on the initial inoculum showed a significant trend ($p < 0.01$) at the expense of production due to the pathogen (Table 1), but low relationship ($r = -0.12$) with the density of nematode in roots in 2013 (Figure 2). The regression of the average of the three years showed a similar trend, but no significant difference ($p < 0.05$).

The last harvest showed a detrimental effect close to 9% on average, associated with the initial inoculum of *M. exigua*. The data indicate that the latest crop production decreased 1% for every 20 000 individuals in 100 g of root, although the dispersion is very high. Research is developing and the next harvest data provide more precision about the effect of *M. exigua* under these conditions.

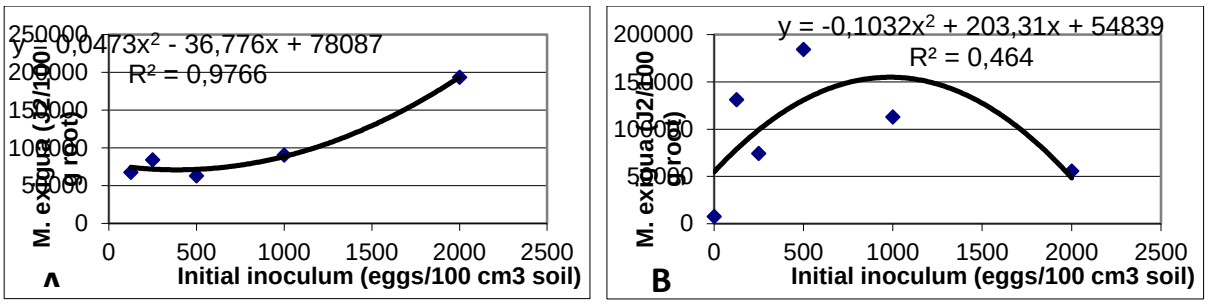

Figure 1. Response curve of the density of *M. exigua* in roots, relative to initial inoculum. September 2011 (A) and 2013 (B).

Table 1. Coffee fruit production, according to the initial inoculum of *M. exigua*.

Initial inoculum	Production (Kg/plant)			
	2011/2012	2012/2013	2013/2014	Mean
0	7.82	4.76	5.68	6.09
125	6.73	5.18	5.17	5.69
250	8.39	3.50	6.69	6.19
500	5.97	5.30	4.78	5.35
1000	6.81	4.47	4.78	5.36
2000	7.09	4.36	4.31	5.25
Regression	y=-0.00016x+7.3	y=-0.0003x+4.7	y= -0.00077x+5.73	y=-0.00039x+5.91
R^2	0.0518	0.0031	0.25	0.1
p value	0.4745	0.7473	0.0018	0.0664

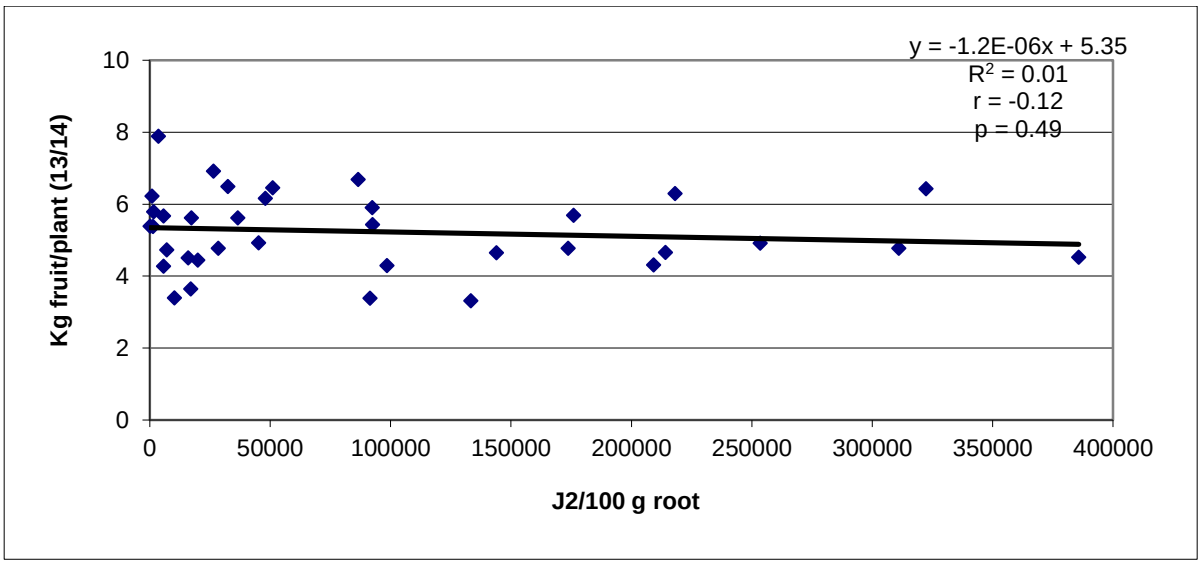

Figure 2. Relationship between the density of M. exigua in 100 grams of root in September 2013 and production of fruit per plant at harvest 2013/2014.

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Effect of Soils Properties on the Quality of Compact Arabica Hybrids in Tanzania

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SUMMARY

For decades Tanzanian Arabica coffee has been popular in the international market due to their top grade fine flavour quality. But there is lack of information on how soils properties influence attributes of cup taste in the country. A study was therefore conducted to assess the beverage of Compact Arabica varieties as influenced by soil properties in Tanzania. Samples for the study were collected from altitudinal range of 1200 to 1800 m a. s. l.; soil types described as clay, loam and silt loam; type of rainfall bi-modal (1500 mm per annum) and uni-modal (1700 mm per annum), and flat and or sloppy terrain. Forty (40) samples of coffee green beans collected from descriptive area of the study were considered in this study. Red ripe coffee cherries were handpicked, wet processed, dried, and graded with only AAs, As and PBs considered in the study. For beverage assessment green beans were roasted allowed to rest for 4 hr, grinded, then dry fragrance of the samples were evaluated by sniffing. Proteins, caffeine and mineral contents of the coffee beans were analyzed. Soils samples were collected from three depths: 0-30 cm, 30-60 cm, and 60-90 cm. Analysis was done to measure pH, cation exchange capacity (CEC), and available soil nutrient and texture. Design for the experiment was Randomized Completely Design, and data were analyzed using Genstat statistical package. Out of 40 samples, 2 of the compacts scored class 4 for specialty coffee. N39 traditional Arabica cultivar scored specialty description. These findings indicate that, to attain specialty or premium coffee from prevailing soil properties in Arabica coffee areas the best cup with excellent aroma is supposed to be prepared from green beans of; Calcium (g/Kg) 0.8-0.9, Potassium (g/Kg) 2.9 to 6.0, proteins (%m/m) 8.0 to 9.0 and caffeine (g/Kg) 10.8 to 13.7. It was also found out that soils with adequate P, K, Clay-loam and silt influences positively the cup taste. Positive correlation coefficient r was found between the cup taste and pH, and soil nutrients.

INTRODUCTION

Tanzanian coffee from both Arabica and Robusta is currently ranked among the best coffee in the world (TCB, 2012). Decazy *et al.* (2003) observed, the types of varieties cultivated, post-harvest processes applied to coffee, geographical areas (soils and climatic conditions), and agronomic practices to a greater extent influence the taste of coffee. Slagle *et al.* (2004) reported that concentrations of nutrients in different soils would also have an impact on the variations in cup quality of coffee. This is because nutrients are required for both vegetative growth of coffee trees and production of high quality beans, and thereby coffee quality (Njoroge, 1998). For example volcanic soils often produce a potent acidity and a fine flavour and for that matter such soils can lead to a more balanced cup. Areas of wet and dry cycles and precipitation which does not exceed 1500 mm have a tendency of producing high quality coffee due to regular cherry ripening and proper bean drying conditions after harvesting (van der Vossen, 1985). The objective of this study was to assess the influence of soils on the cup taste of coffee genotypes established in different ecosystems in Tanzania.

MATERIALS AND METHODS

Coffee cherries were handpicked when fully red ripen between May to September 2013 at Lyamungu (Slopes of Mount Kilimanjaro) and Ugano (Southern Highlands). Wet processing of cherries, followed by fermentation of the parchments and drying of coffee green beans were performed as per the method described by Robinson (1964). Coffee green beans were graded, and beans with AA and A grade selected, weighed in 100 gm. Coffee green beans were analyzed for organoleptic taste and mineral composition. The caffeine content and mineral contents of the coffee beans were determined as per the method adopted by Tanzania Food and Drug Agency (TFDA, 2010). For protein analysis, Kjeldal method was used. To determine sodium and potassium, the filtrate was subjected to spectrometer flame emission. To determine sodium, a wavelength of 589.6 nm was adopted with a special band width of 1.0 nm, and potassium at a wavelength of 769.9 nm with a special band width of 1.0 nm.

Soil sampling from Lyamungu and Ugano was done following the criteria suggested by Cordingley (2010), which include the size of the plot and the level of homogeneity within the plots. This included varying positively the number of auguring within a plot size, and auguring guided on "W" root of sampling within a plot. Soils were collected in three depths (0-30 cm, 30-60 cm, and 60-90 cm) then transported to Lyamungu for analysis.

The cup taste of the coffee samples were assessed by experts' liquorers using sensorial criteria: aroma, body, acidity, bitterness, astringency, sourness and grassy taste. The flavour class assigned; score fine, Good to fine class1; Fair to Good class 3; Fully Fair class 4; Fair Average Quality class 5; About Fair class 6; Poor to Fair class 7; and Poor class 8.

RESULTS AND DISCUSSION

Results for the samples collected from different locations on description of the cup, mineral, proteins and caffeine contents are as summarized in Table 1.

Table 1: Description of the cup, mineral, proteins and caffeine contents of compact coffee genotypes

Location	Genotype	Cup taste	Class	Description	Mineral content			
					Calcium (g/Kg)	Potassium (g/Kg)	Protein (% m/m)	Caffeine (g/Kg)
Lyamungu	CVT1	Medium acidity, body & flav	4	Fruity aroma, dark choc	0.9cde	4.1c	10.2a	9.7efg
	CVT2	Medium acidity, body & flav	6	Chocolate	1.1bc	4.0c	9.2c	9.7efg
	CVT3	Light medium acidity, body	6	Chocolate	1.2ab	6.3c	9.0cd	11.8abc
	CVT4	Medium body, light med acid	5	Aroma taste tones	0.5f	12.7ab	9.1c	10.5cdef
	CVT5	Light medium acidity, body	5	Sweet aroma	0.7ef	3.1c	9.5bc	11.3bcde
	CVT6	Medium acidity, body	4	Dark chocolate	0.8de	6.0c	8.5de	12.8ab
	CVT7	Light medium acidity, body	6	Aroma coffee pulp tones	1.1bc	4.6c	9.2c	11.5abcd
	CVT8	Medium body, light med acid	5	Chocolate	0.9cde	4.8c	9.5bc	9.6efgh
	CVT9	Light medium acidity, body	5	Fruity aroma strawberry	1.1bc	4.9c	9.5bc	13.1a
	CVT10	Medium body, light med acid	5	Fruity aroma strawberry	1.2ab	4.5c	9.0cd	9.8defg
	CVT11	Medium body, light med acid	5	Dark chocolate	1.1bc	5.4c	8.4e	78.9fgh
	CVT12	Light medium body, acidity	5	Fruity taste	0.9cde	16.1a	9.2c	8.7gh
	CVT13	Light medium body, acidity	6	Harsh aroma	1.2ab	4.6c	8.5de	11.8abc
	CVT14	Light medium acidity, body	5	Aroma berry tones	1.4a	5.4c	9.4bc	9.1fgh
	CVT15	Light medium body	5	Harsh aroma	0.9cde	4.5c	9.4bc	8.8fgh
	CVT16	Light medium acidity, flavour	6	Dark chocolate	0.9cde	5.7c	9.0cd	11.6abc
	N39-6	Medium body, light med acid	5	Lemonish citrus aroma	1.0bcd	5.3c	9.2c	7.9h
	KP423-2	Light medium body, acid, flav	6	Lemonish aroma	1.1bc	5.5c	9.9ab	10.5cdef
	PNI088	Light medium acidity, flv, bod	6	Strawishy aroma	1.4a	2.9c	9.3c	10.5cdef
	N39	Light medium acidity, body,fl	5	Aroma berry tones	0.9cde	10.5b	7.6f	8.9fgh
	Mean				1.0	6.0	9.1	10.3
	Tukey's				0.04	0.7	0.1	0.3
Ugano	CVT1	Light medium acidity, body,fl	6	Sourish	1.3abc	5.9bcde	7.9efg	10.7gh
	CVT2	Light medium acidity, body,fl	6	Sourish	1.4ab	7.0bc	9.5ab	11.7efgh
	CVT3	Light medium acidity, body,fl	6	Sourish	0.9bcd	6.5bcd	8.8bcdefg	15.1b
	CVT4	Medium body, light med ac/fl	5	Cean cup	1.1abcd	3.0f	8.8bcdefg	14.8bc
	CVT5	Light medium acidity, flavour	5	Sweet aroma	1.4ab	3.5f	8.9bcdefg	11.6efgh
	CVT6	Medium body, acidity, flavour	5	Fruity taste aroma	1.2abcd	3.0f	9.3bc	12.2defg
	CVT7	Light medium acidity, bod, flv	6	Sourish	1.5a	4.7cdef	8.8bcdefg	12.7cdefg
	CVT8	Medium body, light acidity, fl	5	Chocolate	0.7d	7.7ab	8.8bcdefg	12.6cdefg
	CVT9	Light medium body, acidity, fl	6	Sourish	1.3abc	3.1f	8.2cdefg	12.1efg
	CVT10	Medium body, light acidity, fl	6	Lemonish aroma	0.7d	3.0f	7.7g	17.7a
	CVT11	Light medium body, acidity,fl	6	Sourish	0.9bcd	6.5bcd	7.8fg	11.5efgh
	CVT12	Light medium body, acidity,fl	6	Lemonish	1.2abcd	2.7f	8.0defg	11.4efgh
	CVT13	Light medium body, acidity,fl	6	Sourish	0.8cd	3.4f	10.5a	11.2fgh
	CVT14	Light medium body, acidity,fl	6	Sourish	1.5a	9.5a	7.9efg	11.5efgh
	CVT15	Medium body, light acidity, fl	5	Chocolate	0.9bcd	6.0bcd	9.1bcde	11.1fgh
	CVT16	Light medium acidity, flavour	5	Fruity aroma	1.1abcd	4.4def	7.8fg	9.5h
	N39-6	Medium acidity, flavour, body	4	Dark chocolate	0.9bcd	4.8cdef	9.0bcde	13.7bcde
	KP423-2	Medium body, light acidity, fl	5	Sourish	0.8cd	2.7f	8.0defg	14.5bcd
	PNI088	Light medium acidity, fl, body	5	Dark chocolate	1.3abc	3.6ef	8.1defg	13.2bcdef
	N39	Medium acidity, body, flavour	4	Fruity aroma, d/ch	0.9bcd	2.9f	8.0defg	10.8gh
	Mean				1.1	4.7	8.5	12.5
	Tukey's				0.1	0.4	0.2	0.4

Genotypes with the most outstanding cup taste scores at Lyamungu were CVT1 and CVT6, at Ugano were N39-6 and N39. Almost all coffee genotypes scored class 5 to 6, described for export value. N39 and N39-6 both commercial coffee cultivars maintained their genetic ability in showing excellent cup taste. The position of the contents analyzed which gave the best cup taste results of class 4 at Lyamungu site was on genotype CVT6 whereby Calcium levels in the sample were 0.8 g/Kg, Potassium 6.0 g/Kg, Proteins 8.5% m/m, and caffeine 12.8 g/Kg. Coffee genotype CVT1 at the same site the levels of the contents were Calcium 0.9 g/Kg, Potassium 4.2 g/Kg, Proteins 10.2% m/m, and caffeine 9.79 g/Kg. The description of the cup for CVT1 was fruity aroma, dark chocolate; and CVT6 was dark chocolate. At Ugano, the analysis for the contents of N39-6 were Calcium 0.9 g/Kg, Potassium 4.8 g/Kg, Proteins 9.0% m/m and caffeine 13.7 g/Kg. N39, calcium 0.9 g/Kg, Potassium 2.9 g/Kg, Proteins 8.0% m/m and caffeine 10.8 g/Kg. This could translate that the best cup of coffee with excellent

aroma flavour is supposed to be prepared from green beans with contents of Calcium 0.8 g/Kg to 0.9 g/Kg, Potassium 2.9 g/Kg to 6.0 g/Kg, Proteins 8.0% m/m to 9.0% m/m and caffeine 10.8 g/Kg to 13.7 g/Kg.

Table 2 summarizes result on soil analysis collected from Lyamungu and Ugano where the compact genotypes were established. Soils pH levels at Lyamungu site falls under neutral positions (4.7 to 5.9), and at Ugano slightly alkaline; 5.3 to 7.4. This may influence the availability of soil nutrients to be available for absorption by the coffee plants. Soils with free draining loams with a good water retention capacity and a pH of 5 to 6, and contain proportions of clay usually produces high quality of Arabica (van der Vossen, 1985). But at the same time soils with excessive calcium and potassium produce hard and bitter tasting liquor. Lyamungu soils had good proportions of loam and clay-loam compared to Ugano (Table 2), this could be possibly a reason for 14 samples scoring class 5-4, viz. a.viz 9 of the same class at Ugano.

Table 2. Analytical results for soil samples collected from Lyamungu and Ugano

Site	Depth (cm)	pH (H20)	Exchangeable cation in me/100 sample						Total % N	Trough (ppm P)	Soil type
			Ca^{2+}	Mg^{2+}	K^{+}	Na	%BS	CEC			
Lyamungu	0-30	4.7	8.1	0.3	2.2	0.5	77	16	0.14	1.32	Loam
	30-60	5.2	9.4	0.7	1.7	0.6				1.95	Loam
	60-90	5.6	10.2	1.2	1.8	0.6				0.99	Clay loam
Ugano	0-30	6.2	1.4	0.2	0.7	0.3	23	12	0.13	2.28	Silt clay
	30-60	5.8	0.9	0.5	0.4	0.3				1.07	Clay loam
	60-90	5.4	0.8	1.6	0.3	0.3				1.02	Clay

Results show that there is positive correlation between cup taste and some of the soil nutrients (Table 3).

Table 3. Correlation coefficients between pH, Ca2+, K+, Na+, Mg2+ versus cup taste

	pH	Ca^{2+}	Mg^{2+}	K^{+}	Na^{+}
Cup taste	0.49	0.45	0.13	0.46	0.48
	**	**	ns	**	**

$r_{t\ at\ 0.05\ (0.40)}$

When studying the influence of soil properties on cup quality of wild Arabica coffee in forest ecosystem of Ethiopia, Yadessa et. al. (2008) reported that better cup quality was detected form soil properties with higher levels of available P, K, clay and silt. Potassium increases bean density and augment flavour, and phosphorus contributes to a balanced flavour.

CONCLUSION

This study demonstrated that soil properties have an effect on compact coffee genotypes. It has been shown that the best cup with excellent aroma of the compact coffee genotype is supposed to be prepared from green beans with contents of Calcium 0.8 g/Kg to 0.9 g/Kg, Potassium 2.9 g/Kg to 6.0 g/Kg, Proteins 8.0% m/m to 9.0% m/m and caffeine 10.8 g/Kg to 13.7 g/Kg. As most of soils are deficiency of these nutrients, it is recommended to supplement from application of the farm yard manure or inorganic fertilizer.

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Aggressiveness of *Phoma* and *Colletotrichum* Isolates in Los Santos Region of Costa Rica

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SUMMARY

Climatic variations experienced by the coffee culture of Costa Rica in recent years have also modified the typical progress of some of the major diseases affecting this crop. Therefore, it became necessary to increase knowledge about the aggressiveness of the pathogen *Phoma sp.* and *Colletotrichum sp*; in addition to evaluate the response to different chemical control options available.

During the year 2013 was collected in the field, leaves with symptoms of Derrite and Anthracnose diseases. In the laboratory of plant pathology of CICAFE, the pathogens of the isolations were identified using molecular and morphological techniques. A sample of 10 isolates of *Phoma* and 10 isolates of *Colletotrichum*, were evaluated by the amount of infection, the lesion area, the amount of reproductive structures, the incubation period and the latency period. Moreover, the biological efficacy of various fungicides under laboratory conditions at 8, 15, 22 and 30 days after application of the treatments in the field was determined.

The frequency of the Aggressiveness Index in populations of *Phoma costarricensis* and *Colletotrichum acutatum* showed a normal distribution; with individuals with aggressiveness between 22 and 35% higher than the average. Best efficacy to control these pathogens was obtained with fungicides of Triazol group in mixture with fungicides of Strobilurin group. Also discusses relationships of height and climate associated with the aggressiveness of each pathogen in the study region

INTRODUCTION

In Costa Rica the leaf spot caused by the fungus *Phoma costarricensis* is kwon as "derrite or quema". Disease causes problems especially in growing areas located about 1400 meters above sea level. However, the disease also emerges in places where prevail favorable climatic conditions such as heavy condensation or frequent rain, moderate winds, low luminosity and average temperatures around 20°C (Echandi 1957). On the other hand, Anthracnose disease on coffee leaves and fruits is associated with nutrition problems; and in the tropics the disease is favored by changes in weather.

In recent years, there has been an increased occurrence of these diseases, even causing unusual damage, such as in the flowering times. Because of large variation in climate occurred in the last years, it was necessary to determine the progress curve of these diseases, the aggressiveness of their population and the effectiveness of fungicides for control (ICAFE, 2011).

MATERIALS AND METHODS

Pathogens Isolation

- Leaves with symptoms of both diseases were collected in 10 plantations localized at 1550 meters above sea level in the towns of Dota, Tarrazú and León Cortes, in 2013.
- 2 sections of diseased tissue of 0,5cm size were grown in PDA medium at 21-23 °C in the dark.
- Finally 10 isolates of each pathogen were selected and were identified by PCR method.

Aggressiveness determination

- Each isolate was cultured in vitro for 2 weeks. 2mm sections of medium with the fungus were placed on 6 healthy leaves without fungicides application, 4 segments per leaf were inoculated.
- Every 7 days were evaluated the amount of lesions (IF), lesion size (AL), reproductive structures (ER), incubation period (PI) and the latency period (PL).
- The aggressiveness index (AI) was calculated by the formula: $$IA = \frac{(\mathrm{IF} * \mathrm{AL} * \mathrm{ER})}{(\mathrm{PI} * \mathrm{PL})}$$

Biological efficacy of fungicides

- The study consisted of 8 treatments of contact fungicides, systemic and translaminar, which were applied on 3 plants 3 years old of the Caturra variety each .
- Leaves of the second node in branches of the middle stratum of plants were taken 8, 15, 22 and 30 days after treatment application. In the laboratory, 6 leaves of each treatment, were inoculated with 4 disc of PDA with *Phoma* and were incubated in humid chambers at 21 °C and photoperiod of 12 h.
- Also green fruits were collected and infected with *Colletotrichum*-PDA discs in humid chambers at 23 ºC the same way.

Disease progress curves

- Monitoring was performed in 10 plants (one plagiotropic branch per plant) located in Dota, Tarrazú and León Cortes cities in 2013.

RESULTS

Results so far, indicate that Derrite disease shows an annual average of severity of a 3% nationally; nevertheless the disease increases by 20% in August, November, December and January. Whereas in September and October, the largest increase occurs, up to 90%, corresponding to the months with greater frequency and intensity in rainfall, minor luminosity and temperature. On the other hand Anthracnose disease presents an annual infection rate of 4 %, with the greater increase in September (up to 100 %) and remains a moderate infection level from July to December.

The study of aggressiveness, made during the 2013, in the regions of León Cortes, Tarrazú and Dota, determined that 17% of the population of *Colletotrichum acutatum* is 35% more aggressive than the average of the population; whereas a 30% of *Phoma costaricensis* population are 22% more aggressive than the average of the population. The greater aggressiveness of some of isolates is due to its capacity to form more reproductive structures

more in less time and the development of larger lesions. The first signs of the Antracnose infection are observed after 9 days, whereas in Derrite after 4 days.

The study on the biological effectiveness of different fungicides, indicate that the best control of both diseases obtains when using fungicides of Triazol group in mixture with fungicides of Strobilurin group. A second group, with average effectiveness against pathogens was composed of fungicides with the molecules: Cyproconazol, Epoxiconazole and Carbendacin, alone or mixed. Finally, in a third position, and with less effectiveness, the treatment with the protective type fungicides (copper and ziram), with similar incidence that the treatment without fungicide application.

CONCLUSION

The greatest increase of Derrite and Anthracnose diseases in Los Santos Region during 2013, corresponding to the months of higher frequency and intensity of rainfall, lower brightness and temperature.

Morphological and molecular identification of isolates recovered found that correspond with pathogens *Phoma costaricensis* and *Colletotrichum acutatum*.

The evaluation of 10 isolates from Los Santos Region, found that 17% of the sample of *Colletotrichum acutatum* was 35% more aggressive than the average population; while 30 % sample of *Phoma costarricensis*, was 22% more aggressive of average population.

Fungicides with greater control efficacy against the tested pathogens combine two modes of action: inhibition of ergosterol synthesis and mitochondrial respiration. The least amount of Derrite and Anthracnose diseases, evaluated in this study with the fungicides evaluated; was obtained with the fungicidal mixture: Pyraclostrobin - Epoxiconazole and Cyproconazol - Azocystrobin.

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Epidemiological Surveillance System for Coffee Rust Disease (*Hemileia vastatrix*) in Mexico: a Regional Approach

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SUMMARY

The Mexican approach to face the recent coffee rust epidemic outbreak was to establish a Regional Surveillance System operated by specifically trained personal at state level and centrally coordinated by phytosanitary officials. The System integrates in a web platform field monitoring and restricted-moving sampling with spatial and temporal data analysis based on simple algorithms. In 342 sites, a total of 12 variables are weekly/biweekly measured related to disease intensity, plant phenology and clime. Upon these variables, 12 indexes are estimated to define early warnings at county level. Restricted spatial interpolations to coffee growing sub-regions and four related indexes are also generated to identify risk-management areas. In addition to the official scheme, spore trapping and disease assessment are also conducted in selected areas using a research approach for purposes of data validation and forecasting modeling. The web platform allows certified official to generated customized graphics at all time to establish the epidemic status at state, county and site level. In addition, indexes per county are weakly estimated to alert officials and state policy makers. In 2014, nine additional coffee pests were included in the surveillance system.

INTRODUCTION

The recent coffee rust epidemic outbreak in Central and South America in 2010-2012 warned coffee producing countries due to the lack of updated scientific information regarding the biology and epidemiology of disease that showed a low prevalence since its arrival in the 80`s in the American continent. One of the countries attempts was to generate and exchange information to set up early warning systems for purposes of effective disease management. This paper describe de surveillance system develop in Mexico to address the status of coffee rust problem for purposes of prevention as well as the regional situation of endemic and quarantine coffee pest. The information presented is focused on *Hemileia vastatrix*.

MATERIALS AND METHODS

The Mexican approach was to establish a Regional Surveillance System operated by specifically trained personal at state level and centrally coordinated by phytosanitary officials (Fig. 1).

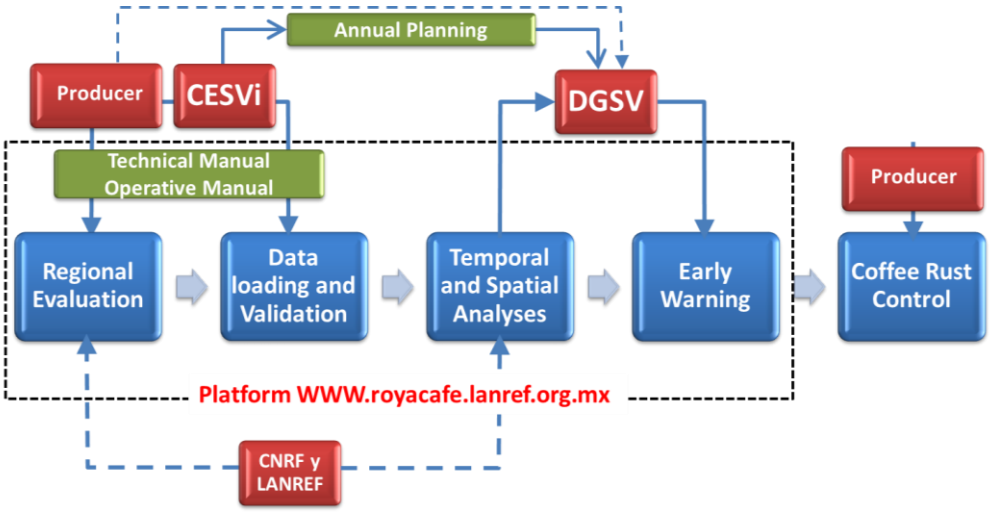

Figure 1. The epidemiological surveillance system model integrates field assessing, analyses and risk management with emphasis on coffee rust.

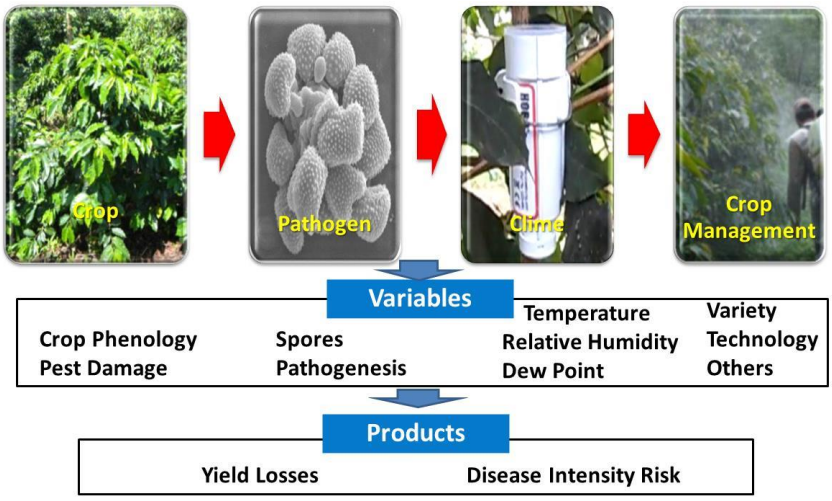

Figure 2. The epidemiological system is the main framework to define the assessed variables and expected surveillance products.

The System integrates in a web platform field monitoring and restricted-moving sampling with spatial and temporal data analysis based on simple algorithms. In 261 sites, a total of 12 variables are weekly/biweekly measured related to disease intensity, plant phenology and clime (Figg. 2, 3). Upon these variables, 12 indexes are estimated to define early warnings at county level. Restricted spatial interpolations to coffee growing sub-regions and four related indexes are also generated to identify risk-management areas. In addition to the official scheme, spore trapping and disease assessment are also conducted in selected areas using a research approach for purposes of data validation and forecasting modeling (1-4,5-7). The overall methodology required to perform the survey is generated by a scientific group (LANREF) and officially validated and released by the phytosanitary division of the Department of agriculture. The required technical information related to the pests as well as protocols, manuals, and capacitation documents are directly placed on the platform public section. Formats, both for field evaluation and for data uploading are also available online in a restricted section especially developed for the surveillance operative purpose (Fig. 4)

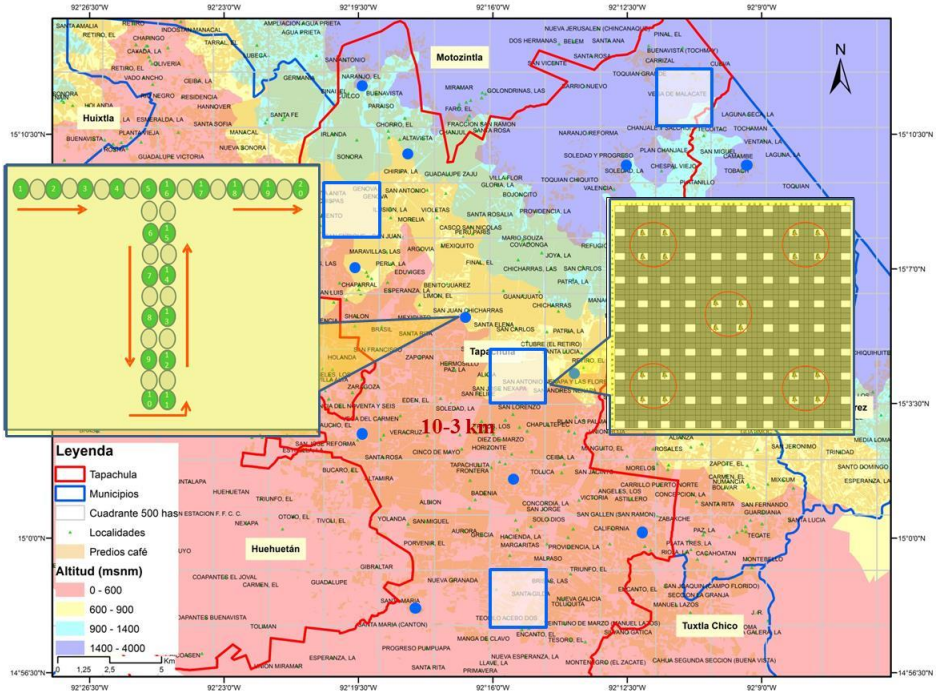

Figure 3. Exemplification of surveillance system at county level (defined by boundaries on red line), of coffee rust and nine coffee pests. One hundred and 20 plants are selected on quadrats of 500has for fixed monitoring (☐) and restricted mobile sampling (●). Inserted squares indicates transect (T), row (☐) and cluster (○) systematic sampling. The total number of sampling-monitoring sites per county and state is fixed through the year but varies among then depending on weighted epidemic factors. This type of maps are loaded weekly to the web platform directly from the planning staff to meet the survey requirements.

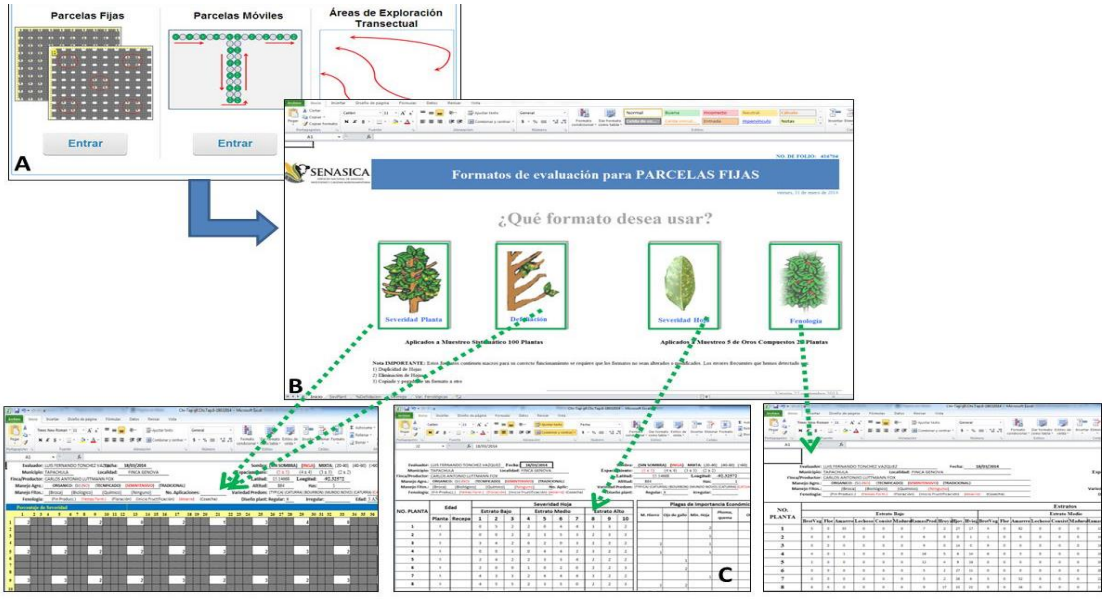

Figure 4. Selected screen captures of the surveillance web platform used by field evaluators accessed through the private interface. A. Visual representation of the sampling-monitoring menu used at regional level. B. Evaluation format menu specific to the disease and phenology variables. C. Macro MS Excel formats loaded with a single field data ready to generate resident graphics and to be online uploaded to the platform.

RESULTS AND DISCUSSION

The web platform allows to the public access technical and risk information on coffee pest currently under surveillance in Mexico (Fig. 5). In addition, in a private platform section, certified official are able to generate customized graphics at all time to establish the epidemic status at state, county and site level (Fig. 6).

Figure 5. Public interface of the surveillance web platform (www.royacafe.lanref. org.mx). A. User friendly menu for customizing maps at state, county and national level over a period of interest to show surveying sites. B. Example of a user generated map for Chiapas State showing mobile sampling sites (blue dots), monitoring sites (red dots) and transect sites (orange dots) in the 25August2013-18July2014 period. C. Risk communication section with information and documents ready to be downloaded. D. Menu for freely technical information handed in manuals, videos and presentations.

Temporal-spatial maps at state level can also be found at the platform with the aim of represent the regional rust status. Identify early foci is the main objective for early warning. In addition, an epidemiological index per county are weakly estimated to alert officials and state policy makers. This index is obtained base on disease, crop phenology and inoculum estimations which are in turn used estimate indexes. The Epidemic index is relatively categorized per state in a red, yellow and green color, similar to the traffic light, to associate with high, moderate and low regional risk. Monthly epidemiological reports are generated and posted on the official web for public awareness. In addition to that, special risk communication to the production sector of any particular region can take place to activate a disease management program. Since the system started on august 2013 in Chiapas and Veracruz, the larger coffee producer estates, a total of 11571 field evaluations have been performed totalizing 173565 disease/plant data entries and 1151280 temperature and RH observations by the end of March.

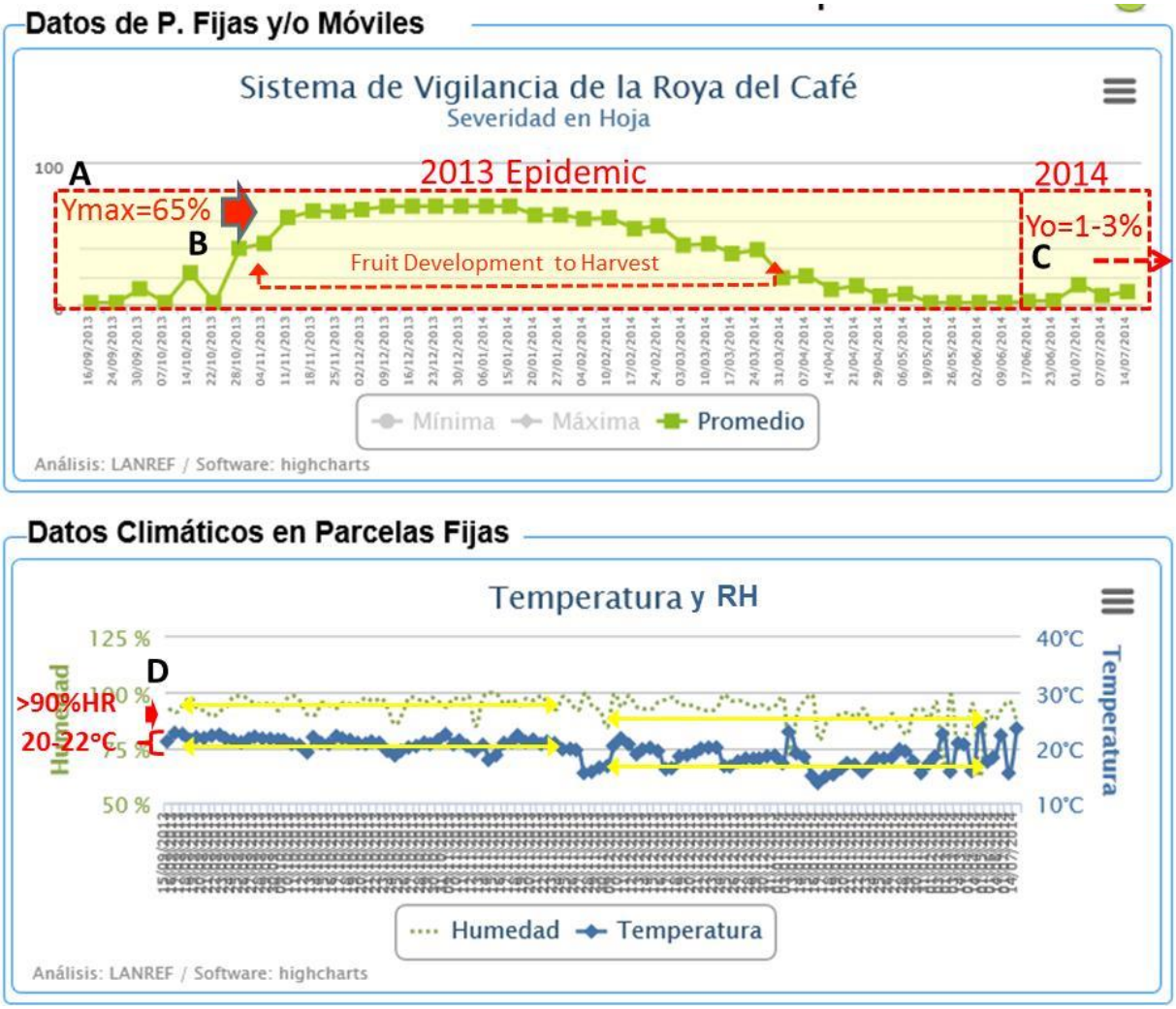

Figure 6. Temporal module to generate customized plots of disease and crop phenology it the private interface of the surveillance web platform (www.royacafe.lanref.org.mx). A. Example of the 2013 disease progress curve of coffee rust base on leaf severity (Y) and the initial epidemic onset for 2014 at San Juan Cancuc County. B. 2013 epidemic onset in relation to fruit development and the Ymax-value. C. 2014 epidemic onset and Yo-value. D. Weather data showing the steady temperature and relative humidity close to the inductive conditions to spore germination at the beginning of the assessment period.

Base on the data platform, in March 2013, Chiapas and Veracruz exhibit 5.5% and 4.3% leaf severity with 33% and 15% of defoliation, respectively. On these states, the maximum severity in some fields reached an average of 70% and 48% on early autumn. In Chiapas, the most affected state, yield loss estimation at county level ranged from 0.5% to 33%. Analysis of residual inoculum, new foliar tissue and inductive weather are fundamental to prevent counties for the new epidemic season. In 2014, Puebla, another large coffee producer state, was included on the System. In addition, using enhanced sampling-monitoring protocols, six additional pests were added for surveillance purposes: three endemic pests and three quarantine pest non-present in Mexico. This new approach has the aim to optimize the System evolving onto an integrated phytosanitary model to effectively contribute on crop sustainability.

The surveillance system also requires the investigation of some aspects that may enhance the data analyses and the comprehension of the pathosystem for purposes of management.

Regarding the coffee rust, information on the pathogenicity process (4,6) spore dispersion (2,5,8) and the implication on the epidemic are fundamental (1,7).

The Coffer Rust System can be accessed on www.royacafe.lanref.org.mx/index.php.

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Beverage Quality and Biochemical Components of Shaded Coffee

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SUMMARY

Coffee beverage quality is an important sensory attribute and is used as a measure for price determination. A study was conducted to investigate the effect of shading levels on biochemical components and beverage quality of coffee variety, K7. The trials were set up at the Coffee Research Foundation (CRF) demonstration farm in Namwela, Bungoma County. The results showed that shade levels significantly affected the bean size and biochemical components of the coffee. Shaded coffee had significantly higher levels oil, caffeine while coffee in full sun had significantly higher levels of trigonelline and sucrose. Generally, shaded coffee recorded higher acidity and balance. The results indicate that shade has potential to improve coffee quality.

INTRODUCTION

Coffee beverage quality often referred to as cup or liquor quality is an important attribute of coffee (Muschler, 2001; Agwanda *et al*, 2003) and is used as a measure for price determination. Though quality is an inherent factor, environment and genetic diversity can play the major roles in determining the coffee physical, organoleptic and bean biochemical quality attributes expression (Leroy *et al*., 2006). The beverage quality is based on the characterization of numerous factors including taste and aroma (Kathurima *et al*., 2009) which are related to biochemical contents of roasted beans whose presence could be favourable or unfavourable (Clifford, 1985). Several authors have reported the positive effect of shade on coffee quality in terms of bean size and biochemical composition (Geromel *et al.* 2008; Vaast *et al*., 2006; Muschler, 2004). Shading led to an increase in size and improvement in the biochemical composition of the coffee bean through delayed ripening by one month (Vaast *et al*., 2006). It has also been noted that shade reduces the portion of rejects which include diseased, mummified or dried berries. In Costa Rica, Muschler, (1998) reported that rejects accounted for up to 10% in the un-shaded samples and less than 1% under shade. Kathurima *et al*., (2012) observed no clear gain on the sensory quality parameters due to shade but the contribution of the shade to the increased premium grades (AA and AB) which are highly valued in the coffee trade in Kenya was significant.

In Kenya, however, there is little information on the effect of shade on quality of Kenyan local coffee varieties.

MATERIALS AND METHODS

Study site

This study was conducted at the CRF demonstration plot in Namwela. Namwela is located in Bungoma County at 0o 45'43N 34o 33'42E, 1641 metres above sea level, an average rainfall of 1329 mm. The experimental design was a randomized complete block. The different shading levels (per cent) were based on the distances from the shade tree trunk: 0 – 1.5 m (80%), 1.5 – 3 m (70%), 3 – 4.5 m (50%), 4.5 – 6 m (30%) and full sun (0%). The shade measurements were taken using a Line Quatum sensor (LI-COR, USA).

Biochemical and Beverage quality

The coffee samples for analysis were taken from composite of fully ripe cherries from four trees in each of the five treatments. The samples were processed using the wet method (pulping, fermentation, drying and de-husking). Seven sensory variables namely; fragrance/aroma, flavour, aftertaste, acidity, body, balance and overall were evaluated and scored together with three process control variables (uniformity, clean cup and sweetness) by a panel of seven trained cuppers on a 10-point scale. Caffeine, oil, trigonelline, total chlorogenic acids (CGA), and sucrose were analyzed in green coffee samples using specific methodologies and quantified on percent dry weight basis. The sensory and biochemical data obtained were subjected to analysis of variance using Costat version 6.400 (1998-2008, Co Hort Software) statistical program.

RESULTS AND DISCUSSION

Analysis of variance showed that all biochemical components, except chlorogenic acid, were significantly ($p<0.05$) affected by shade levels (Table 1). The contents of trigonelline and sucrose were significantly higher in full sun while oil and caffeine content were significantly higher in the shaded coffee (Table 1). This partly agrees with Morais et al., (2006) who found that shade also enhanced the chlorogenic acid content. The positive effect of shade on biochemical components that enhance quality has been attributed to the larger bean size brought about by delayed ripening and hence better bean filling (Bote and Struik, 2011; Vaast et al., 2007 and Morais et al., 2006). The shade levels had a significant ($p<0.05$) effect on acidity and balance (Table 2). Similar results have been reported by Vaast et al., (2007) who further observed that overall preference for coffee grown under shade was significantly ($p<0.05$) higher than that under full sun. The rest of the sensory attributes were largely unaffected by the treatments (Table 2). These results, though preliminary indicate the potential of shade in enhancing the quality of coffee.

Table 1. Mean biochemical components (% dry weight basis) analyzed in green coffee under different shade levels.

Shade level – Distance from tree (m)	Biochemical components				
	Oil	Caffeine	Trigonelline	Sucrose	CGA
0 – 1.5	16.75	1.22	0.77	8.22	5.37
1.5 – 3.0	16.59	1.03	1.01	8.42	6.19
3.0 – 4.5	16.54	0.99	0.93	8.09	6.05
4.5 – 6.0	16.57	0.94	0.94	8.67	6.26
Full sun	16.30	0.87	1.11	8.76	6.28
LSD (Shade level)	0.21	0.05	0.06	0.29	0.40
CV (%)	1.05	3.79	5.44	2.75	5.35

Table 2. Sensory characteristics of coffee under different shade levels

Shade level – Distance from tree (m)	Sensory variables						
	Fragrance	Flavour	After taste	Acidity	Body	Balance	Overall
0 – 1.5	7.55	7.62	7.62	7.77	7.57	7.57	7.54
1.5 – 3.0	7.52	7.62	7.57	7.61	7.60	7.54	7.49
3.0 – 4.5	7.54	7.52	7.54	7.70	7.58	7.56	7.50
4.5 – 6.0	7.57	7.63	7.56	7.79	7.70	7.58	7.61
Full sun	7.56	7.48	7.52	7.56	7.57	7.54	7.54
LSD (Shade level)	NS	NS	NS	0.11	NS	NS	NS
CV (%)	1.85	2.61	2.44	2.38	2.65	1.91	2.26

The sensory characteristics are measured on a scale of 1-10, with 10 being the best score.

CONCLUSION AND RECOMMENDATIONS

These results indicate the potential of shade in enhancing the quality of coffee. However, further studies need to be conducted on appropriate shade tree selection and their management.

ACKNOWLEDGEMENTS

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Global Warming: Empirical Evidence from Eastern Africa and Its Implications on Coffee Production

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SUMMARY

Over the last decade, phenomenal changes in global weather patterns have been observed. According to Intergovernmental Panel on Climate Change (IPPC), the projected global average surface temperature would increase by between 1.4 – 5.8°C over the period 1990 – 2100.Temperatures have been assumed to be increasing in the major coffee growing areas. Empirical data from the coffee growing areas have been analysed and confirmed the trend. These increases are bound to have a significant effect on coffee production.

Arabica coffee *Coffea arabica* originated from the Ethiopian highlands where it grows naturally as an under storey plant. It therefore developed under shade conditions with low temperature range. This determines to a greater extent the adaptability, productivity and quality of the crop under commercial conditions. Any change in temperature may have a profound effect on coffee production in terms of field management, diseases and pests processing and quality. Management of global warming phenomenon will determine whether consumers will continue enjoying a cup of good quality coffee or not.

The paper presents and discusses over 45 years' data in some major coffee growing areas of eastern Africa, the implications on coffee production and potential adaptation strategies.

INTRODUCTION

Over the last decade, phenomenal changes in global weather patterns have been observed.

According to Intergovernmental Panel on Climate Change (IPPC), the projected global average surface temperature would increase by between 1.4 – 5.8°C over the period 1990 – 2100. Temperatures have already increased by an average of 0.6°C (IPCC, 2001) although higher increases have been observed. Benisten (2007) indicated that the mean temperatures in Basel, Switzerland are 6°C higher than the 1961 – 1990 average, and hotter than any other time since 1901.

Arabica coffee Coffea arabica originated from the Ethiopian highlands where it grows naturally as an under storey plant. It therefore developed under shade conditions with low temperature range. This determines to a greater extent the adaptability, productivity and quality of the crop under commercial conditions. Any increase in temperature may have a profound effect on coffee production.

Coffee is a crucial crop to over 26 countries in Africa and Central America. An increase in temperatures will have detrimental effect on the coffee production in these countries. In Uganda, a warming of only 2°C would massively cut the land suitable for coffee production (IPCC, 2001). In Brazil, if mean temperatures were to increase by 5.8°C the suitable areas for

farming coffee will decrease and even an increase in precipitation may not counter the damage of the increased temperature (IPCC, 2001).

MATERIALS AND METHODS

Coffee Research Foundation has been in existence since 1944. In 1944 a meteorological station was set up at Jacaranda farm Ruiru (1.09^0 S, 36.9^0 E, 1623 m above sea level) and data recording started. Temperature data was has been collected all these years. A 67 year temperature data (1945 – 2013) was compiled and trend analysis done.

RESULTS AND DISCUSSION

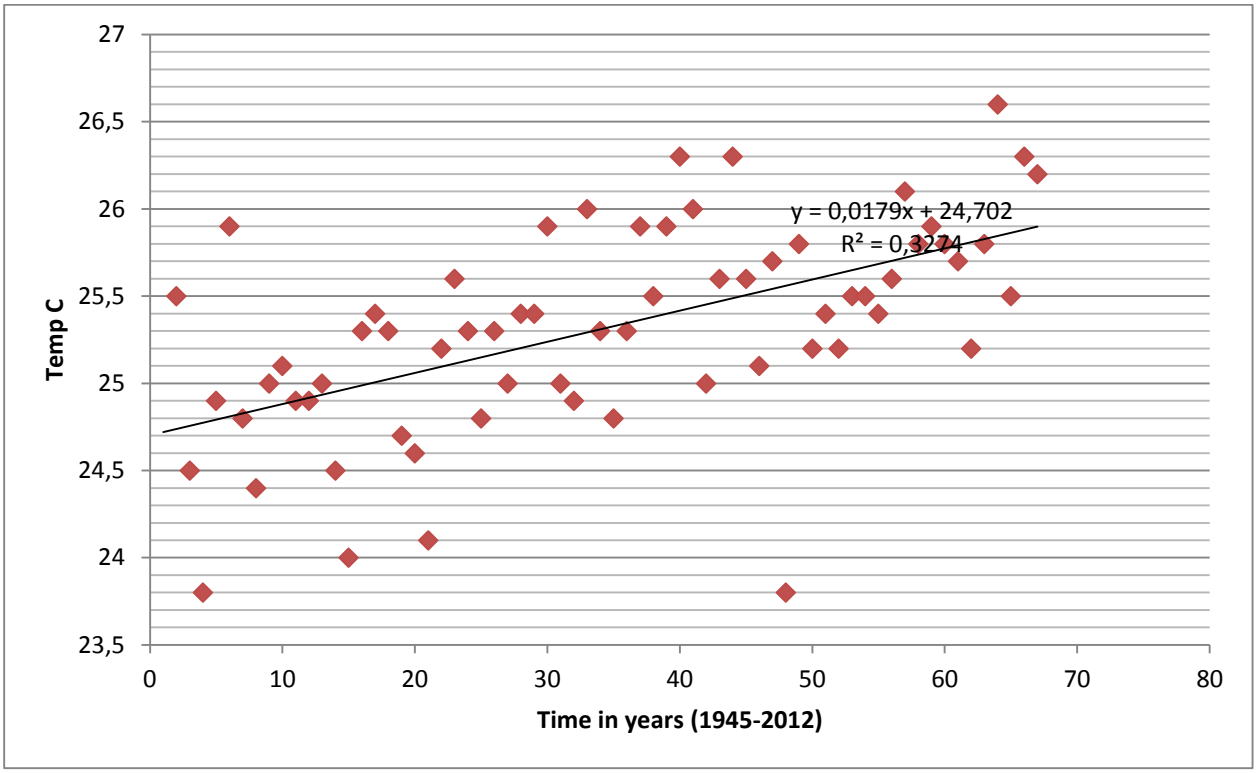

Figure 1. Maximum temperature trend at Ruiru 1945-2012.

The 67 years data indicate very clearly an increase in temperature which averages 0.0179^0 C annually (Fig 1). The maximum mean temperatures has risen from 24.7^0C in 1945 to 25.9^0C in 2012 a mean increase of 1.2^0C. The fluctuations have also been in on the increase. Upto around 1970 the maximum temperature fluctuations were within one standard deviation (+1 SD) above the long term mean. By the year 2000 this has increased to more than two standard deviations above the long term mean (Fig 2).

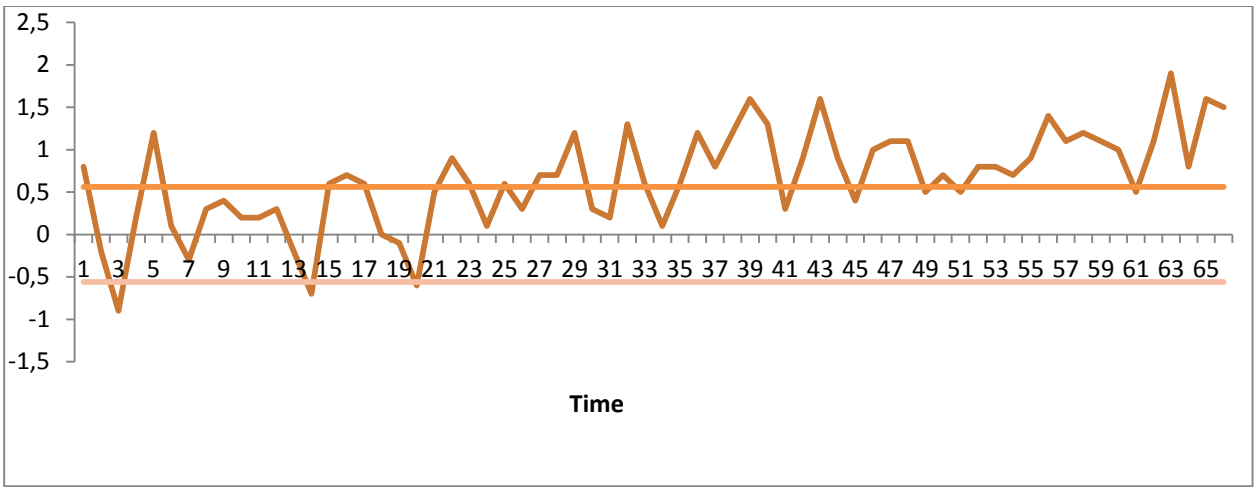

Figure 2. Maximum temperature variations along the long term mean.

From the early 1960s, Kenya has generally experienced increasing temperatures over vast areas. Over inland areas, the trends in both minimum (night-time/early morning) and maximum (daytime) temperatures depict a general warming through time (GoK 2010). The Kenya Meteorological Department has also recorded increase in maximum temperatures as follows: western region 0.5 – 2.1^{0}C; Northern and North Eastern 0.1 – 1.3^{0}C; Central 0.1 - 0.7^{0}C; South Eastern 0.2 -0.6^{0}C and Coast 0.2 – 2.0^{0}C (GoK 2010). This rise in temperatures will have profound effect on the economy. One of the major impacts is a decline in agricultural productivity. This may be attributed to variation in the diurnal temperature range, which has profound effects on agricultural production systems because crops have specific range of temperatures within which they grow optimally. In fact a recent study has estimated that the direct costs of climate change damage in Kenya will potentially amount to between one and two billion US Dollars annually by the year 2030 and considerably greater if indirect costs are included (GoK, 2007).

Arabica coffee does better at a temperature range 15°C – 24°C. Temperatures over 25°C reduce photosynthesis and above 30°C leaf damage occurs (Wilson, 1999). Besides this temperature range, arabica coffee does not tolerate a diurnal range of more than 19°C (Mwangi, 1983).

It has been shown that high leaf temperatures reduce the rate of photosynthesis. Kumar and Tieszen (1980) observed that photosynthetic rates decreased above 25^{0}C.

High soil temperatures may also increase the rate of evaporation from the surface soil layer and the rate of organic matter breakdown. This may lead to poor soil structure and increased susceptibility to erosion (Willey, 1975).

An increase in temperature will also affect the rainfall, both amount and variability. For coffee to flower, it requires a 4 – 6 weeks moisture stress followed by rainfall. An increase in temperature may mean prolonged stress that affects the coffee trees adversely, variable rainfall that will not coincide with the peak moisture demand for coffee resulting in light 'hungry' beans of lower quality, faster breakdown of organic matter and vaporization of the nitrogenous compounds of decomposition resulting in reduced organic matter in the soil.

Coffee is attacked by a number of pests. The behaviour and life cycle of insects is greatly affected by temperatures. A change in temperature would therefore affect the dynamics of

these pests with some increasing in number and voracity, some minor insect pests becoming major while some may be wiped out.

Arabica coffee is attacked by numerous diseases but those of economic importance are Coffee Berry Disease (CBD) caused by *Colletotrichum kahawae* and Coffee Leaf Rust (CLR) caused by *Hemileia vastatrix*. CBD is favoured by high precipitation and is more common in the upper coffee zones. It causes losses of up to 100%. On the other hand, CLR is favoured by high temperatures and is more common in the lower altitude coffee areas. A rise in temperature will therefore increase significantly the leaf rust incidences. Already leaf rust has been observed in the higher altitudes of Kenya where leaf rust was not a problem in the past Kairu (2007). This could be attributed to global warming and the costs of protecting coffee from these two diseases would be very high and may render coffee production uneconomical.

To realize the high quality potential of Arabica coffee, the coffee undergoes wet processing. The coffee is then dried through a strict and controlled process lasting over one month. The coffee should never get into contact with water during this drying period. As already indicated, high temperatures will also result in sporadic and unexpected rains. Rewetting of coffee encourages mould growth rendering the coffee unsuitable for human consumption.

CONCLUSION

There is adequate evidence of global warming in Kenya. This will have profound effect on agricultural systems more so on coffee. It is therefore imperative that clear and specific adaptation strategies are put in place if Kenya has to continue producing and marketing the high quality coffees is known of.

ACKNOWLEDGEMENTS

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Factorial Design of Leaf Spectral Properties of Four *Coffea arabica* Genotypes

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SUMMARY

Measurements of main pigment contents, as chlorophylls and anthocyanin, have many applications in agriculture, ecology, and remote sensing. The aim of this study was to separate the effects of self-shading and water supply by analyzing the spectral data referent to the leaf optical properties of four genotypes of *Coffea arabica*. LICOR 1800 spectral data in the near infrared and visible regions (350 – 1100 nm) were obtained for cv. IAPAR 59, cv. Catuaí IAC 99 and two accesses of the IAPAR Ethiopian collection (entitled E083 and E027). Two level-four factor calculations were carried out for the spectral absorptions at three strategic wavelengths: 520 nm (anthocyanin), 554 nm (maximum variation) and 650 nm (chlorophyll) to differentiate the optical properties of genotype/ water supply/ space orientation/ stratum/ leaf surface. Larger average absorbance values for chlorophyll and the anthocyanin were observed for the adaxial compared with abaxial leaf surfaces in four genotypes. E027 and E083 showed larger quantities of chlorophyll and anthocyanin when plants were irrigated, while the opposite effect in anthocyanins was found in cv. Catuaí 99. North-oriented leaves in Catuaí 99 contained more anthocyanin, although South-oriented ones in E027 contained more chlorophyll. No differentiation was found among the inferior (self-shaded) and superior strata of the four genotypes. Factorial design analysis is seen to be very useful for finding small but significant spectral differences for coffee genotype leaf absorbance between 500 and 700 nm to differentiate the main pigment contents.

INTRODUCTION

Measurements of main pigment contents, as chlorophylls and anthocyanin, have many applications in agriculture, ecology, and remote sensing. The role of the chlorophylls is essentially related to photosynthesis and assimilation. Under water stress, a decrease in chlorophylls content is noticed in many species. Anthocyanin biosynthesis may be even induced by stresses, such as deficiencies in nitrogen and phosphorus, wounding, pathogen infection, desiccation, low temperature, and UV-irradiation. The protective effects of anthocyanins are related to their ability, via screening and/or internal light trapping, to reduce the amount of excessive solar radiation reaching the photosynthetic apparatus. Considering the possibility of non-destructive analysis of pigment content based on spectral data, the aim of this study was to separate the effects of self-shading and water supply by analyzing the spectral data referent to the leaf optical properties of four genotypes of *Coffea arabica*.

MATERIAL AND METHODS

Coffee seedlings of two cultivars (IAPAR 59 and Catuaí IAC 99) and two accesses of the IAPAR Ethiopia collection (entitled E083 and E027) were planted in 2009, in the IAPAR experimental fields, in Londrina (-23° 18' 37" S, 51° 09' 46" W, 585 m of altitude), Brazil. Plants were cultivated under two water supply regimes – field conditions and irrigation.

Measurements of leaf optical absorbance were performed with the LI-COR 1800 spectroradiometer with 2 nm resolution . Spectral data in the near infrared and visible regions (350 – 1100 nm) were obtained for leaves collected from coffee plants of the four genotypes under two water supply regimes, considering their position on the plant (upper and lower layers), their cardinal orientation (North and South) and leaf surfaces (abaxial and adaxial). Leaves were situated at the 4^{th} or 5^{th} metamer counting from the tip of the 2^{nd} order axes. Three plants (replicates) were observed, in August 2012, for each genotype and water regime.

Two methods were applied to differentiate the optical properties of genotype/ treatment/ orientation/ stratum/ leaf face - principal component analysis (PCA) and 2^4 factorial design calculations. The second method was carried out for the spectral absorptions at three strategic wavelengths for coffee leaves: 520 nm (anthocyanin), 554 nm (maximum variation that also could include anthocyanins) and 650 nm (chlorophyll). The PCA matrix was pretreated by finding common base lines for the spectra (software Origin) and preprocessed by centering on the average, normalizing to unit area or uniform vector length or taking the second derivative.

RESULTS AND DISCUSSION

The PCA permitted only clear separation between the adaxial and abaxial faces. Figure 1A contains the PCA results for the raw (unprocessed) spectral data for the IAPAR 59. The discrimination between the adaxial and abaxial surfaces was very clear, however no discrimination could be found for the irrigated *versus* field conditions, the superior *versus* inferior stratum and different cardinal orientations. PCA performed on all the above types of preprocessing also discriminated only between adaxial and abaxial surfaces. Figure 1B shows the corresponding spectra where the adaxial results had higher absorbance values than the abaxial ones, but with no differentiation in pigment absorbance between the irrigated and field grown plants.

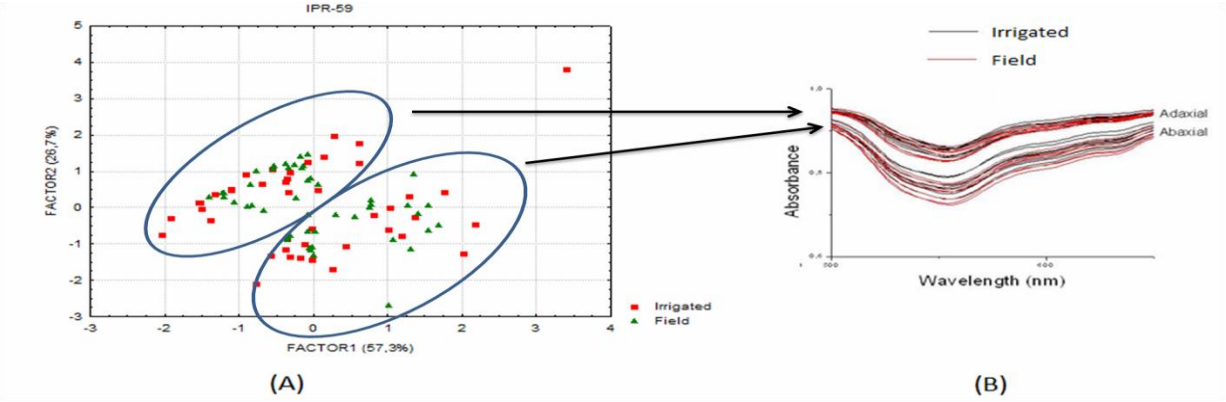

Figure 1. A) PCA score plot containing 84.0 % of the total data variance showing discrimination between spectra obtained on the adaxial and abaxial faces of the coffee leaves and B) the corresponding spectra between 500 and 650 nm of irrigated and field grown plants.

Four factorial design main effect values were calculated for level changes of each factor: 1) non-irrigated (-) to irrigated (+), 2) inferior (-) to superior (+) strata, 3) South (-) to North (+) and 4) adaxial (-) to abaxial (+) faces. Another set of effect values was calculated changing the levels of the third factor from East (-) to West (+). Table 1 contains the principal effect values and their significance levels for the four coffee genotypes. An analogous table (not presented) was compiled changing the cardinal space orientation 'North-South' factor to 'East-West'.

Table 1. Effect values and p-values for the 2^4 factorial designs for spectral data analysis of leaf absorbance at three strategic wavelengths.

Wavelength	520 nm (anthocyanin)		554 nm (maximum)		650 nm (chlorophyll)	
Factors	Effect	p-values	Effect	p-values	Effect	p-values
IAPAR 59						
Irrigation	-0.0072	0.2993	0.0106	0.3450	-0.0043	0.6134
Stratum	0.0012	0.8628	0.0059	0.6038	0.0046	0.5925
Orientation	0.0016	0.8121	0.0034	0.7633	0.0006	0.9451
Surface	**-0.0415**	**<0.0001**	**-0.0703**	**<0.0001**	**-0.0371**	**0.0001**
Catuaí IAC 99						
Irrigation	**-0.0093**	**0.0215**	**-0.0168**	**0.0065**	**0.0030**	**0.0138**
Stratum	0.0067	0.0916	0.0087	0.1418	-0.0002	0.8416
Orientation	**0.0116**	**0.0049**	**0.0170**	**0.0061**	0.0017	0.1450
Surface	**-0.0504**	**<0.0001**	**-0.0741**	**<0.0001**	**-0.0170**	**<0.0001**
E027						
Irrigation	**0.0135**	**0.0012**	**0.0243**	**0.0014**	**0.0057**	**0.0411**
Stratum	0.0046	0.2312	0.0035	0.6182	0.0005	0.8603
Orientation	-0.0049	0.2032	**-0.0194**	**0.0086**	**-0.0063**	**0.0238**
Surface	**-0.0518**	**<0.0001**	**-0.0832**	**<0.0001**	**-0.0249**	**<0.0001**
E083						
Irrigation	**0.0233**	**0.0002**	**0.0547**	**0.0007**	**0.0303**	**0.0017**
Stratum	0.0052	0.3501	0.0055	0.7055	0.0015	0.8678
Orientation	0.0056	0.3124	0.0079	0.5874	0.0045	0.6095
Surface	**-0.0381**	**<0.0001**	**-0.0622**	**0.0002**	**-0.0254**	**0.0071**

Significant effects at the 95% confidence level are in bold type. Significance levels are indicated as p-values.· Critical t-value at the 95% confidence level for 32 degrees of freedom was 2.04.

The adaxial/ abaxial factor levels for the cv. IAPAR-59 presented spectral differences as indicated by their 95% confidence level significant effect values (Table 1). Significant negative effect values at all three wavelengths with an average of –0.048±0.007 were calculated for the effect of changing from the adaxial to the abaxial surface of the leaf. This indicates larger average absorbance values for chlorophyll and the anthocyanin occurred for the adaxial side of the leaf. This was true for all studied genotypes. Anthocyanins in leaves are localized in vacuoles of epidermal cells or those just below adaxial epidermis, but occasionally, also in the cells of abaxial epidermis and the palisade and spongy mesophyll. This fact, together with knowledge that chlorophyll palisade rich cells are localized in the upper part (adaxial surface) of C_3 species leaves, could explain this strong differentiation between the abaxial and adaxial leaf surfaces.

The irrigation effect was significant for the Catuaí 99, E027 and E083 genotypes (Table 1). Significant positive irrigation effects were calculated for the E027 and E083 coffee genotypes (averaging 0.0250±0.007) indicating larger quantities of chlorophyll and anthocyanin in leaves when plants were irrigated. Although the differences between irrigated and non-

irrigated spectra were much smaller compared to differences between the leaf surfaces, there was a clear tendency for the spectra of the irrigated leaves to fall above the non-irrigated ones for E083 and E027. In a case of the two accesses of the Ethiopian collection this response indicates more capacity for assimilation in more favorable water supply conditions.

When leaf spectrum responses of cv. Catuaí 99 were analyzed, negative significant effects were noted for anthocyanins, but positive ones for chlorophylls (Table 1). In this cultivar, it is clear that higher quantities of anthocyanins were synthetized in leaves of unirrigated plants as the response to abiotic stress, where anthocyanins perform their protective role under less favorable conditions [8], while the higher chlorophyll content in leaves of irrigated plants reflects their increased capacity for photosynthetic activity under more favorable conditions. Comparing the two coffee cultivars, the lack of differentiation in pigment content under water stress in IAPAR 59 (Figure 1 and Table 1) could indicate its lower sensibility to water fluctuations compared to Catuaí 99, that has also been indicated by other ecophysiological parameters, such as stomatal conductance, transpiration or/and water potential components [9].

The coffee plants were planted in East-West lines. Northern-oriented leaves in Catuaí 99 contained more anthocyanin, although Southern-oriented ones in E027 contained more chlorophyll, which calls for more comparative analyses for obtaining plausible biological or agronomical explanations. No differentiation was found for inferior (self-shaded) and superior strata.

Factorial design analysis has been very useful for finding small but significant spectral differences for coffee genotype leaf absorbance between 500 and 700 nm to differentiate the main pigment contents.

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Diagnostics, Identification and Control of Coffee Root Mealybugs in Colombia

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SUMMARY

In the last years, in Colombia, there has been an increase of Coffee Root Mealybug populations causing damage to coffee crops. Root Mealybugs are affecting about 50% of new coffee plantations and there are about 23 species identified so far; however, *Puto barberi* (Cockerell) is the predominant species that causes the most severe damage.

INTRODUCTION

Coffee Root Mealybugs are the second most important coffee pest in Colombia, affecting coffee plantations during renovation. Root Mealybugs are a group of insect pests that attack roots, sucking sap and delaying plant growth, which also favors fungal pathogen entrance that ultimate would kill the plants. Villegas and Benavides (2011) conducted a recognition of species of Coffee Root Mealybugs in Colombia and reported the genera *Puto*, *Neochavesia*, *Dysmicoccus*, *Geococcus*, *Rhizoecus* and *Pseudococcus*; however, *Puto barberi* (Hemiptera: Putoidae) was recorded in 86% of more than 5,000 evaluated trees, which make this species as the most predominant and devastating in Colombia. The biology of *P. barberi* was assessed in order to propose control strategies and a diagnostics of the current situation in Colombia was measured in two departments through a random sampling.

MATERIALS AND METHODS

Species identification of Coffee Root Mealybugs in Colombia

Sampling root mealybugs were conducted in seven departments of Colombia: Caldas, Cauca, Cundinamarca, Norte de Santander, Risaralda, Santander and Tolima, selecting 30 randomly affected coffee crops and 30 coffee per crop in order to identify the species.

Assessing the life cycle of *Puto barberi* (Cockerell)

A morphological description and the duration of the life stages of *Puto barberi* was carried out in lab conditions.

Diagnostics of Root Mealybugs infested coffee crops from two coffee producing departments in Colombia

In the departments of Valle del Cauca and Norte de Santander, a random sampling of around 100 coffee farms having coffee plantations less than two years old, was achieved in order to determine the percentage of farms with young coffee crops under Root Mealybugs attack. Root Mealybugs species were identified in each affected coffee farm and parasitism, if at all, was recorded.

Validating control strategies in field conditions

More than 120 Root Mealybugs infested coffee crops in Valle del Cauca and Norte de Santander were visited and evaluated in order to propose control strategies.

RESULTS

Species identification of Coffee Root Mealybugs in Colombia

The most important species of coffee root mealybugs in Colombia were found to be: (1) *Puto barberi* (Cockerell) (Fig. 1) the most abundant and limiting species in Colombia, registered in 86% of the total infested evaluated trees (Table 1), which presents a random distribution in the field; (2) *Dysmicoccus texensis* (Tinsley), causing outbreaks on isolated coffee areas and becoming important because are covered by simbiotic fungi which constrain the neck and tree roots, making it difficult to control (Fig. 2). In addition, we report for the first time a scale insect, *Toumeyella coffeae* Kondo 2014, attacking coffee roots in Norte de Santander and Valle del Cauca in Colombia (Fig. 3).

Table 1. Genera of coffee root mealybugs registered in the departments of Caldas, Cauca, Cundinamarca, Norte de Santander, Risaralda, Santander and Tolima (N = 30) and percentage of trees affected by batch (N = 30).

Departments	***Puto***	***Dysmicoccus***	***Neochavesia***	***Geococcus***	***Rhizoecus***	***Pseudococcus***
Caldas	85,29	2,69	3,34	1,02	1,03	0,52
Cauca	100	2,33	0	2	0	0
Cundinamarca	94,86	1,79	0,94	0	0,09	1,52
Norte de S.	93,48	1,83	9,24	0,41	0	2,9
Risaralda	64,64	2,84	23,67	0,74	0,7	9,6
Santander	87,6	1,53	13	0,34	0,28	1,8
Tolima	76,17	2,44	0	7,3	2,5	1,5
Average	**86%**	2,2%	7,17%	1,6%	0,66%	2,54%

Figure 1. Giant mealybug *Puto barberi,* attacking coffee roots.

Figure 2. *Dysmicoccus texensis* and simbiotic fungi.

Figure 3. *Toumeyella coffeae* Kondo (2014) attacking coffee roots.

Assessing the life cycle of *Puto barberi* (Cockerell)

Puto barberi exposes thelytoky parthenogenesis and ovoviviparous behavior. This species completes its life cycle from nymph I to adult in 141 ± 0.99 days (Table 2).

Table 2. Life cycle of *Puto barberi*, in the laboratory (25±2 C° and 70 ±10 % RH) CENICAFE, Colombia.

Developing Stages		Sample size	Duration in days (mean ± S.E)
NYMPH	Nymph I	99	17.8 ± 0.17
	Nymph II	97	24.4 ± 0.77
Nymphal stage duration			42.2 ± 0.46
ADULT	Pre-deposition	40	42.2 ± 2.74
	Nymphs deposition	30	51.4 ± 6.80
	Post-deposition	30	5.3 ± 0.82
Duration adult			98.9 ± 1.61
LIFE CYCLE			141 ± 0.99

Diagnostics of Root Mealybugs infested coffee crops from two coffee producing departments in Colombia

The diagnosis of mealybugs in Colombia determined that 43% of coffee farms in the department of Norte de Santander are infested by coffee root mealybugs (n = 86). Around 15% revealed the presence of the new scale insect species, *Toumeyella coffeae*. For the department of Valle del Cauca, 56.4% of coffee farms were infested by mealybugs (n = 85).

Validating control strategies in field conditions

We have validated control strategies of coffee root mealybugs during nursery and the first 12 months of crop establishment. Thus, destructive seedlings sampling after 1.5 months of planting the emergent seeds must reveal the presence of the insect pest in order to recommend the use of selective insecticides. In planted coffee trees, there should be planted at least 360-400 extra plants per hectare in order to check 30 growing plants monthly. These are used as indicators and will be destroyed instead of the definitive plants. The presence of the insect pest in the roots are a condition to control. In order to control mealybugs on coffee crops, more than 20 pesticides were experimentally tested. We recommend Silex® and Engeo® in nursery, as well as Silex® and Verdadero® in growing coffee plots. Spraying must be performed in moist soil (field capacity).

Faced with the diverse and complex situations that may occur in attacked coffee crops, and the difficulty in decision making to recommend the appropriate control, we designed a table to better assist control strategies.

STATE OF COFFEE CROP	ROOT CONDITION	PHYTOSANITARY CONDITION	COFFEE CROP APPEAREANCE
Nursery **NU**	Straight **STRA**	Infested with Mealybugs **INFE**	Damage (chlorotic – nonproductive – dead) **DA**
Establishment or productive crop **PRO**	Twisted **TWI**	Non-infested with Mealybugs **NON**	Apparently healthy (With production and without chlorosis) **HEAL**
NURSERY NUSTRAINFEDA = Discharge , replace NUSTRAINFEHEAL = Control NUTWI = Discharge, replace NUSTRANON = Ideal state		**ESTABLISHMENT AND PRODUCTION** PROSTRAINFEDA = Discharge , replace PROSTRAINFEHEAL = Control PROTWINFEDA = Discharge , replace PROTWINFEHEAL = Control, harvest, discharge then replace PROSTRANON = Ideal state	

Nursery

Establishment or productive crop

Straight

Twisted

Infested with Mealybugs

Non-infested with Mealybugs

Damage
(chlorotic – nonproductive – dead)

Apparently healthy
(With production and without chlorosis)

During the course of these six year continuous research in lab and field conditions, we report for the first time the presence of newly discovered hymenopteran species parasitoids of *Puto barberi* in Colombia, all from the family Encyrtidae. Therefore, five individuals of *Puto* genus were parasitized by four species, two of which belonged to the genus *Hambletonia* and *Aenasius* closed to *bolowi* (Fig. 5).

Aenasius closed to *bolowi*

Hambletonia sp.

Figure 5. Parasitoids naturally attacking *Puto barberi* in field conditions in Colombia.

CONCLUSION

Puto barberi is the most abundant species of Coffee Root Mealybug and the most limiting insect species in Colombia.

Control strategies were designed in order to face this limiting insect pest during coffee renovations in Colombia.

We report for the first time at least four species of parasitoids of *P. barberi* in Colombia, which states a clear possibility for biological control approaches in the near future.

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Some Aspects of the Ecology of *Hypothenemus Hampei* Related to Climate Variability Scenarios: Dispersal, Colonization and Population Dynamics

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SUMMARY

In order to understand the population dynamics of the Coffee Berry Borer (CBB), *Hypothenemus hampei* (Ferrari), and their behavior in the context of phenological crop in diversed scenarios of climate variability on coffee production, research was carried out to describe the movement of CBB in the field and to compare its population dynamics between shaded and unshaded coffee crops in an area where never before had planted coffee.

INTRODUCTION

The coffee berry borer (CBB), *Hypothenemus hampei* (Coleoptera: Curculionidae), is the most economically important coffee pest worldwide. The biology and population dynamics of this pest is strongly influenced by climatic events like "El Niño" and "La Niña". The occurrence of climate changes is evident from increase in global average temperature, changes in the rainfall pattern and extreme climatic events. This range of variation in temperature can cause adaptive changes in populations and incidence of insect pests between different altitudinal ranges, which can affect the growth and abundance, reproduction, number of generations per year, dispersal patterns, feeding rates and distribution. In order to understand the population dynamics of CBB and their behavior in the context of phenological crop in diversed scenarios of climate variability on coffee production, research was carried out to develop a methodology to describe the movement and population dynamics of CBB in the field.

MATERIALS AND METHODS

In the locality of La Catalina (Risaralda, Colombia) in a plot of 2,353 *Coffea arabica* var. Colombia plants distributed over an area of 6,644 m^2 we evaluated allele variation of a microsatellite marker on polymorphic Colombian CBB populations to describe the movement of this insect for several generations. A release-recapture technique was evaluated under field conditions using the CBB marked population. Furthermore, in the locality of Naranjal (Caldas, Colombia), each month we studied the dynamics of dispersal, density and population growth of CBB during four consecutive years in relation to climate variables between shaded and unshaded coffee crops in an area where never before had planted coffee.

Allele variation estimation of the microsatellite HHK.1.6 in Colombian coffee berry borer populations

The microsatellite molecular marker HHK.1.6 was synthesized, and used for the amplification of over 100 DNA samples from CBB adults from more than 80 municipalities in Colombia. Subsequently, 100 CBB beetles were field collected at "La Catalina" Experimental Station, in order to determine the genotype of the borer beetles there present, so the absence of the marked populations could allow the monitoring of the genetically different individuals released in a mark-release-recapture strategy.

The amplification of the microsatellite HHK. 1.6 loci was performed in a final volume of 20 µl; each reaction contained 4 µl of DNA (approximately 20 ηg), 0.2 µl of the "forward + reverse" (CGGCACGAATAATCCCTAC + CCTGAATTATCGACGTCGG respectively) primers (0.5 µM), 1.6 µl of 0.2 mM dNTP, 4 µl of 1 X buffer, 1.2 µl of 1.5 mM $MgCl_2$, 0.1 µl of Taq DNA polymerase (0.5 U) (Promega) and 8.9 µl of autoclaved MilliQ water.

Design, development and evaluation of a device for releasing coffee berry borer adults

We developed a device to release CBB populations and to ensure that the individuals flied simultaneously. The main components of the device were light, temperature and wind effectors. The device efficiency was evaluated by measuring the average percentage number of borer beetles that flew at different times (8:00 h, 11:00 h, 14:00 h and 17:00 h) after releasing 10 groups containing 100 individuals.

Releasing and recapturing marked coffee berry borer populations

The movement of *H. hampei* was evaluated at different times and distances, after releasing 5,000 adults of a polymorphic population, at 14 hours, in an epicenter of a coffee crop (at within a radius of 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80 m). For the recapture of the released marked borer beetle population, the next day after the release, in each quadrants of the experimental plot, we counted the number of coffee berries bored by the CBB beetles in each tree. The infested berries were then marked using white indelible ink. We repeated the same procedure at 5, 15, 30, 45, 60, 75 and 90 days after the release (sampling times), marking with a different indelible ink color at each time. This bioassay was performed between October 2012 and February 2013. All coffee trees of the experimental plot were harvested so borer beetle infested fruits were removed and the initial population reduced to a minimum. The polymorphic borer beetles used in this study were reared on artificial diet following the methodology described by Portilla, selecting the 5,000 most active females. In order to confirm that the recaptured populations were the marked released, 95 days after, in each quadrant, we collected 100 CBB infested coffee fruits and 20 *H. hampei* individuals as control population. A CBB adult was extracted from each infested coffee berry collected and genomic DNA was isolated. The allele of interest, using the microsatellite molecular marker HHK.1.6, was amplified following the methodology described above so confirmation of the marked population was assessed.

Dispersal, colonization and population growth of CBB on shaded and unshaded coffee crops

Two coffee plots of 7000 trees each (shaded and unshaded) of 120 days old were selected. Each month we studied the dynamics of dispersal, density and population growth of CBB during four consecutive years in relation to climate variables. Every month 30 trees were randomly selected, and in each of them the number of productive branches was recorded; two

branches were randomly selected and the total number of fruits present and fruits bored were counted. With this information the average infestation per tree was estimated. In the trees selected for estimation of infestation 100 bored fruits were collected from the branches and soil. These fruits were dissected to record the number of individuals of CBB at each stage of development. With the record number of states of CBB in the fruits of the tree and on the ground, was estimated the total population, identifying the proportion of them that were in the tree and on the ground. To assess colonization of CBB in the plot, maps were constructed to evaluate each month, the presence or absence of CBB in each tree.

CONCLUSION

Allele variation estimation of the microsatellite HHK.1.6 in Colombian coffee berry borer populations.

The microsatellite molecular marker HHK. 1.6 amplified a band of 175 bp in all samples of the CBB genomic DNA from Colombia, except those collected in the municipality of Guapotá (Santander), which showed genetic differences amplifying an alternative allele of smaller molecular size, with 173 pb. With this evidence, the borer beetles of the Guapotá region were useful as the marked population in a mark-release-recapture strategy.

Design, development and evaluation of a device for releasing coffee berry borer adults

The efficiency of the release device was measured by estimating the average percentage of borer beetles individuals that flew in two seconds in four releasing times. The values obtained were between 88.3 and 89.1%. According to Student's *t* test ($p < 0.05$), the mean values of this variable at the different release times were statistically similar. Thus the environmental conditions implicit at each time did not affect the insect flight. Therefore, we conclude that the device allows the fly of 88.8% of the insects, with a confidence interval of 2.4 and a coefficient of 95%. This device ensures a homogeneous release of CBB adults from a central point, as a basic condition to assess the movement in field conditions.

Releasing and recapturing marked coffee berry borer populations

To confirm that the released marked insects were in fact the target population, we amplified the allele fragment that recognizes the population from Guapotá. The percentage of individuals recaptured containing the 173bp alternative allele on 100 borer beetles in each quadrant of the experimental plot was obtained. The proportion was between 68.1 and 100% across distances; Finally, the mathematical expression that described the movement of CBB in space over time was $\hat{y} = \alpha\beta^{xi}$ being $\hat{Y}$ the average number of borer beetles recaptured per tree, and x the distance in meters. This method will allow to determine the movement of *H. hampei* from different environmental and ecological scenarios.

Estimation of the coffee berry borer flight distance

The flight distance of the CBB was evaluated through the bored coffee berries after the release. It was noted that the CBB flew up to 65 m, recorded in the northern quadrant on the fifth day of the evaluation. Interestingly, borer beetles were observed colonizing coffee fruits after day 5, only in the north quadrant, where the largest recaptures were recorded. The average of recaptured borer beetles per tree at 5 m from the releasing point was about 50% higher than to 10 m.

Dispersal, colonization and population growth of CBB on shaded and unshaded coffee crops

Colonization and dispersal of CBB in coffee fields are very dynamic, occurring in less than six months in an aggregated form from the edges towards the interior of the coffee plots (Figure 4). This last according to the maps of dispersion and the linear relationship between the variance and the mean for the variable percentage of infested fruits with the Taylor´s Power Law for dependence of variance in animal populations. Shade provided conditions for the highest levels of population values recorded of 2,674 CBB individuals per tree, compared to sun exposed that showed values of 1,326 CBB per tree. Most CBB captured with baited traps in a month (23,643 adults) occurred during a climatic event El Niño (mean temp > 21°C) in contrast to La Niña period with 814 adults captured (mean temp < 21°C). The population growth of CBB was related to high temperatures (> 21 ° C), low soil moisture (<30%), low precipitation (<200 mm), water deficit of at least two continuous months and high sunshine (230 h). The results here exposed are allowing to recommend control strategies according to stratification of the Colombian coffee areas for climate zones.

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Value Chain Interventions in the Indonesian Specialty Coffee Sector

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SUMMARY

Indonesia is world's third largest coffee producing country. Therefore, Indonesia has more opportunities to improve the national economy and livelihoods of coffee farmers. However, Indonesia faces many limitations to produce high income and sustained smallholders' livelihood. This has led several actors to conduct interventions on agro commodity products. Value chain intervention becomes a popular rural development strategy applied by various actors, such as international development agencies, private sectors and governments,which majority aims were to improve the development of business and economy and to alleviate poverty in developing countries. This strategy has improved the linkage between smallholder farmers and lead firms. The interventions have increased buyers' involvement in the producing countries including Indonesia. This led the quality improvement and farm-gate price. This research aims to understand the value chain interventions in the Indonesian specialty coffee sector applied by development agencies, private sectors and the government of Indonesia. This study uses scholarly literature analysis and in-depth interviews.

INTRODUCTION

In the agricultural sector, Indonesia plays a significant role as one of the world's largest coffee producing countries. As such, it has more chances to increase the state economy and the livelihoods of smallholders. However, Indonesia faces many restrictions to participate in higher-valued market because of several reasons, such as limited market information and low quality products. These limitations have led many actors including development agencies, the private sector, and the government to conduct intervention programs in the coffee commodity. According to Neilson (2014), the particular development approach being used by donors in the interventions has been influenced by the work of Gereffi and Porter.

The major aims of the value chain interventions were to encourage the development of business and economy and to alleviate poverty. The aim of this study is to understand the value chain interventions in the Indonesian specialty coffee sector that conducted by development agencies, private sector and the government.

DEVELOPMENT AGENCIES AND VALUE CHAIN INTERVENTIONS

Global value chain (GVC) framework has been embraced by many global development agencies because this concept has proven a powerful tool in identifying the limitations on geographic settings and development in economic (Rodrik, 2006).Several global development agencies have supported the Indonesian coffee sector development. According to Neilson (2014),at least ten global development agencies have supported the Indonesian coffee sector's development

In 2000, according to the National Cooperative Business Association (NCBA, 2000), the NCBA provided technical assistances for operating, managing and marketing the processing and exporting of coffee to a local cooperative, KUD Sane in Toraja. This intervention has increased the farm-gate price in Toraja and Enrekang.

In 2005, USAID provided funding as part of its economic rehabilitation programme in Aceh following the Indian Ocean earthquake and Tsunami in December 2004 (Blackmore et al., 2012). De Wolf (2013) argues that the idea behind the establishment of a coffee buying station was to create employment opportunities for the community in Takengon, Aceh. Then, in the same year, the United Nations Development Programme (UNDP) with others supported the development of a Coffee Forum as part of the Emergency Response and Transitional Recovery Program in Aceh. The Coffee Forum initiated activities including facilitation of farmers' cooperatives and linking them to global market; and the formation of a society protecting *Gayo* Coffee (*MasyarakatPerlindungan Kopi Gayo* or MPKG).

AMARTA, the central program of the USAID program, was established uses the terminology 'value chain': "The project will utilize and strengthen the private sector-led agribusiness environment across the coffee, cocoa and horticulture value chains" (Neilson, 2014: 54) to support value chain and encourage economic prospects, particularly in Papua and North Sumatera (De Wolf, 2013).

In recent years, IFC has worked collaboratively with Ecom Agroindustrial Corp Ltd, an international trading company to set up a coffee buying station, provide technical assistance and training to farmers in Northern Sumatera. The development of coffee in this region aimed to encourage smallholder farmers to develop extended supply contracts with Ecom and to encourage them to become involved in certification (IFC, 2012).

The IDH (Sustainable Trade Initiative) partnership with Sara Lee, Nestlé, Tchibo, Mondelez, and others implemented the Sustainable Coffee Program, the aim of which was to increase the volume of sales of sustainable green beans coffee from 8% to 25% by 2015.

Since 2008, Centre for International Agricultural Research (ACIAR) has worked in collaboration with ICCRI and the private sector e.g. Campos, a roaster company in Australia, initiating value chain intervention in Flores and Enrekang, their main aim being to increase the farm-gate price by producing higher quality coffee by encouraging smallholder farmers' to produce pulped natural and fully-washed coffee and by linking coffee organization with international buyer and roaster company from Australia.

VECO has supported the Indonesian coffee sector since 2010, particularly in the Eastern Indonesian such as Flores and Sulawesi. VECO's focus has been on capacity building of coffee farmer organizations and local NGOs. The aims of value chain interventions have been to support sustainable certification and to expand coffee activities in the future (Neilson, 2014).

PRIVATE SECTOR AND VALUE CHAIN INTERVENTIONS

There are several private sectors that involved in value chain interventions in the Indonesian specialty coffee sector, e.g., Nestlé, Toarco Jaya (Key Coffee of Japan) and Ecom. These coffee companies conduct interventions among smallholders in Lampung, Toraja and North Sumatera, respectively. Increased competition among buyers in Indonesia has led the private sectors to conduct VCIs through providing extension services and direct buying. The main

aims of the private sector-led VCIs have been to promote the coffee business by maintaining sustainability and traceability, and to guarantee regular supply in the long term.

Private sector-led intervention is highly likely to shape captive governance along the value chain because producers who have many limitations have to fulfill the buyers' complex demand specifications (Gereffi et al., 2005). The private sector actors commonly collaborate with other actors to provide training and capacity building. The interventions exercised by the private sector bring several benefits to smallholder farmers in the forms of more opportunities to access information and achieve higher prices.

In the specialty coffee market, consumers, particularly in the developing countries have increasingly appreciated high quality coffee and differentiated coffee products, e.g., certified coffee and single origin coffee, factors that have led intermediaries to offer high quality-based premium coffee prices. The quality of coffee is absolute in the international coffee market: premium prices are paid by consumers for high quality and differentiated coffee products; providing more opportunities for producers to improve the quality of their coffee and to increase coffee prices. However, smallholder farmers in coffee producing countries such as Indonesia face several limitations when producing high quality coffee and differentiated products. This has seen the private sector directly engage upstream, i.e., quality, in closer relationships with farmers, certification, supply certainty, sustainability and competitive advantage. The private sector willingly engage with the upstream because it is keen to secure high quality coffee supplies from smallholder farmers. Increased consumer demand for high quality coffee and increased competition among the exporters and international trading companies is linked to the growth and maturity of the global specialty coffee market. Furthermore, the private sector is willing to maintain the sustainability of the coffee business by engaging in closer relationships with smallholder farmers, relationships that will ultimately improve the quality of communication and coordination between the two actors. This enables the farmers to easily understand the buyers' demands; and, the buyers are able to comprehend the challenges faced by the farmers. In coffee producing countries, increased consumers demand for certified products, in a time when smallholder farmers face limitations vis-à-vis arranging certification, has forced the private sector to become directly involved.

THE GOVERNMENT OF INDONESIA AND VALUE CHAIN INTERVENTIONS

The government of Indonesia also play important role in conducting value chain intervention that can be considered as small business development. In 2001, the government of Indonesia's collaborative work with Indonesian Coffee and Cocoa Research Institute (ICCRI) and the private sector designed and initiated value chain interventionprogramin several coffee producing areas especially Arabica coffee producing areas including Kintamani, East Java, Flores and Enrekang.The value chain interventions mainly encouraged smallholder farmers to conduct a combination between product and functional upgrading and to enhance the use of coffee processing machines in order to obtain higher farm-gate price.The product upgrading conducted by improving the quality of coffee through producing full-washed coffee, while functional upgrading conducted by producing green beans coffee instead of parchment coffee. The value chain interventions also proposed to improve the implementation of sustainability coffee for improving farmers' livelihoods; to improve the supply chain's efficiency; to facilitate the private sector to develop agribusiness across the commodity value chain; and, to provide extension services and technical assistance to smallholder farmersfor off-farm management, particularly by diffusing the full-washed coffee processing method (Virgiano, 2012), encouragingfarmer organizations to produce higher qualitycoffee (fully-washed coffee)through locally farmer-managed processing units (UPHs), enhancing the use of processing equipment at the farm-level (pulpers, washers and hullers) and encouraging farmer

organizations to directly trade coffee to both local and internationalexporters or roasters companies.

This intervention provides several benefits for farmers. However, the risk management is frequently ignored by value chain interventions.

CONCLUSION

Rural developments that commonly conducted through the implementation of VCI in the Indonesian specialty coffee sector conducted by various sectors including international development agencies, private sector and the government. Although these VCIs adopted different approaches, includingvalue chain linkage and upgrading, the major aim of the rural development interventions was to improve the smallholders' livelihoods and reduce poverty. However, there has been very little evaluation of the VCIs in Indonesian coffee sector, such that the effectiveness of these interventions is unclear.

The case of Indonesian specialty coffee sector strongly suggest the significant role played by development agencies, private sector and the government of Indonesia. The increasing interest of these actors to implement value chain intervention in the Indonesian specialty coffee sector has increased farmers' opportunities to improve quality coffee and obtain higher farm-gate price.

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Coffea liberica Bull. Ex Hiern: Seed Morphological Observations

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SUMMARY

Coffea liberica Bull. ex Hiern contributing less than one per cent of the marketed coffee, is the third commercially exploited coffee species well known for its larger cherries, when compared with those of *C. arabica* or *C. canephora*. This species, originally found near Monrovia in Liberia, is characterized by tolerance of drought, ability to grow in poor soils and capacity for resisting horticultural treatment, but unfortunately, it is inferior to Arabica as far as beverage quality is concerned. From the taxonomic point of view, all *Liberica* taxa were grouped in one species, *C. liberica* Bull ex Hiern, with two varieties: *liberica* (vernacular name Liberica) and *dewevrei*, (vernacular name Excelsa).

The present study is aimed at characterizing *Liberica* seeds, with emphasis to the silverskin and the endosperm tissue. As far as we know, it is the first time an ultrastructural characterization of *C. liberica* seeds is reported. The microscopic observations carried out by means of optimized histochemical techniques were discussed in comparison with those obtained on Arabica seeds.

Silverskin morphology reveals significant differences between Liberica and Arabica, whereas endosperm tissues does not offer the opportunity to differentiate the two species. Endosperm cells were also morphologically described, with a particular emphasis to oil bodies area. The distribution and the size of oil bodies show a slight difference in *Liberica* varieties.

INTRODUCTION

Liberica coffee are known in the Philippines and in parts of Africa for their large cherries, distinguished from other coffee species for their trees with the large, dark green, leathery leaves. This species is indigenous in the neighbourhood of Monrovia in Liberia, described first by Bull in 1874.

Chevalier defined *C. liberica* seed (13-15 mm in lenght and 8 mm in width) as ellipsoid-flat with a right furrow, from brown to greenish silverskin, very tight to the seed. Recently, from the systematic point of view, all the *Liberica* taxa were assembled in a single species with two varieties: var. *liberica* and var. *dewevrei*. The vernacular names ‘Liberica’ and ‘Liberian’ coffee refer to *C. liberica* var. *liberica* and ‘Excelsa’ to *C. liberica* var. *dewevrei*, but there are numerous intermediate hybrids between C. *liberica* and C. *excelsa*.

The economic importance of *Liberica* varieties is first related as gene sources for disease resistance for arabica coffee, as a carrier of the SH3 gene transferred by hybridization and, secondary, there are several natural hybrids derived of crosses with *C. arabica* and with *C. canephora*.

The aim of this paper is focused to discover possible morphological differences between *Liberica* varieties seeds with the same geographical origin in comparison with Arabica. This work could partially clarify their systematic based on silverskin tissue and increase the information about liberica seeds, a coffee species few studied from now on.

MATERIALS AND METHODS

Seeds of *Coffea excelsa* A. Chev. (synonymous of *Coffea liberica* var. dewevrei) and *Coffea abeokutae* Cramer (synonymous of *Coffea liberica* var. liberica) with the same geographical origin (Karnataka, India) were send to Illycaffé, Biolab (Trieste, Italy).

Measures of length, width and thickness of all samples received were made with a Mitutoyo Absolute Digimatic Caliber (CD 6”CSX, Mitutoyo Corp., Japan).

Portions of silverskin were treated with the basic fuchsin stain (1%, diluted in water) and observed by a standard optical microscope (Leica Leitz DMRXE, Leica microsystems Wetzlar GmbH, Germany). Because of the few quantity of material, 2-3 seeds were put in a fixative solution (formaldehyde 37% in a buffer phosphate pH 7.2) for several days, than samples were cut with a cryostat (Leica 3100 Reichert-Jung, Leica microsystems) at -24°C in transversal section (12-14 µm). Sections were stained with Toluidine blue O solution (TBO) in a buffer phosphate (pH 4.4) and with UV- Schiff method.

Samples for electron microcopy were treated following the protocol of Spurr’s resin. Oil body diameters were measured on each image (from 10 to 16 images per sample type) obtained by TEM at the same magnification (x5600), using the program TESI Imaging µImage (ImageNT ver. 2.0 CASTI Imaging s.r.l., Venice, Italy).

CONCLUSION

C. liberica seeds have a great size variability and generally they are known to be bigger than arabica beans. Liberica beans could reach 14 mm in length versus Arabica that reached maximum 10.5 mm, both Liberica and Arabica seeds maintained the same width (about 7 mm). *C. excelsa* beans are really similar to Arabica.

The characteristic of Liberica silverskins (Fig.1a,b) are summarised in Table1. On the basis of the Chevalier studies, the silverskin observations in coffee species reveal differences in size fibers, end fibers, cell wall punctuations and cell wall thickness. The presence of both, long and short fibers, can distinguish *C. liberica* from *C. arabica* (Fig.1c), characterized by long fibers and thinner cell walls (5-8 µm). Some differences has been observed also intraspecies, between the two liberica varieties: var. *dewevrei* is characterized by a greenish silverskin, long fibers with ovales punctuations and thick cell walls. Var. *liberica* shows a yellowish color and some short fibers with irregular shape. Therefore, the punctuations type and the cell wall thickness seems to be the main morphological characters that could be considered to distinguish var. *dewevrei* from var. *liberica.* Further samples must to be observed to confirm this hypothesis.

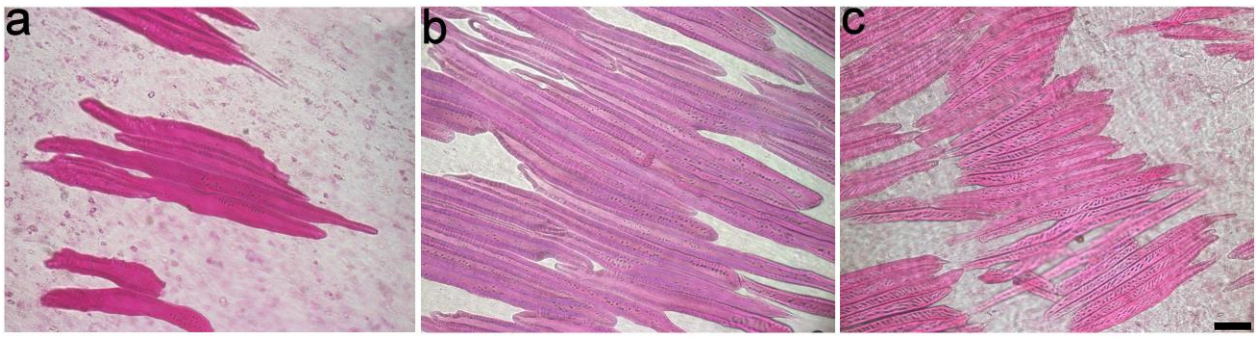

Figure 1. silverskin fibers of var. *liberica* (a), var. *dewevrei* (b) and Arabica seeds (c) (basic fuchsin), bar: 50 µm.

Table 1. silverskin description of *C. liberica* varieties and *C. arabica* seeds; values of average and standard deviations

species	**Color**	**description**	**fibers lenght (µm)**	**fibers end**	**punctuations**	**channels**	**Cell wall thickness (µm)**
var. *liberica*	Yellowish	loosely packed with the predominance of amorphous tissue	long (449 ± 75) short and irregular	rounded or pointed	oval	numerous	10 ± 1
var. *dewevrei*	Greenish	clustered and tightly packed	long (453 ± 119) short (253 ± 34)	rounded or pointed	oval	numerous	10 ± 2
C. arabica	Yellowish	clustered and tightly packed	long (364 ± 67)	rounded or pointed	linear	numerous	7 ± 1

All Liberica samples have a typical coffee endosperm tissue, generally characterized by polygonal cells, with no interspaces between them (Fig.2a). As Arabica, there are cell walls with nodular structures, produced by the secondary wall. The major components of cytoplasm are essentially lipids, proteins (Fig.2b) and carbohydrates. Lipids in Liberica seeds, as other coffee species, are stored in 'oil bodies' (Fig.2c,d), functioning as energy reserve and mainly composed by triacylglicerols, surrounded by a phospholipids monolayer and oleosins. The oleosins quantity could determine the size of oil body.

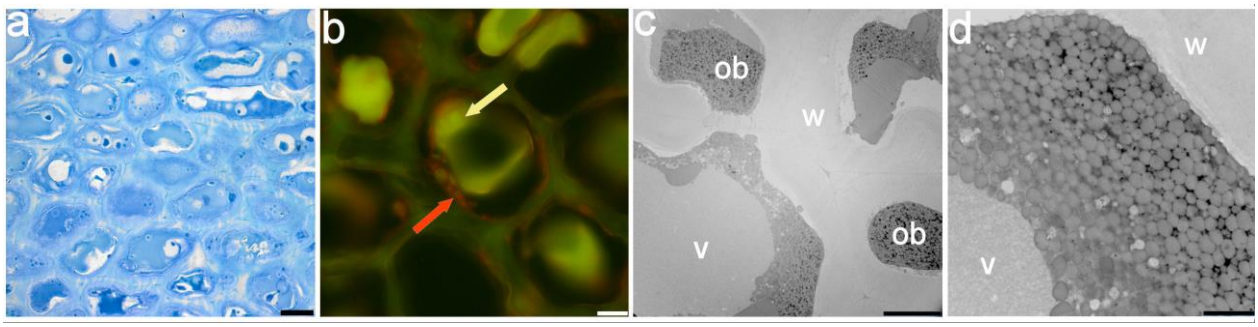

Figure 2. *C. liberica* var. *dewevrei*. a. endosperm cells (TBO, optical microscope), bar: 25 µm; b. lipids (red) and proteins (yellow) inside a cell (UV-schiff, optical microscope), bar: 10 µm; c, d. oil bodies (ob) in endosperm cells, (v) vacuole, (w) cell wall (electronic microscope), bars: 10 and 2 µm, respectively.

Endosperm cells show a lateral oil bodies distribution in a thin layer close to the cell walls (Fig.2d), forming sometimes oil drops. A large presence of whitish vesicles indicate the beginning of lipids digestion. In var. *dewevrei*, the external endosperm cells are strongly

stained, showing dense vacuoles and phenolic substances (Fig.2a), particularly rich in oil bodies. Values of oil bodies diameter of *liberica* samples show a small intraspecies variability, 0.56 ± 0.13 (var. *liberica*) and 0.71 ± 0.12 (var. *dewevrei*), the same range than Arabica seeds.

C. arabica and *C. liberica* with the same origin have no morphological differences in endosperm cells. If we consider that these samples have similar oil content and a different oil body size, we could hypothesize that they have a different expression and quantity of oleosins and so, a different strategy to degrade lipids during the germination stage. It could be interesting to deepen this molecular aspect to validate this hypothesis.

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Advances in Use of Fungicides to Manage Coffee Leaf Rust and Coffee Berry Disease in Kenya

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SUMMARY

Since sulphur was first used as a fungicide to control powdery mildew on grapes, chemical control of plant diseases, including Coffee Leaf Rust (CLR) caused by *Hemileia vastatrix* and Coffee Berry Disease (CBD) caused by *Colletotrichum kahawae*, has proved to be inevitable. Additionally, new races of these diseases have developed overtime thus increasing the need for rapid chemical control as genetic resistance is broken and cultural control rendered less effective. Climatic changes have also affected the effectiveness of spray programmes especially those of protective nature. There have been a lot of advancements in the chemical industry with manufacturers developing new molecules in this dynamic field. This review focuses on the major advances in the type of molecules, formulations, spray intervals, challenges and future perspectives in the control of Coffee berry Disease (CBD) and Coffee Leaf Rust (CLR) with special focus to the Kenyan situation.

INTRODUCTION

Coffee is an important cash crop for both small-scale and large scale farmers in Kenya. Kenya being an agricultural country, coffee plays an important role in income generation, creation of employment opportunities, production of raw materials and earning foreign exchange .The two major diseases that constrain coffee production in Kenya are Coffee Leaf Rust *Hemileia vastatrix* Berkeley and Broome and Coffee Berry Disease (CBD) *Colletotrichum kahawae* Waller and Bridge. Adequate control of both CBD and CLR can be achieved through prompt application of fungicides (Alwora and Gichuru, 2014). However, the cost is prohibitive and most farmers either apply at lower rates, higher intervals or do late applications. This often results to reduced efficacy or no control at all because the success of chemical control depends on the disease pathogenesis stage at the moment when the fungicide is applied (Ricardo, 2010) and in the long run may result to pesticide resistant fungi. Fungicides can be applied either as protective/preventive or curative depending on ability to be translocated into the plant.

Effective management of CBD and CLR is greatly influenced by the type of fungicide applied, rate of application, time of application, equipment used and technical know-how of the applicant. This review therefore seeks to highlight the advances in the type of molecules, formulations, spray intervals, challenges and future perspectives in the control of CBD and CLR in Kenya.

TYPES OF MOLECULES

There has been a slow but gradual change in the type of molecules used for control of CBD and CLR in Kenya. The first molecules to be introduced were inorganic fungicides. Then followed the organic fungicides which are more effective and less toxic than inorganic ones

(Sherman and Scott, 1998). Recently there has been an increase in the number of biological fungicides being presented for the control of CBD and CLR.

Inorganic fungicides

These are fungicides that contain metals such as copper, sulphur or mercury in their chemical structure (McGrath, 2004). The first molecule to be used was Copper as Bordeaux and Burgundy Mixtures way back in the last decade of the 19th century. Three decades later, cuprous oxide (Red copper) was quickly adapted as Peronox. Copper hydroxide (blue copper) and Copper oxychloride (Green copper) were later introduced to manage both CLR and CBD (Okioga, 1978).

Organic fungicides

These are fungicides that contain carbon in their chemical structure (McGrath, 2004). The use of these fungicides began in 1934 and has since played a major role in the world wide control of plant diseases (Sherman and Scott, 1998). The first molecules in this group included carbamates which have been the most important, versatile and widely used fungicides under trade names such as Mancozeb and Maneb. They were followed by Dicarboximides such as Captan, Folpet and Captafol which are wide-spectrum fungicides. Later, new molecules were introduced; Iprodione, Vinclozolin and Chlorothalonil; which have a narrow spectrum and may be prone to development of resistance by the target fungi (Sherman and Scott, 1998). Other molecules introduced later include Carbendazim (Bavistin and Derosal), Folicidin (Cypendazol) and Benlate (Benomyl). The most recently developed organic fungicides used to manage CBD and CLR include Benzimidazoles (Thiabendazole), sterol inhibitors (Triadimefon, Cyproconazole, Hexaconazole and Propiconazole) and the most recently introduced Strobulins (Trifoxystrobin, Pyraclostrobin and Azoxystrobin).These fungicides are absorbed by the plant and carried by translocation to various parts of the plant. Most of them are eradicative.

Combined molecules

There has also been a rise in the number of two fungicide molecules packaged as one. This is a key aspect in reduction of the risk of pathogens developing resistance to the pesticides. Such formulations include Chlorothalonil + copper, Anilazine + copper, Azoxystrobin + chlorothalonil, and the most recent fluopyram+ trifoxystrobin.

FORMULATIONS

This is the form in which the fungicide is presented. A formulation comprises of the active ingredient and the carrier material. Effectiveness of the fungicide, wastage, safety of applicants and the environment, ease of application, handling, storage and type of spray equipment used informs the development of new formulations of new or previously existing fungicides. The first formulation of fungicides was a dry formulation which was then followed by introduction of liquid formulations.

Dry formulations

The first chemical was adapted as Peronox was a wettable powder (WP) formulation of cuprous oxide. Peronox 50WP has since disappeared from the commercial distribution but cuprous oxide is still being used in many formulations such as Nordox 50 WP, Nordox Super 75 WP and Nordox 75 WG (wettable granules). Later there was introduction of dry flowables

(DF) such as Kocide 40 DF. Manufacturers are also addressing the size of the particles by introducing encapsulated fungicides with the aim of reducing the sizes.

Liquid formulations

This formulation was adopted with the introduction of new fungicide molecules such as chlorothalonil, strobulin and benzimidazoles. They are presented as emulsifiable concentrates (EC) such Anvil 5% EC and Cabrio 250 EC, solutions (S), soluble concentrates (SC) and soluble flowables (SF).

SPRAY RATES AND INTERVALS

The change in the molecules and formulations of fungicides has led to significant changes in the rates and interval of application of these fungicides. The first recommended rate for cuprous oxide as a straight application was 7.7kg/ha for control of CBD and 3.8kg/ha for control of CLR. Chlorothalonil was first sprayed at a rate of 4.4l/ha for control of CBD. The introduction of tank mixes led to new rates of (copper + chlorothalonil) 5.5kg/ha + 2.2l/ha in order to reduce the risk of resistance development and control both diseases. Development of newer formulations resulted to lower rates of 2.75kg/ha+ 2.2L/ha of copper + chlorothalonil.
The introduction of new molecules has also led to introduction of much lower rates of application such as 0.33l/ha + 3.3kg/ha (Pyraclostrobin+ copper) and 0.22l/ha + 3.3kg/ha (Azoxystrobin+ copper).

CHALLENGES

Despite the advances highlighted in this review, the Kenyan coffee industry has also had a fair share of challenges in regard to chemical control of CBD and CLR. The major challenge is development of resistance by the target organism. (Okioga, 1976 ; Javed, 1980) found out that certain strains of *Colletotrichum coffeanum* (*now C. kahawae*) in coffee plots sprayed with Carbendazim formulations (Bavistin and Derosal) and with Folicidin (Cypendazol) were resistant to these fungicides as well as Benlate (Benomyl). The isolates retained the resistance even after stoppage of usage of the fungicides and this led to withdrawal of the molecules.
The second challenge is the upsurge of non target organisms on sprayed coffee trees. Studies by Kairu and Muthamia (1990) showed that the use of Chlorothalonil as a straight spray to control CBD caused an upsurge of Bacterial Blight of Coffee (BBC), caused by the bacterium *Pseudomonas syringae* pv *garcae*. It was therefore recommended to be used as a tank mix with copper and Maneb to control both CBD and BBC (Alwora and Gichuru, 2014).
Thirdly, the coffee farmers have to deal with the high costs of the new fungicides. The use of fungicides poses hazard to all living organisms and the environment. Studies have also shown there are traces of copper based fungicides in underground waters in coffee growing areas of Kenya (Loland *et al.,* 2004). In addition, consumers of agricultural products are now more concerned about the quality and safety of what they consume including presence of residues of agrochemicals used in production, processing and preservation of the products. This aspect has led to establishment of Maximum residue limits (MRLs) for all the chemicals used in coffee and other crops.

FUTURE PERSPECTIVES

With the use of fungicides to manage coffee diseases being inevitable, there is a dire need to develop environmentally friendly and sustainable strategies to manage the diseases. Integrated disease management combining cultural, biological and chemical control methods, presents a wholesome strategy that will cater for resistance risks, environmental and health risks,

sustainable production as well as exploration of new market niches such as low pesticide coffee and organic coffee.

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Fruit Load, Yield, Vegetative Growth and Photosynthesis in Coffee (*Coffea arabica* L.)

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SUMMARY

Coffee plants flower heavily resulting in large yields of smaller sized fruits which on the other hand reduces shoot growth and new leaf development. In severe cases of abundant fruit set branches may die and growth of the plant can totally be jeopardized. For understanding processes and conditions determining the balance between fruit load and shoot growth, it is necessary to experimentally determine the relationship between fruit load reduction and shoot growth. In the experiment reported about in this paper, coffee fruits were manually thinned to impose four levels of fruit loads, leaving 100, 75, 50 and 25% of the fruits. The study is done at Jimma, south western Ethiopia and aimed at underpinning possible management interventions promoting yield stability. Data on yield, bean properties, vegetative growth and photosynthetic capacity were taken over two successive years (no fruit load manipulations done in the second year). The results indicated that shoot growth, leaf development, leaf photosynthetic capacity and coffee bean size were inversely associated with percentage fruit load. Coffee bean yield in the first year was increased with fruit load. In the second year, however, yield declined drastically and no yield was harvested from coffee trees with full fruit load in the first experimental year. In coffee trees with higher fruit load this is probably due to strong competition between fruits and vegetative parts so that trees could not conserve nutrients and carbon for the next production season. In the conditions of study maintaining fruit load at the level of 50 % might keep fruit yield stable from year to year and rescues the plant from premature aging. However further study is needed to underpin practical management measures.

INTRODUCTION

The number of flowers per tree influences fruit quality, quantity and stability of production from year to year. In coffee, plants tend to flower heavily resulting in the production of high fruit load without a concomitant balance in vegetative growth leading to a phenomenon called ‘biennial bearing’, characterized by large yields of small sized fruit in “on” years, and low sometimes even no yields in ”off” years. In years of profuse flowering and high fruit loads average bean size is smaller than for trees with lower fruit numbers, growth of new branches is reduced due to competition for growth substrates between fruits and branches. In severe cases of overbearing with fruits, branches may even die (‘die back’). Insufficient production of new branches in a given year results in fewer flowers and fruits, and hence, in reduced bean yield in the following year. We manipulated experimentally fruit load per tree so as to understand processes and conditions determining the balance between fruit load and branch growth. We manipulated fruit load on coffee (*C. arabica*), early after flowering and measured yield, bean properties, vegetative growth and photosynthetic capacity over two successive years (no fruit load manipulation in the second year) to underpin possible management interventions promoting yield stability.

MATERIALS AND METHODS

The study was carried out in two consecutive years (2012/13 and 2013/14 production cycles) on eight-years old Arabica coffee (*C. arabica* L.) trees of cultivar 74-40 at two different sites in Jimma zone, south western Ethiopia. Sixteen coffee plants, arranged in a randomized complete block design, were selected and four fruit loads (full fruit load = 100%, 75, 50 and 25% of full fruit load) were manually imposed in the 'pinhead' stage on all fruit bearing branches of the plants. Data on vegetative growth, gas exchange variables, leaf nitrogen, bean yield and size were collected and effects of these treatments on each of the variable were analysed using SAS statistical software vr. 9.2.

RESULTS AND DISCUSSION

Shoot growth, number of leaves per branch and leaf dry weight per individual leaf of field grown coffee trees were inversely related to fruit load percentage (Figs. 1a - d). The effects were initially large, restraining branch growth between day 100 - 150 after fruit thinning, but later in the season the differences between treatments were preserved (Figs 1 a and b). As compared to growth with 25 % fruit load, branch extension growth of coffee trees with 100 % fruit load was suppressed by about 20 cm, i.e. approximately 20% (Fig. 1a). The development of new leaves decreased as the percentage of fruit load increased from 25 to 100%. Coffee berries are strong sinks and have priority demand for assimilates from branches and adjacent leaves. In heavily loaded trees, this significantly restrained vegetative growth and reduced the number of newly developing leaves and their weight. Removing some fruits probably makes additional resources available to individual organs and thus reduces competition among them. This helps the tree develop additional leaves and increase partitioning of photo-assimilates toward them.

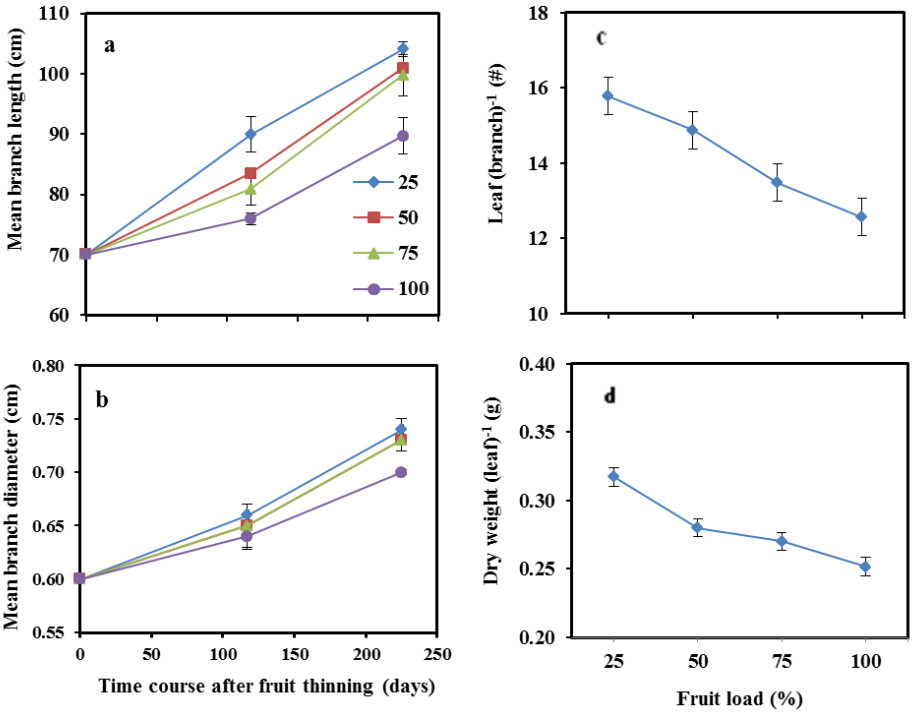

Fig. 1. Effect of fruit load on branch length (a), branch diameter (b), leaf number per branch (c) and leaf dry weight (d) of field grown coffee trees. Data in panel c and d were collected during fruit ripening. Vertical bars indicate mean ± 1SE. When not shown, SE was smaller than the symbol.

Light-saturated rate of leaf photosynthesis, A_{max}, was significantly affected by the number of fruits per tree. Trees with 25 and 50% fruit load had significantly greater A_{max} than trees with 75 and 100% fruit load (Fig. 2a). This finding is, however, in contrast to the results observed from previous fruit load studies in coffee, e.g.. Accumulation of soluble carbohydrates was indicated as a process inhibiting photosynthetic rate in coffee trees with reduced fruit load, but this was not universally observed to cause an effect on leaf photosynthetic rate as the change in photosynthetic rate of leaves depends not only on the carbohydrate status alone but also on the active pools of other substrates, for example, leaf N. In this experiment it was observed that leaf photosynthetic rate was positively and significantly correlated with leaf nitrogen (Fig. 2b).

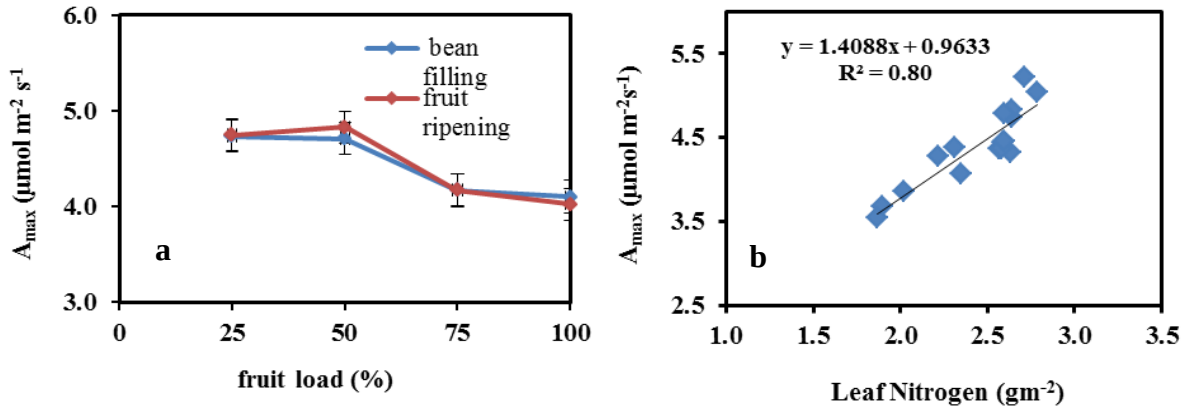

Figure 2. Effect of fruit load on the light-saturated rate of leaf photosynthesis, A_{max} (a) and relationship between A_{max} and leaf N (b).

Fruit thinning decreased coffee bean yield in the first experimental year. But, in the second year bean yield was higher in thinned trees but no fruit was harvested in the second year from coffee trees with full fruit load in the first year (Fig. 3a). In coffee trees with 25 and 50% fruit load, bean yield was observed to be higher in the second year than in the first year. As such the combined yields of both years were higher in the trees that had been thinned in the first year. Similarly, fruit thinning increased individual bean weight and size. Bean size distribution is an important determinant of price. Different bean sizes have different pay scales across different bean size categories. The result, on the other hand, showed greater weight variability among beans of coffee trees with lower fruit load than in trees with 100% fruit load. Thinning some fruits from coffee trees enables the trees to maintain a better balance between vegetative and reproductive growth. This stabilizes the amount of yield produced from year to year and contributes for the development of good quality beans.

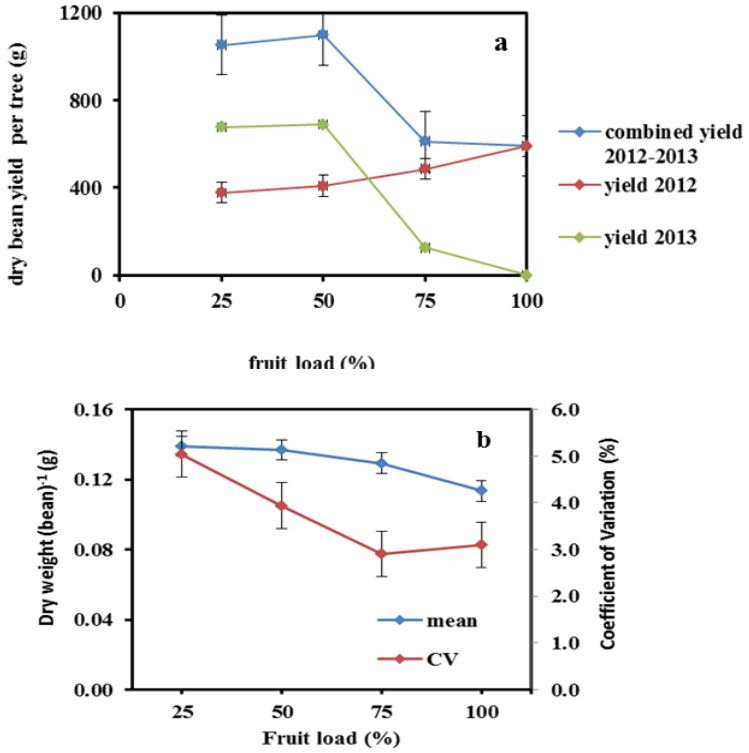

Figure 3. Dry bean yield for 2012, 2013 and aggregated (a), and mean bean weight and its coefficient of variation versus fruit load (b).

CONCLUSION

The experiment clearly indicated that higher fruit load in coffee plants strongly suppresses vegetative growth, and reduces bean yield and size. To rescue the plant from premature aging and keep the yield stable from year to year, the current data indicate coffee fruits need to be thinned to a medium crop load (50%). This reduces tree stress and balances photo-assimilates distribution between fruits and vegetative parts. However, further study is needed to underpin practical management measures.

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Therblig Analysis of the Coffee Picking Process with Canguaro 2M

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SUMMARY

The Canguaro 2M is a tool developed by CENICAFE that assists in manual harvesting. The picking basic cycle analysis with this tool was performed using engineering techniques. The Therbligs cycle with Canguaro 2M consists of micro-movements Search-select (Sh-St), Transport Empty (TE), Grasp (G), Release load to the palm (RLp), Hold (H), Transport loaded (TL) and Release load (RL).

INTRODUCTION

The coffee harvest in Colombia is carried out selectively and manually, in more than 16 rounds per year, which are characterized by low load and concentration of ripe fruits. In Colombia, coffee picking represents the largest part in the coffee production cost, from 35% to 40% of the total cost (1). Consequently, the application of engineering techniques, especially the study of micromovements or therbligs could improve the manual processes (2).
The Canguaro 2M (Figure 1) was developed by CENICAFÉ to assists in the manual harvesting and enhance the performance of the picking process. This tool consists of a bag that is supported on the waist and shoulders of the picker, with two legs that carry the picked fruits to the container or bag. With this harvesting tool, the micro-motion that involves the fruit transportation to the basket or plastic container is avoided (3).

METHODS

The picking basic cycle analysis was performed employing engineering techniques (2). This analysis was carried out by monitoring the picker during manual coffee harvesting using both traditional method and Canguaro 2M. This monitoring was conducted on 13 pickers in the Central Station of Cenicafe, Naranjal, localized in Chinchina, Caldas, Colombia. Videos with a resolution level of 60 frames per second were made. Image analysis techniques were applied to identify the micro-movements or therbligs and their participation in the basic harvesting cycle. During the monitoring, the yield in kg per hour was registered for the efficiency indicator. ANOVA Analysis, parametric-t test- and no parametric test- U-Mann Whitney- were employed to identify significant differences (α=0,05) between methods.

Figure 1. Picking process with Canguaro 2M.

Figure 2. Picking process with traditional method

RESULTS

The therbligs cycle with traditional method (Figure 3) includes the following micro-movements: Search-select (Sh-St), Transport Empty (TE), Grasp (G), Release load to the palm (RLp), Hold (H), Transport loaded (TL) and Release load (RL).

The basic harvest cycle of the coffee picking process with Canguaro 2M is represented in Figure 3. Initially, the collector search and select (Sh-St) the mature fruit, then transport empty (TE) with his hands up to the selected fruit; grasp (G) the fruit; release load (RL) into the Canguaro sleeve.

However, the pickers did not follow this basic harvest cycle because of additional unnecessary micromovements such as release load to the palm (RLp), hold (H) and transport loaded (TL). These therbligs form a harvest subcycle as shown in Figure 3.

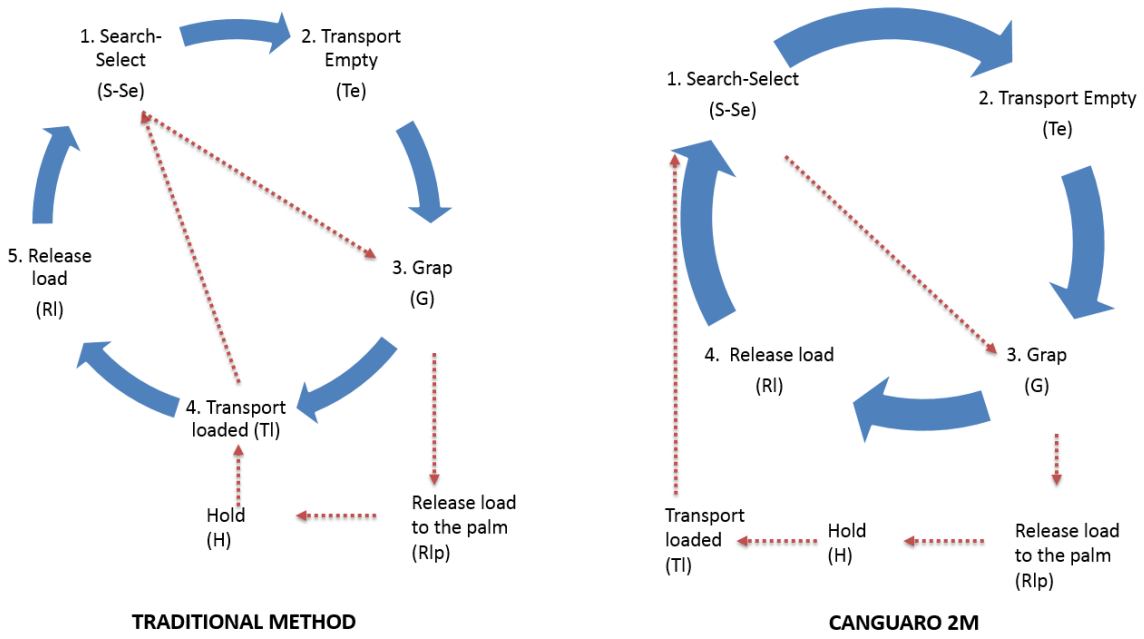

Figure 3. Therbligs analysis of the coffee picking process with different methods.

The cycle time to pick a fruit was estimated in 0,512 ± 0,098 seconds with Canguaro 2M and 0,608 ± 0,0048 seconds with the traditional method. Nevertheless, with Canguaro 2M, a reduction in picking time of only 15,8% (confidence interval 1,8%) would be possible.
However, no significant differences were observed in yield (kilograms per hour) with the picking method. Only improvements in picking efficiency with Canguaro 2M were detected in experienced pickers.

Table 1. Cycle time to pick a fruit by method

Time for picking a fruit in seconds				Significance	
Method	**Cycle time (s)**	**Standard deviation**	**Standard mean error**	**T Test**	**U-Mann Whitney**
Traditional	0,61	0,015	0,002	0,00*	0,00*
Canguaro 2M	0,52	0.023	0,003		

On the other hand, the time by micro-movements with Canguaro 2M is shown in Table 2, which include the percentage of the time cycle. The therbligs Transport Empty and Release load were the micro-movements with greater variation among the pickers. ANOVA analysis indicated that there were significant statistical differences in the picker´s time in the following therbligs: Search-Select (significance 0,040) and release load (significance 0,000).

Table 2. Time of the micro-movements with Canguaro 2Md.

Therblig	Descriptive Analysis			Percentage of the time cycle, %		
	Time of Therblig (s)	Confidence Intervale	Coefficiente of Variance	Upper limit	Mean	Lower Limit
Search-Select	0,048	0,0057	1%	10,03%	9,40%	8,65%
Transport Empty	0,096	0,0057	14%	19,00%	18,80%	18,47%
Grasp	0,08	0,0018	6%	15,28%	15,60%	16,00%
Release load to the palm	0,064	0,0017	1%	12,28%	12,50%	12,75%
Hold	0,08	0,0022	7%	15,36%	15,60%	15,92%
Transport loaded	0,08	0,0022	7%	15,36%	15,60%	15,92%
Release load	0,064	0,0044	18%	12,78%	12,50%	12,19%

CONCLUSION

Study results support the conclusion that training of pickers with Canguaro 2M is required to raise the efficiency indicators, in response to a time reduction of the harvest cycle.

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How Density and Planting Pattern Affect Coffee Plant Structure and Berry Distribution in First Production Year?

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SUMMARY

This study aimed to analyze the berry location and maturing within the plant architecture for two planting densities combined with two planting patterns in a first production year. Non destructive measurements of coffee plant architecture (cv. IAPAR 59) were performed on plants grown in two plant densities (6,000 and 10,000 plants ha^{-1}) and planting patterns (square – Q or rectangular - R). The number of phytomers and length of the plagiotropic axes were linearly related to their rank on the trunk. In rectangular PP, the competition between plants in the row promoted the apical dominance more than in square PP. In first production year, the berries were situated close to the trunk in horizontal directions, and in lower layers in the vertical plant profile. Our results of total berry production per ha^{-1} argue in favor of very dense plantation with a square planting pattern. However, further observations are needed to check that canopy closure during the following years, and Arabica coffee biennial production will not reverse the observed trends.

INTRODUCTION

In the Arabic coffee (*Coffea arabica* L.) the entire phenological cycle of the berry production takes two years, unlike most of the other crops that complete the reproductive cycle in one year. Six phenological phases have been schematized to describe the coffee reproductive cycle of two years.

The productive year for coffee culture, under the subtropical conditions of Paraná, Brazil, starts with flowering in September and lasts until August the next year (last berry collection). During this period, two-three large and two-three small flowering flushes occur. In this most subtropical region of coffee cultivation in the world (>23°S), when fertile soils are available, high density plantation has been recommended as the most stable and efficient system of production.

Assuming that the flowering dynamics reflects in berry ripening within the plant structure, this study aimed to analyze the berry location and maturing. Growth parameters such as the number of phytomers emitted, berry distribution along orthotropic and plagiotropic axes, and total leaf area were compared between two high densities combined to two planting designs, in the first production year.

MATERIALS AND METHODS

The experiment was set up at the Agronomical Institute of Paraná (IAPAR), Londrina, Brazil (23°18'S and 51°17'W), in 2010 with 15-year-old *Coffea arabica* trees (cv. IAPAR 59). The seedlings were planted in 1995 and pruned close to the ground (1st in 2000 and 2nd in 2008). Two high plant densities (6,000 and 10,000 plants ha^{-1}) were combined to two planting patterns, square (Q) and rectangular (R), for defining four treatments identified as Q_{10}, R_{10} (3 m x 0.33 m), Q_6 (1.29 m x 1.29 m) and R_6 (3 m x 0.55 m).

Non destructive measurements of coffee plant architecture were performed in June of 2010, *i.e.* in the second year after the 2nd pruning. Visually mature fruits were collected in June, July and August of 2010. The June collection concerned mature berries (*mb*), while second and third collections concerned the immature (*ib*) ones.

Before the first fruit collection, the whole plant topology and geometry was described at phytomer scale and coded as multi-scale tree graphs (MTGs). Length of each internode, length and width of each leaf, *mb* and *ib* number per phytomer were recorded. Total leaf area (LA) per plant was computed with different softwares – VPlants, PlantGLViewer and VegeSTAR, for 3D mock-up construction and LA calculation, respectively. Data extraction was performed using AMAPstudio - Xplo software. Two-way ANOVA, with density and planting pattern considered as factors, was performed withe R was performed with R.2.14.2.

CONCLUSION

Length and number of phytomers of plagiotropic axes, as well as the zones containing *mb* and *ib*, were studied in relation with their rank along the trunk and field treatments. The number of phytomers and length of the plagiotropic axes were linearly related to their rank on the trunk (Fig. 1).

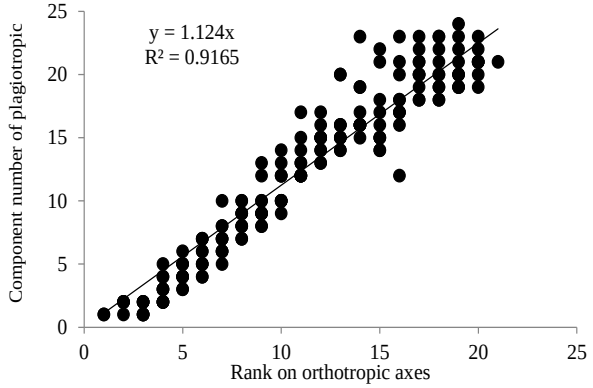

Figure 1. Linear regression between the phytomer number of plagiotropic branches and ranks on orthotropic trunk coded from top to the bottom (example of Q_{10}).

Table 1. Coefficient of linear regressions established between the orthotropic bearer and phytomer number / length of plagiotropic branches. Plants were grown in two plant densities (6,000 and 10,000 plants ha^{-1}) and two planting patterns (square – Q and rectangular - R).

Treatment	Phytomer number	Length of plagio-tropic axes
Q_{10}	1.124	2.607
Q_6	1.000	2.327
R_{10}	0.871	2.142
R_6	0.974	2.352

Regression coefficient was generally near to 1 (Table 1) and corresponds to the slope of the regression, i.e. the ratio between the number of emitted plagiotropic and orthotropic phytomers. The growth pattern in Table 1 is similar to that previously defined for various coffee cultivars, where the primary branches show the same growth rate as the trunk, *i.e.* they share approximately the same phyllochron. However, the slope varied significantly between treatments, showing lower values for rectangular than square PP. Consistently, the phytomer number in orthotropic axes was higher in rectangular PPs than in square ones, whereas the phytomer number was lower in higher density (Table 2). Also, the average internode length of plagiotropic axes was higher in R than in Q planting patterns and in lower density. Altogether these results suggest that in rectangular PP and higher density, the competition between plants in the row promoted the apical dominance more than in square PP and lower density.

Table 2. Mean and p-values values of structural parameters for coffee trees grown in two plant densities (6,000 and 10,000 plants ha^{-1}) and two planting patterns - PP (square – Q and rectangular - R).

Treatment	Phytomer number in orthotro-pic axes	Length of orthotropic axes	Internode length in orthotropic axes	Phytomer number in plagiotropic axes	Length of plagio-tropic axes	Internode length in plagiotropic axes
Q_{10}	20.8	82.58	2.54	12.32	28.24	2.17
Q_6	21.0	80.62	2.53	12.47	28.49	2.20
R_{10}	22.4	88.96	2.86	11.57	28.10	2.30
R_6	23.0	84.20	1.89	12.29	28.88	2.20
ANOVA						
Density	0.5820	0.4314	0.2514	**<0.0001**	**0.0025**	0.5393
PP	**0.0257**	0.2508	0.1148	0.1452	**0.0003**	**<0.0001**
Dens. x PP	0.7821	0.7403	0.4517	**0.0429**	0.464	**0.0224**

p-values <0.05 were considered significant and marked in bold.

The plagiotropic axes bearing *mb* were situated from the 1st to the 13th ranks along the trunk (counting from the bottom to the top), while those bearing *ib* were situated from the 1st to the 14th orthotropic ranks (Fig. 2). Along the plagiotropic axes (counting from the bottom to the top), *mb* and *ib* were located in a zone near the trunk, from 1st to the 13th metamer in square PP, and from the 1st to the 14th metamer in rectangular PP. The average *mb* number per metamer, within the zone of berry appearance previously described, was lower in rectangular than in square PP, with the lowest *mb* number in R_6 and highest in Q_{10} (Table 3). In contrast, the average *ib* number per metamer was not influenced by neither density nor planting pattern.

Generally, the highest concentration of berries was localized at the zone from 2nd to 6th orthotropic rank and from 3rd to 8th plagiotropic metamer (Figure 2).

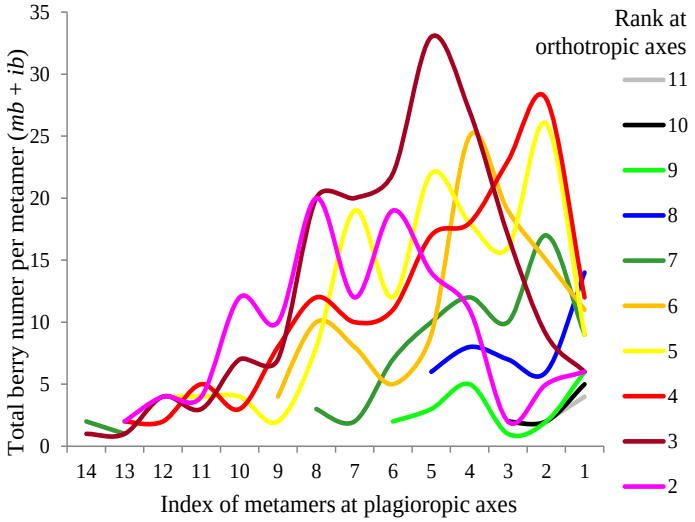

Figure 2. Distribution of the total berry number (*mb* + *ib*) along the trunk and plagiotropic axes. Axes ranked from bottom to the top (example of data set for R_{10}).

Table 3. Mean and p-values for distribution of mature (*mb*) and immature berries (*ib*) in coffee trees grown in two plant densities (6,000 and 10,000 plants ha^{-1}) and two planting patterns - PP (square - Q and rectangular - R).

Treatment	Number of berries per metamer at the zone of appearance (1st - 14th)	
	mb	*ib*
Q_{10}	0.357	0.349
Q_6	0.391	0.325
R_{10}	0.288	0.405
R_6	0.221	0.292
ANOVA		
Density	0.0843	0.8937
PP	**0.0007**	0.2061
Dens. x PP	0.6399	0.7150

p-values <0.05 were considered significant and marked in bold.

In the coffee structures described in the first production year, leaves occupied the upper canopy/ plant layers only (from 15th to 23th trunk ranks). The LA per plant did not differ between treatments (Figure 3), but the final LAI and berry production per ha^{-1} were significantly higher for 10,000 than for 6,000 density. The average LA per harvested berry was about 100 cm^2 in square PP, and about 150 cm^2 in rectangular PP. Considering that an average LA per harvested berry of 20 cm^2 is a minimum to reach a desirable quality, leaf area is not supposed to be a limiting factor for berry production in the present study.

Finally, in first production year, the average *mb* number per metamer (assumed to be the product of the first big flowering flush) was higher in square PP than in rectangular PP, whereas the total number of berries from posterior flushes (*ib*) was not influenced by PP. Our results of total berry production per ha^{-1} argue in favor of very dense plantation with a square planting pattern. However, further observations are needed to check that canopy closure during the following years, and Arabica coffee biennial production will not reverse the observed trends.

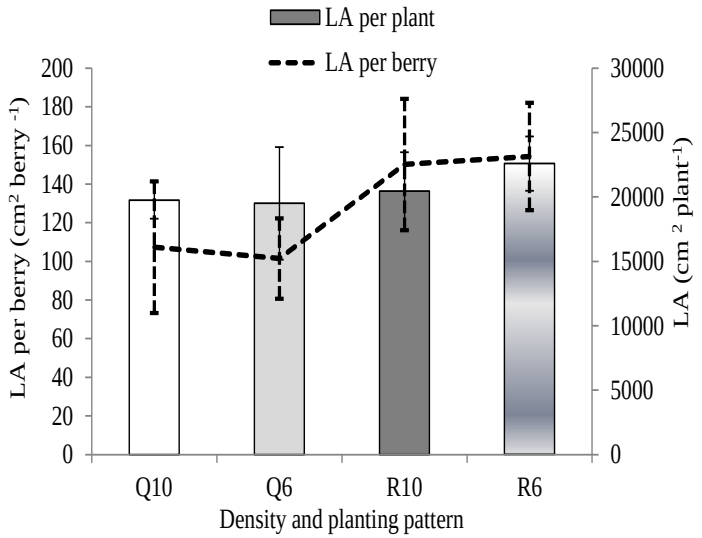

Figure 3. The total leaf area (LA) per plant and LA responsible for one berry produced.

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Using GIS in the Selection of Quality *Coffea canephora* in Nigeria

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SUMMARY

Quality improvement of *Coffea canephora* which accounts to 70% of coffee produced in Nigeria will help in enhancing its productivity. Coffee production in Nigeria had served as a source of income for rural farmers; however abolition of marketing board has led to decline in the production of coffee due to scarcity of information about marketing of the commodity. Improved cup qualities in Nigeria Coffee would be a linkage to prosperous marketer with a specific preference to consumers demand. To make coffee production more sustainable in Nigeria, improved quality coffee plantations must be established. This study was aimed to obtain a coffee cultivation location for selection of quality coffee and subsequent establishment of coffee quality plantation.

Ten locations were mapped with ArcGIS 10.1 software to determine their altitude. The altitude of cultivated areas ranges from 133m to 522m above sea level, and Iyamoye in Kogi state has the highest (552m) altitude as compared to other two locations. This location may habour genotypes of coffee with high quality traits since high elevation improves the quality of the bean and potential cupping quality. These genotypes will further be analyzed for biochemical precursors of coffee cup quality.

INTRODUCTION

To make coffee production more sustainable in Nigeria, high quality coffee plantations needs be established. This study was aimed to obtain location for selection of quality *Coffea canephora* and subsequent establishment of high coffee quality plantation. The shortage in the supply of volumes of high quality arabica coffee is posing a challenge in meeting current need of high quality coffee. And this makes it necessary to improve robusta coffee on quality. Since green coffee is graded and classified for export with the aim of producing the best cup quality and thereby securing the highest price. And this is based on some of the following criteria: altitude and/or region, botanical variety, preparation (wet or dry process), bean size (sometimes also bean shape and colour), number of defeats, roast appearance, cup quality (flavor, characteristics, cleanliness, etc) and bean density. There is need to study the altitude at which Nigeria robusta coffee is been produced. Also altitude is known to shape coffee flavor profile. High elevation improves the quality of the bean and potential cupping quality.
To date very little research has been conducted to understand and therefore improve coffee cup quality in *Coffea canephora*. This research was conducted to select quality clones of Robusta coffee with respect to altitude at which they are cultivated.

MATERIALS AND METHODS

Ten locations which comprise of 8 farmers' plots and 2 germplasm plots were mapped with ArcGIS 10.1 software to determine their altitude (Fig 1). These locations may habour genotypes of coffee with high quality traits since high elevation improves the quality of the

bean and potential cupping quality. These genotypes will further be analyzed for biochemical precursors of coffee cup quality.

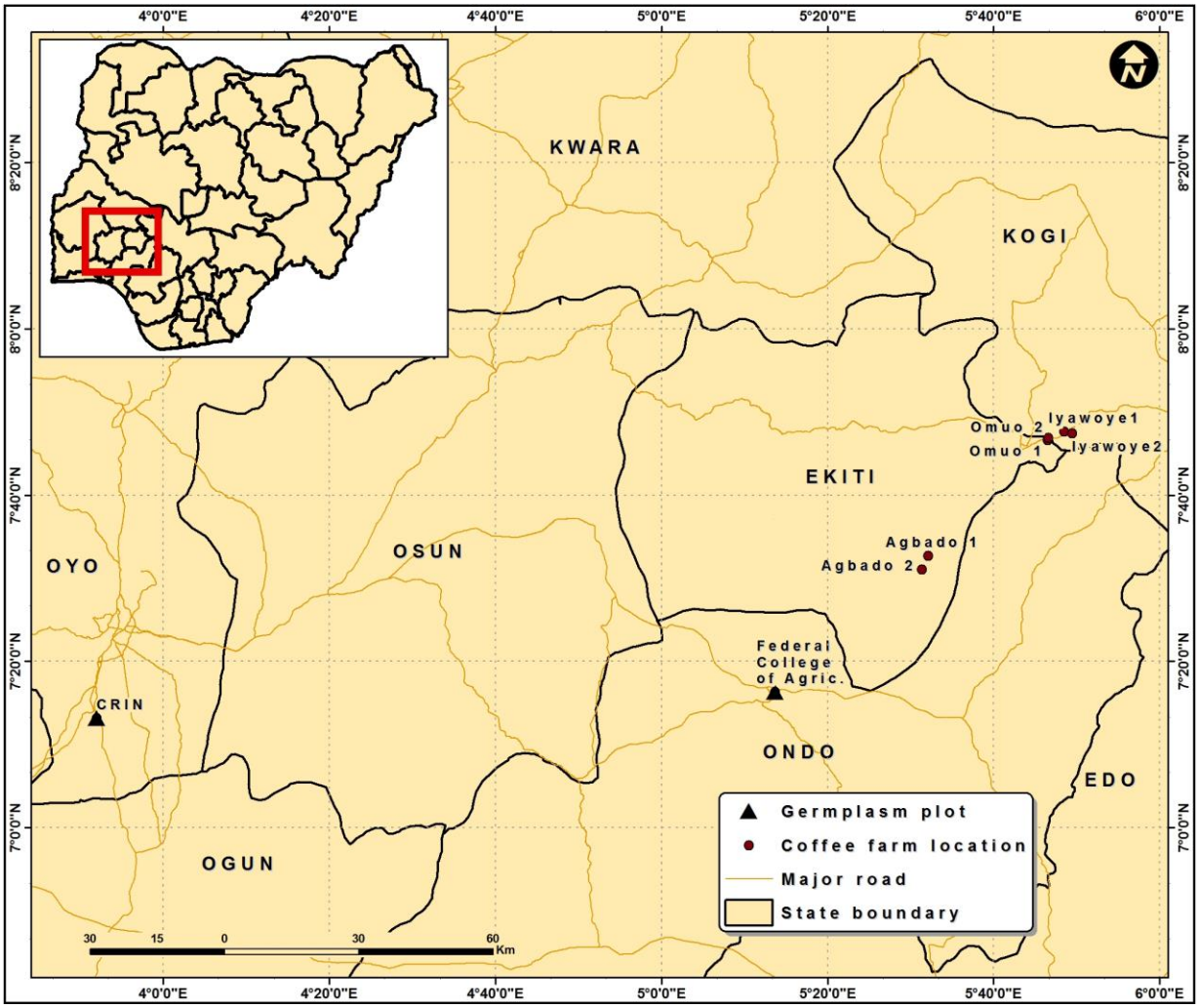

Figure 1. The map of some locations in Nigeria where Robusta coffee is produced and conserved.

RESULTS AND DISCUSSION

The altitude of cultivated areas studied for *Coffea canephora* ranges from 133m to 522m above sea level (Table1), and Iyamoye in Kogi state has the highest (552m) altitude as compared to other locations. *Coffea canephora* has been known to be cultivated at altitude which usually ranges between 0 to 700m (http://www.ico.org/botanical.asp) unlike *Coffea arabica* which thrives best at higher altitude. Geographical Information System can aid coffee production by identifying and mapping factors that influence crop productivity (Ellen, 2009) and quality (Leonel and Philippe, 2007). It may be deduced that Iyamoye is more suitable for the establishment and production of good quality coffee plantation since it has higher elevation, knowing very well that as high the elevation the better the quality.

Table 1. The latitude, longitude and altitude of coffee producing locations

Location	Latitude	Longitude	Altitude
AGBADO 1	7.5449449	5.5352401	207
AGBADO 2	7.5174732	5.5218614	381
AKURE North	7.271845	5.2275437	357
CRIN	7.2192851	3.8659008	133
ISE-Ekiti	7.3985756	5.4006514	407
Iyawoye1	7.7938429	5.8075505	429
Iyawoye2	7.7904024	5.8243691	522
Iyawoye3	6.4160156	3.4277344	522
OMUO 1	7.7770255	5.7748171	506
OMUO 2	7.7812977	5.7762922	508

CONCLUSION

Selection of those genotypes of Robusta coffee that can be produced at different altitudes will be useful in the improvement of cup quality in Robusta coffee. These coffee genotypes grown at 522m above sea level may harbor genes responsible for high quality traits. This will assist in decision making in choosing a good location for coffee processing plant establishment and further breeding for high quality *Coffea canephora* in Nigeria.

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Ortet Selection in Half-Sib Progenies of *Coffea canephora* Var. Guarini

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SUMMARY

Brazil is the second largest producer of *Coffea canephora* var. Conilon. *C. canephora* is a diploid species (2n = 2x = 22 chromosomes), self-incompatible, and divided into two groups called Guinean and Congolese. The Congolese group has four subgroups. Coffee plants of *C. canephora* known in Brazil as Conilon, in belong partly to the Guinean group and mostly to the Congolese subgroup 1 (probably hybrids of Conilon x Robusta). The *C. canephora* var. Guarini belongs to the Congolese subgroup 2. Since 1970, the IAC develops a breeding program for *C. canephora* var. Robusta, var. Guarini and var. Conilon types. The aim of the current work was to select superior Guarini trees with high yield, resistance to rust**,** large beans, high percentage of flat beans and others desirable agronomic and technological characteristics in order to obtain promising ortets for selection of clonal cultivars of C. *canephora* var. Guarini. The experiment included 41 half-sib progenies of Guarini coffee and was established in Mococa, SP, Brazil, in 1994, using a randomized block design with a plant per plot, 16 repetitions and spacing of 4 x 3 m. The parental trees of the 41 progenies had been selected previously in several experiments. The average production of fresh coffee berries and green coffee per plant and per year over a period of three harvesting years for individual plants and per progeny was analyzed. In 2013 the vigor and production were assessed visually using a 1 – 10 point scale, fruit maturity, characteristics of beans and the caffeine and soluble solids levels. The average yield of the 41 half-sib progenies was 2,809 kg of green coffee per ha and per year. The average production of 35 selected trees ranged from 19.5 to 29.8 kg of fresh coffee berries per plant, corresponding to 3.89-5.96 kg/year of green coffee. The average yield of the 35 selected ortets was 4,037 kg of green coffee per ha and per year. Regarding technological characteristics, the average outturn of green coffee in relation to fresh coffee berries was 20.0%. The 35 selected coffee plants were vigorous, high resistant to rust and fruit maturation was medium and medium to late. The percentage of flat type beans ranged from medium to high (67.7 to 92.1). The 100 seed weight ranged from 14.8 to 23.8 g and average sieve size ranged also from medium to high (15.6 to 19.1). The percentage of caffeine of the analyzed 20 trees varied from 1.9 to 2.8 and soluble solids from 27.0 to 33.0. The average production of the six most productive ortets were 5.96; 5.79; 5,78; 5,74; 5.58 and 5,51 kg of green coffee per ortet and per year. The results obtained demonstrate the possibility to select in Brazil superior clones of the *C.canephora* var. Guarini (Congolese Robusta coffee type), with high production, high percentage of flat beans and high average sieve size. These data will be further used for the *C.canephora* breeding program aiming at clonal selection at the IAC.

INTRODUCTION

Brazil is the second largest producer of *Coffea canephora* var. Conilon. *C. canephora* is a diploid species (2n = 2x = 22 chromosomes), self-incompatible, and divided into two groups

called Guinean and Congolese. The Congolese group has four subgroups. Coffee plants of *C. canephora* known in Brazil as Conilon, in belong partly to the Guinean group and mostly to the Congolese subgroup 1 (probably hybrids of Conilon x Robusta). The *C.canephora* var. Guarini belongs to the Congolese subgroup 2. Since 1970, the IAC develops a breeding program for *C. canephora* var. Robusta, var. Guarini and var. Conilon types. The aim of the current work was to select superior Guarini trees with high yield, resistance to rust**,** large beans, high percentage of flat beans and other desirable agronomic and technological characteristics in order to obtain promising ortets for selection of clonal cultivars of C. *canephora* var Guarini.

MATERIALS AND METHODS

The experiment included 41 half-sib progenies of var. Guarini coffee and was established in Mococa, SP, Brazil, in 1994, using a randomized block design with one plant per plot, 16 repetitions and spacing of 4 x 3 m. The parental trees of the 41 progenies had been selected previously in several experiments. The average production of coffee berries and of green coffee per year over a period of three harvesting years for individual plants and per progeny was analyzed. In 2013 the vigor and production were assessed visually using a 1 – 10 point scale, fruit maturity, resistance to rust, characteristics of beans and the caffeine and soluble solids levels.

RESULTS AND DISCUSSION

The average of the 41 half-sib progenies was 2,809 kg of green coffee per ha and per year. The data obtained of the 35 best ortets in relation the average production of three harvests of cherries coffee and green coffee (kg/ortet/year), the grains types (flat, peaberry and eleplant beans), the weight of 100 dry beans and the average sieve size were found in table 1. The average production of 35 selected trees ranged from 19.5 to 29.8 kg of fresh coffee berries per plant, corresponding to 3.89-5.96 kg/ortet/year of green coffee. The average yield of the 35 selected ortets was 4,037 kg of green coffee per ha and per year. Regarding technological characteristics, the average outturn of green coffee in relation to fresh coffee berries was 20.0%. The 35 selected coffee plants were vigorous, high resistant to rust and fruit maturation was medium and medium to late. The percentage of flat type beans ranged from medium to high (67.7 to 92.1). The weight of 100 dry beans ranged from 14.8 to 23.8 g and average sieve size ranged also from medium to high (15.6 to 19.1). The percentage of caffeine of the analyzed 20 trees varied from 1.9 to 2.8 and soluble solids from 27.0 to 33.0. The average production of the six most productive ortets were 5.96; 5.79; 5,78; 5,74; 5.58 and 5,51 kg of green coffee per ortet and per year.

Table 1. Average production of three harvests of cherries coffee and green coffee in kg/ortet/year, grain types, weight of 100 dry beans and average sieve size of 35 ortets from cv. Guarini in Mococa, SP, Brazil.

Ortets	Average production of cherries coffee kg/ortet/year	Average production of green coffee kg/ortet/year	Grains types			Weight of 100 dry beans g	Average sieve size
			Flat %	Peaberry %	Elephant beans %		
57	22.4	4.48	69.80	30.20	0.00	19.5	17.3
73	19.5	3.89	84.00	16.00	0.00	21.0	18.3
79	24.8	4.96	82.20	17.80	0.00	16.8	16.6
85	20.7	4.14	89.20	10.80	0.00	18.9	17.7
87	22.8	4.55	85.00	15.00	0.00	21.3	18.1
89	29.8	5.96	77.60	22.40	0.00	16.3	17.0
98	21.8	4.37	85.80	14.20	0.00	15.1	16.2
99	25.7	5.14	86.70	13.30	0.00	16.3	17.7
105	27.2	5.43	89.20	10.10	0.60	16.2	16.6
106	23.5	4.70	87.20	12.30	0.50	16.0	17.2
112	27.9	5.58	80.60	19.40	0.00	15.7	15.7
115	22.0	4.39	79.30	20.70	0.00	18.2	17.3
116	27.5	5.51	76.60	23.40	0.00	18.8	17.6
127	21.5	4.29	75.50	22.50	1.80	19.3	17.1
136	22.8	4.56	87.90	12.10	0.00	15.9	16.4
160	22.9	4.59	84.70	14.90	0.40	18.0	16.8
161	20.5	4.10	86.10	13.90	0.00	15.1	17.5
499	21.4	4.28	80.30	16.80	2.90	16.0	16.5
533	23.0	4.60	67.70	31.40	0.90	14.9	17.1
575	25.8	5.16	89.90	10.10	0.00	16.8	17.6
628	25.4	5.08	88.00	12.00	0.00	20.4	19.1
631	22.6	4.52	90.40	8.50	1.10	23.8	18.4
633	29.0	5.79	90.50	8.70	0.80	21.6	18.4
636	21.7	4.35	89.40	10.60	0.00	21.3	18.3
682	26.0	5.20	92.10	7.90	0.00	15.7	16.5
695	28.9	5.78	90.80	9,20	0.00	15.3	15.6
704	23.2	4.63	83.20	16.80	0.00	19.3	17.3
708	27.5	5.49	87.40	12.60	0.00	18.7	18.0
712	26.1	5.22	89.20	10.80	0.00	14.8	16.6
738	22.5	4.50	89.80	9.70	0.50	19.6	17.9
750	28.7	5.74	81.10	18.90	0.00	17.9	16.2
761	24.8	4.96	85.00	15.00	0.00	16.2	16.9
767	23.1	4.61	87.70	12,30	0.00	17.8	17.1
769	21.6	4.31	83.30	16,70	0.00	18.0	17.6
812	23.5	4.70	75.40	24,60	0.00	16.3	16.6
Average	24.3	4.84	84.25	15,47	0.27	17.79	17.2

CONCLUSION

1. The average of the 41 half-sib progenies was 2,809 kg of green coffee/ha/year.
2. The average production of 35 selected trees ranged from 19.5 to 29.8 kg of fresh coffee berries per plant, corresponding to 3.89-5.96 kg/ortet/year of green coffee.
3. The average yield of the 35 selected ortets was 4,037 kg of green coffee/ha/year.
4. The percentage of caffeine of the analyzed 20 trees varied from 1.9 to 2.8 and soluble solids from 27.0 to 33.0.
5. The average production of the six most productive ortets were 5.96; 5.79; 5,78; 5,74; 5.58 and 5,51 kg of green coffee per ortet and per year.
6. The results obtained demonstrate the possibility to select in Brazil superior ortets of the *C. canephora* var. Guarini (Congolese Robusta coffee type), with yield, percentage of flat beans and average sieve size very high.
7. The data obtained in this work will be further used for the *C. canephora* breeding program aiming at clonal selection at the IAC.

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Bean Traits of Ten Clones of Cultivar Conilon Vitória of *Coffea canephora* var. Conilon

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SUMMARY

Clones of cultivar Conilon Vitória (*Coffea canephora* var. Conilon) are being recommended for cultivation in Brazil, mainly so in the States of Espírito Santo, Bahia and Rondônia. It is well established that clones of the cultivar Conilon Vitória are highly productive in the States of Espírito Santo and Bahia, averaging 3,600 kg of green coffee per hectare and per year. In the State of São Paulo there is a vast area with high average temperatures, which is suitable for cultivation of *C. canephora*. Therefore, an observation trial was established in Mococa, SP, in 2010 with ten clones of cv. Conilon Vitória that were selected in the State of Espírito Santo. The objective was to study adaptability of cv. Conilon Vitória to the growing conditions in São Paulo, and to evaluate agronomic and technological traits in this region. The layout of the observation trial in Mococa was with the clones planted in single rows of 40 plants each, spaced at 3.8 x 1.5 m. Observations were made on these clones in 2013 on technological traits of the coffee beans. Samples of 1.5 kg of cherries of each of the ten clones were collected and processed to assess the following bean traits: outturn (% dry bean weight/fresh cherry weight), percentage of flat beans, peaberries and elephant beans, weight of 100 dry beans and average bean size (medium sieve size). The average outturn of the clones was 25%. The percentage of flat beans ranged from 27.8 to 69.0, of peaberries from 31.0 to 72.2 and of elephant beans from 0.0 to 0.5. These data show low percentages for the elephant beans defect and high percentages of peaberries for many of the clones. The weight of 100 dry beans ranged from 11.7 to 19.5 g and the average sieve size varied from 13.4 to 16.9. In September 2013, the flowering of clones was excellent. Thus, in 2014 further yield data was collected. The data obtained in 2014 confirmed the high yields of clones Conilon used in the experiment, averaging 3,000 kg of green coffee per hectare.

INTRODUCTION

Clones of cultivar Conilon Vitória (*Coffea canephora* var. Conilon) are being recommended for cultivation in Brazil, mainly so in the States of Espírito Santo, Bahia and Rondônia. It is well established that clones of the cultivar Conilon Vitória are highly productive in the States of Espírito Santo and Bahia, averaging more than 3,600 kg of green coffee per hectare and per year. In the State of São Paulo there is a vast area with high average temperatures, which is suitable for cultivation of *C. canephora*. The aim of the current work was to obtain data of clones of cultivar Conilon Vitória in relation to outturn and beans characteristics.

MATERIALS AND METHODS

Therefore, an observation trial was established in Mococa, SP, in 2010 with ten clones of cv. Conilon Vitória that were selected in the State of Espírito Santo by INCAPER. The objective was to study adaptability of cv. Conilon Vitória to the growing conditions in Sao Paulo, and to evaluate agronomic and technological traits in this region. The layout of the observation

trial in Mococa was with the clones planted in single rows of 40 plants each, spaced at 3.8 x 1.5 m. Observations were made on these clones in 2013 on technological traits of the coffee beans. Samples of 1.5 kg of cherries of each of the ten clones were collected and processed to assess the following bean traits: outturn (% dry bean weight/fresh cherry weight), percentage of flat beans, peaberries and elephant beans, weight of 100 dry beans and average bean size (medium sieve size).

RESULTS AND DISCUSSION

The data obtained the ten clones of cv. Conilon Vitória and clone G35 used as control (outturn, percentagens of flat, peaberry and elephant beans, weight of 100 dry beans and average sieve size) are found in table 1. The average outturn of the clones was 25%. The percentage of flat beans ranged from 27.8 to 69.0, of peaberries from 31.0 to 72.2 and of elephant beans from 0.0 to 0.5. These data show low percentages for the elephant beans defect and high percentages of peaberries for many of the clones. The weight of 100 dry beans ranged from 11.7 to 19.5 g and the average sieve size varied from 13.4 to 16.9. In September 2013, the flowering of clones was excellent. Thus, in 2014 further yield data was collected. The data obtained in 2014 confirmed the high yields of clones Conilon used in the experiment, averaging 3,000 kg of green coffee per hectare.

Table 1. Percentage of grains flat, peaberry and elephant beans types, weight of 100 dry beans in grams and average sieve size of 10 clones of cultivar Conilon Vitória selected by INCAPER and G35 clone of Verdebrás, an experiment in Mococa-SP, Brazil.

Clones	Types of grains			Weight of 100 dry beans (g)	Average sieve size
	Flat	Peaberry	Elephant beans		
	----------%----------				
1V	41.3	58.7	0.0	15.6	15.3
2V	52.4	47.1	0.5	19.5	16.5
4V	51.3	48.7	0.0	18.4	15.9
6V	59.7	40.3	0.0	11.7	13.4
7V	60.6	39.4	0.0	14.6	13.7
8V	27.8	72.2	0.0	14.6	14.4
10V	39.3	60.7	0.0	17.2	15.4
11V	67.0	33.0	0.0	18.0	14.9
12V	45.8	54.2	0.0	17.5	16.9
13V	69.0	31.0	0.0	18.0	15.9
G35	70.7	29.3	0.0	18.6	16.1

CONCLUSION

1. The six best clones of cv. Conilon Vitória, in relation the average sieve size were: 12v, 2v, 4v, 13v, 10v and 1v.
2. The ten clones of cv. Conilon Vitória presented high percentagens of peaberry type (31.0 to 72.2).

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Selection of Arabusta Coffee Clones (*Coffea arabica* X *C. canephora* DP)

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SUMMARY

In the present study the average annual yield over four harvests were evaluated as well as several agronomic and technological characteristics of 14 Arabusta coffee clones. Arabusta consists of F1 hybrids obtained by crossing *Coffea arabica* with *C. canephora* trees with duplicated number of chromosomes. The Arabusta clones were observed between 2009 and 2013 in a field trial established in 2007, in Campinas, SP, Brazil. The statistical design of the trial was in randomized blocks with three replications and four plants per plot. The average yield of the trial in kg/ha/year was 3,317 and the average yields of the five most productive clones were 4,528; 4,353; 4,142; 4,019 and 4,002 kg of green coffee per ha and per year. Scores for vegetative growth of the Arabusta clones showed in 2013 high vigor with average visual scores of 7 to 9 and IAV production 6-8 points on 1-10 point scale. The flowerings have been abundant. Fruit maturation was late and the average fruit size was medium to large. All clones showed high levels of resistance or immunity to coffee leaf rust. The clones have large dark green leaves that stay turgid during the dry season, which is associated with the observed high level of drought tolerance of the Arabusta clones during the dry season. Regarding technological characteristics, it was found that the average outturn of green coffee in relation to fresh coffee berries was 15.2%. The mean percentages of flat, peaberry and elephant beans types were 53.2%, 46.1% and 0.8 %, respectively. The average weight of 100 dry beans was 18.7 g and the average sieve size was 17.4. Caffeine content in green beans of the Arabusta clones ranged from 1.51% to 2.29%, whereas *C. arabica* cv. Catuaí Vermelho IAC 99 gave 1.20%. The cup quality of the Arabusta clones was considered as good (intermediate in quality between Arabica and Robusta). The data obtained in this study indicate the possibility of selecting Arabusta coffee clones with high yield, vigorous, high resistance to rust, good cup quality and average levels of caffeine. Arabusta may represent a new option for coffee cultivation on marginal and normal land suitable for arabica and Conilon or Robusta coffee growing regions. Arabusta coffee may also be of interest as a new product for direct use in the soluble coffee industry or as a product to be sold to specific coffee markets used to blends between Arabica and Robusta coffees.

INTRODUCTION

The species *Coffea arabica* and *C. canephora* were known for arabica and robusta coffee, respectively. The F1 hybrids obtained from crosses between the species *C. arabica* and *C. canephora DP* is known as Arabusta. Given the characteristics that plants Arabusta coffee have shown, obtaining clones selected these interspecifics hybrids could be an alternative to coffee planting in Brazil in areas of Conilon or Robusta coffees in areas of São Paulo marginal to Arabica a and more appropriate the cultivation of Robusta coffee. Thus, this study aims to evaluate Arabusta coffee clones with great agronomic characteristics, architecture and adequate size and with the leaf rust resistance among these F1 hybrids.

MATERIALS AND METHODS

The experiment was established in Campinas in 2007, with 14 clones F1 of Arabusta coffee (*Coffea arabica* X *C. canephora DP*) in randomized complete block design with three replications and four plants per plot. Coffee plants were harvested individually and statistical analyzes we used the average of the plots. Yields four crops were analyzed in kilograms of mature coffee, in the period from 2009 to 2012 and in 2012 the index of visual evaluation for vigor (IAV vigor), giving up 10 points to the coffee, with 1 = poor vigor and 10 = great vigor, the index of visual assessment of production (IAV production), with 1 being few productive and 10 very productive plants. Were analyzed either the maturation and size of fruit, the natural rate of infection of rust and characteristics of beans (percentage of grains of flat, peaberry and elephant beans types, weight of 100 dry seeds and average sieve size). The experiment was analyzed to the average production of fresh coffee berries per plant, in kilograms, and the test for comparison of means was Scott-Knott 5%. The content of caffeine from several clones and a preliminary analysis of the drink was made in some clones.

RESULTS AND DISCUSSION

The results referring to the average productions and yields in clones Arabusta coffee and yields in the period 2009 to 2012 in the EP 529 D experiment, are shown in table 1.

Table 1. Average production of four harvests of mature coffee and green coffee in kg/plant/year and average yields in kilograms of green coffee/hectare/year of Arabusta coffee clones in Campinas – SP, Brazil. (1755 plants/hectare).

Clones Arabusta	Average[(1)] production of mature coffee per plant/year(kg)	Average production of green coffee per plant/year(kg)	Yields averages of green coffee (kg/ha/year)
H 2460-3	7.23 b	1.10	1,931
H 9880-7	10.35 b	1.57	2,756
H 9880-9	16.94 a	2.58	4,528
H 15198-3 C 172	11.53 b	1.75	3,072
H15198-4 C 173	12.42 b	1.89	3,317
H 15198-12 C 182	11.79 b	1.79	3,142
H 15198-14 C 184	15.08 a	2.29	4,019
H 15199-1 C 164	15.51 a	2.36	4,142
H 15199-2 C 165	15.04 a	2.28	4,002
H 15199-3 C 166	9.27 b	1.41	2,475
H 15199-4 C 167	10.48 b	1.59	2,791
H 15203-1 C 162	11.89 b	1.81	3,177
H 15210-1 C 155	16.30 a	2.48	4,353
H 15210-2 C 156	10.69 b	1.63	2,861
F	3,13**	-	

*** Significant at 1% by F test. C.V.(%) = 26,09*

1) To compare the means used the Scott-Knott test at 5%. Same letters mean no statistical differences between clones and different letters there are statistical differences.

The average production of mature coffee per plant ranged from 7.23 to 16.94 kg. The analyzed data show significant differences among the 14 clones. The average production of four harvests in kilograms of green coffee, per plant and per year ranged from 1.10 to 2.58

and the average yield in kilograms of green coffee per hectare and per year ranged 1,931 to 4,528. The five best clones were H9880-9; H15210-1C155; H15199-1 C 164; H15198-14 C184 and H15199-2 C 165 with average yields of 4,528; 4,353; 4,142; 4,019 and 4,002 kilograms of green coffee per hectare and per year, respectively. These values can be considered high as well. Also analyzed the quality of the beverage preliminarily of clones Arabustas and it was found that is higher than the Robusta coffee (intermediate drink between Arabica and Robusta). Similar results were either obtained by several authors. Their percentages of caffeine in coffee Arabusta clones ranged from 1.51 to 2.29%, while *C. arabica* cv. Catuaí Vermelho IAC 99 gave 1.20%. The clones Arabusta coffee are very vigorous. In 2012 the index values of visual assessment for vigor (IAV vigor) ranged 7-9 and IAV production 6-8 points. The flowerings have been abundant. Fruit maturation was considered average late, despite having high desuniformity and the fruit size was medium to large. Arabusta clonal coffee was all resistant to rust. These clones presenting large dark green leaves, and turgid during the dry seasons. A preliminary analysis in this experiment during six dry seasons showed that these clones Arabusta coffee are highly drought tolerant. The data obtained in 2012 concerning the performance and characteristics of beans (percentage of grains flat, peaberry and elephant beans types, weight of 100 dry beans in grams and average sieve size) of 14 clones Arabusta coffee, in the experiment EP 529 D, are shown in table 2.

Table 2. Outturn and beans characteristics obtained in 2012 of clones Arabusta coffee in the EP 529 D experiment installed in Campinas – SP, Brazil.

Clones Arabusta	Outturn[1]	Grains types			Weight of 100 dry beans	Average sieve size
		Flat	Peaberry	Elephant beans		
	——————————%——————————				(g)	
H2460-3	37.4	38.1	60.0	2.0	17.0	17.6
H9880-7	36.6	47.0	52.3	0.7	15.7	17.5
H9880-9	39.8	52.1	46.7	1.3	18.2	17.7
H15198-3 C172	38.9	51.3	48.2	0.5	17.7	16.2
H15198-4 C173	37.1	57.9	40.8	1.3	18.4	17.6
H15198-12 C182	39.4	58.0	42.0	0.0	19.8	17.9
H15198-14 C184	41.6	57.7	42.0	0.4	18.1	17.4
H15199-1 C164	39.1	58.0	42.0	0.0	18.1	16.7
H15199-2 C165	33.3	57.4	42.6	0.0	19.3	17.3
H15199-3 C166	38.9	58.4	40.3	1.3	20.9	17.8
H15199-4 C167	35.8	49.7	48.6	1.8	18.2	17.9
H15203-1 C162	37.3	51.5	47.4	1.1	18.6	16.4
H15210-1 C155	38.6	54.1	46.0	0.0	21.9	18.1
H15210-2 C156	37.8	53.1	45.9	1,0	18.7	17.4
Average	38.0	53.2	46.1	0.8	18.7	17.4

1. Outturn = ratio between the weight of green coffee in relation to dry fruit, in percentage, of a sample of 600 g.

Analyzing this table it can be seen that the ranged from 33.3 to 41.6% and the average was 38.0% (lower outturns). Due to low outurns can assume low fertility of hybrids Arabustas. The percentage of flat grains type ranged from 38.1 to 58.4%, the peaberry type from 42.0 to 60.0% and the elephant beans type from 0.0 to 2.0%. Therefore, in clones Arabusta the percentage of elephant beans type is very low and the peaberry type is too high (in relation to

C. arabica). The weight of 100 dry beans ranged from 17.0 to 21.9 g, averaging 18.7, presenting therefore high values. The average sieve size ranged from 16.2 to 18.1 with a mean of 17.4. The data indicate that it is possible to select clones of Arabusta coffee with high weight and sieve size of beans.

CONCLUSION

1. 1 The five best clones of Arabusta coffee were: H9880-9; H15210-1 C155; H15199-1 C164; H15198-14 C184 and H15199-2 C165 with average yields of 4,528; 4,353; 4,142; 4,019 and 4,002 kg of green coffee/hectare/year, respectively.
2. The data from this study indicate the possibility of selecting clones Arabusta coffee with high yield, vigorous, high resistance to rust, drink good quality and average levels of caffeine.

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IAC Catuaí SH_3: a Variety of *Coffee arabica* Showing Good Yield, High Resistance to Coffee Leaf Rust and Good Bean Quality

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SUMMARY

IAC Catuaí SH_3, an dwarf *Coffea arabica* variety developed at the Instítuto Agronômico de Campinas (IAC), SP, Brazil, originated from the F1 cross between the cultivar Catuaí Vermelho IAC 46 and the IAC 1110-8 (BA10) introduction received at the IAC in 1967. Catuaí Vermelho used in the cross is dwarf growing (Caturra gene) and highly productive but susceptible to coffee leaf rust. It is one of the most popular coffee cultivars planted in Brazil. IAC 1110-8 corresponds to an accession (BA10) received from CATIE, Costa Rica, that was selected at the Balehonnur Experimental Station, Mysore, India. There are reports mentioning that BA10 derived from introgression of *C. liberica* into the *C. arabica* species, which could explain some of the characteristics acquired by it. It is a tall-growing variety with high resistance to rust in most of the coffee producing countries, with good yield and excellent cup quality. However, BA10 shows a high percentage of elephant beans, which is a trait that is difficult to eliminate in successive generations derived from BA10. To obtain the IAC Catuaí SH_3 variety, six generations of line selection were carried out for yield, vigor, fruit maturation, rust resistance, outturn, physical bean qualities (percentage of flat, peaberry and elephant beans, weight of 100 dry beans and average bean sieve size), drought tolerance, resistance to *Cercospora coffeicola* (brown eye spot) and cup quality. In the 6^{th} generation of field trials, where IAC Catuaí SH_3 was selected recently, this variety showed average green coffee yield in three cropping seasons of 2,478 kg/ha/year and resistance to prevalent races of rust in the places where IAC Catuaí SH_3 was planted in the states of São Paulo, Paraná and Minas Gerais. This variety is carrying the SH_3 gene, which confers resistance to predominating rust races. This variety is carrying the SH_3 gene, which confers resistance to predominating rust races in homozygous condition (SH3SH3). In tests conducted in the greenhouse it was also found that this variety has also good resistance to *C. coffeicola*. Drought tolerance was evaluated under field conditions after periods of prolonged droughts using the Turgency Index test. The results were promising, indicating that this variety has good drought tolerance. Furthermore, IAC Catuaí SH_3 is a vigorous variety with medium to late maturing fruits. Average outturn (ratio between the weight of green coffee and fresh coffee berries) was 18.2%, with more than 80% of the flat bean type and less than 5% of elephant beans, a 100 bean weight around 12.9 g and average bean sieve size of 16.9. In preliminary cup tests, IAC Catuaí SH_3 gave excellent results with very characteristic flavor and aroma.

INTRODUCTION

Coffee leaf rust is the main disease affecting coffee plantations of Brazil and in the world. The aim of this study was to obtain a variety of arabica coffee with high yield, dwarf stature, red fruits, good agronomic and technological characteristics and high resistance to coffee leaf rust. In Brazil were detected 17 physiological races of *Hemileia vastatrix* from 1972 to 2002.

New physiological races of the fungus could therefore be present in the plantations of Brazil. After 44 years of disease epidemics in this country, the SH3 gene is the only resistance factor not yet overcome by *Hemileia vastatrix* races. The BA10 (IAC 1110-8 introduction) containing the SH3 gene is still resistant to prevalent races of coffee leaf rust in Brazil, conferring therefore durable resistance.

MATERIALS AND METHODS

IAC Catuaí SH_3, an dwarf *Coffea arabica* variety developed at the Instítuto Agronômico de Campinas (IAC), SP, Brazil, originated from the F1 cross between the cultivar Catuaí Vermelho IAC 46 and the IAC 1110-8 (BA10) introduction received at the IAC in 1967. Catuaí Vermelho used in the cross is dwarf growing (Caturra gene) and highly productive but susceptible to coffee leaf rust. It is one of the most popular coffee cultivars planted in Brazil. IAC 1110-8 corresponds to an accession (BA10) received from CATIE, Costa Rica, that was selected at the Balehonnur Experimental Station, Mysore, India. There are reports mentioning that BA10 derived from introgression of *C. liberica* into the *C. arabica* species, which could explain some of the characteristics acquired by it. It is a tall-growing variety with high resistance to rust in most of the coffee producing countries, with good yield and excellent cup quality. However, BA10 shows a high percentage of elephant beans, which is a trait that is difficult to eliminate in successive generations derived from BA10. To obtain the IAC Catuaí SH_3 variety, six generations of line selection were carried out for yield, vigor, fruit maturation, rust resistance, outturn, physical bean qualities (percentage of flat, peaberry and elephant beans, weight of 100 dry beans and average bean sieve size), drought tolerance, resistance to *Cercospora coffeicola* (brown eye spot) and cup quality. The rust resistance was assessed by scores from 0 to 4, being 0 and 1 = resistant; 2 = moderately resistant, 3 = moderately susceptible and 4 = susceptible.

RESULTS AND DISCUSSION

The variety IAC Catuaí SH3 was originated from the F1 cross IAC H6839, between the cultivar Catuaí Vermelho IAC 46 and IAC 1110-8 obtained in 1967 in the Centro Experimental de Campinas at the IAC. In segregating generations, plants were mainly select for high yield, vigor, red fruits, resistance to coffee leaf rust and good agronomic and technological characteristics. After six generations, the name IAC Catuaí SH3 was given to the selection. The variety IAC Catuaí SH3 carry the SH3 gene and is still resistant to prevalent races of rust in Brazil, presenting therefore durable resistance. In the 6^{th} generation of field trials, where IAC Catuaí SH_3 was selected recently, this variety showed average green coffee yield in three cropping seasons of 2,478 kg/ha/year and resistance to prevalent races of rust in the places where IAC Catuaí SH_3 was planted in the states of São Paulo, Paraná and Minas Gerais. This variety is carrying the SH_3 gene, which confers resistance to predominating rust races in homozygous condition (SH3SH3). In tests conducted in the greenhouse it was also found that this variety has also good resistance to *C. coffeicola*. Drought tolerance was evaluated under field conditions after periods of prolonged droughts using the Turgency Index test, on a 1-10 point scale. The results were promising, indicating that this variety has good drought tolerance. Furthermore, IAC Catuaí SH_3 is a vigorous variety with medium to late maturing fruits. Average outturn (ratio between the weight of green coffee and fresh coffee berries) was 18.2%, with more than 80% of the flat bean type and less than 5% of elephant beans, a 100 bean weight around 12.9 g and average bean sieve size of 16.9. In preliminary cup tests, IAC Catuaí SH_3 gave excellent results with very characteristic flavor and aroma.

CONCLUSION

1. The IAC Catuaí SH3 variety show good yield, high resistance to coffee leaf rust and good agronomic and technological characteristics.
2. The IAC Catuaí SH3 variety show either good resistance to *Cercospora coffeicola* (brown eye spot), high drought tolerance and excellent cup quality.

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Evaluation and Selection on F1 Hybrids (BA10 X *Coffea arabica*) with Drought Tolerance and Resistance to Coffee Leaf Rust

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SUMMARY

Drought and susceptibility to coffee leaf rust (*Hemileia vastatrix*) are important factors limiting growth and production of *Coffea arabica* varieties. The aim of this study was to evaluate tall growing arabica hybrids for yield, drought tolerance, technological traits and resistance to coffee leaf rust, in order to select superior F1 trees for clonal propagation. The F1 hybrids were obtained in 1972 at the Instituto Agronômico de Campinas (IAC). The field experiment was conducted at the IAC Experimental Station of Campinas and contained, among others varieties, six crosses of cultivar Mundo Novo and six crosses of cv. Acaiá with a coffee introduction named BA10. Acaiá is a cultivar related to Mundo Novo. The BA10 introduction comes from the Balehonnur Research Station in India and is probably derived from a cross between *C. arabica* and *C. liberica*. Was used a randomized block design, with plots of single plants repeated six times for each cross. The data in yields of 16 harvests, expressed in kg of green coffee per hectare and per year, were analyzed. Yield and vigor were also observed in three years on a 10-point visual scale. Drought tolerance observations were made in three years of prolonged drought on a 1-10 point scale for leaf turgency index (IT). Rust resistance was evaluated in the field in two years by using a 0-4 point scale. Five plants from BA10 x Mundo Novo IAC 471-5 (H8421) and one plant from BA10 x Acaiá IAC 474-7 (H8187) were pre-selected as promising ortets. The selected trees of H8421 had average turgency scores ranging from 7.0 to 8.5 and average yields from 1,885 to 2,658 kg/ha/year. The best plant F1 H8421 - 5 got IT 7.7 points and average yield of 2,658 kilograms of green coffee per hectare and per year. The H8187 - 6 plant got IT of 8.0 points and yield of 3,000 kg of green coffee/ha/year. The best control plant of cultivar Acaiá IAC 474-7 had a turgency score of 6.7 points and an average yield of 1,772 kg of green coffee/ha/year. Another control plant H8216 – 2, sensible to drought, gave IT of 4.7 points and average yield of 1,513 kg of green coffee/ha/year. All the selected plants of H8421 and H8187 were highly resistant to coffee leaf rust. The F1 hybrid H8187-6 showed heterosis of 69.3% compared to more productive parent (cv. Acaiá IAC 474-7). In the final selection, two plants, H8421-5 and H8187-6, were selected.

INTRODUCTION

Drought is one of the most important factors that can limit the growth and production of arabica coffee. The same occurs with rust (*Hemileia vastatrix*). Several studies have been conducted to evaluate coffee when to water stress. In the IAC there are several genetic materials that constitute sources of drought tolerance and resistance to rust. The objective of this study was to analyze the F1 hybrids in the Germplasm Bank of IAC, which combine yield, drought tolerance and resistance to rust, in order to obtain superior F1 plants, with high yield, drought tolerance, resistance to rust and excellent agronomic and technological characteristics of seeds for clonal propagation.

MATERIALS AND METHODS

The F1 hybrids with their parents were obtained in 1972 and their relationship is as follows:

F1 hybrids	Parents
H8187	1110-8-5 (BA10) X CP474-7
H8421	CP471-5 X 1110-10 (BA10)
H8411	CP467-1 X (1109-7 X CP387-17)-1-3
H8431	CP474-1 X 1518-2 (S333)
H8517	CMP386-2 X (C1109 -7 X CP387-17-1-3
H8429	CP474-1 X 1120-35 (X321)
H8396	CP382-14 X (C1109-7 X CP387-1-3
H8414	CP467-1 X 1133-2 (Harar)
H8126	C1107-4-1 (BA21) X CP474-4
H8427	CP474-1 X 1110-4 (BA10)
H8420	CP471-5 X 1110-1-1
H8518	CMP386-2-4-9 x 1110-1-1
CP474-7 (control)	Acaiá IAC 474-7
H8216 (control)	1120-16 X 1125-3

The experiment was conducted at the Experimental Station of Campinas, in randomized blocks, with plots of one plant, repeated six times. The F1 hybrids were obtained in 1972 at the Instituto Agronômico de Campinas (IAC). The field experiment was contained, among others varieties, six crosses of cultivar Mundo Novo and six crosses of cv. Acaiá with a coffee introduction named BA10. Acaiá is a cultivar related to Mundo Novo. The BA10 introduction comes from the Balehonnur Research Station in India and is probably derived from a cross between *C. arabica* and *C. liberica*. Were analyzed the data in yields of 16 harvests in kg of coffee cherry. The mean data from each selected plant were converted to kilograms of green coffee per plant and per year by dividing the mean values obtained for each plant, the outturn of 5.5 (relation of fresh coffee berries with green coffee). Subsequently were estimated the average yield in kilograms of green coffee per hectare and per year from each mother plant selected for production and others agronomic and technological characteristics of seeds. The drought tolerance ratings were made under field conditions in three years of prolonged drought by leaf turgency index (IT) assigning 1-10 points F1 plants, being 1 when the plant showed severe wilting and 10 when he was turgid . It is important to note that a method of visual estimation of drought tolerance has been successfully used in cereals and in coffee. Symptoms of drought stress are gradual and culminate with the winding of the plants leaves. The rust resistance was evaluated in the field in two years by assigning 0-4 points, 0 and 1 for leaf rust free (resistant plants), 2 for leaves with little sporulation (moderately resistant plants), 3 leaves for greater sporulation (moderately susceptible plants) and 4 for leaves with high sporulation (susceptible plants). To make the selection of the best plants were also evaluated in terms of evaluation of vigor (IAV vigor) and the rate of visual assessment of production (IAV production). The heterosis of the F1 hybrid H8187-6 was calculated in relation the more productive parent (cv. Acaiá IAC 474-7).

RESULTS AND DISCUSSION

The data obtained from selected coffee (F1 hybrids) compared with the index of turgency (IT), rust resistance, average production of green coffee per plant and per year (16 harvests) and estimated average yield in kilograms of green coffee per hectare and per year, the 16-year period, are found in table 1.

Table 1. Relation of coffee tree F1 hybrid with high size, selected with their respective average rates of leaf turgency index (IT) observed in the experiment EP 132 in Campinas-SP, Brazil, in three years of severe drought, rust resistance, average production of green coffee per plant and per year (16 harvests) in kilograms and estimated average yield in kilograms of green coffee per hectare and per year in the period of 16 years.

F1 hybrid	Parents[1]	Average leaf turgency index (IT)[2]	Rust resis-tance[3]	Average produc-tion per plant and per year (kg)	Estimated yield (kg/ha/year)
H8187-3	1110-8-5 (BA10) X CP 474-7	8.0	1	0.56	1,594
H8187-6	1110-8-5 (BA10) X CP 474-7	8.0	1	1.05	3,000
H8421-1	CP471-5 X 1110-10 (BA10)	8.3	1	0.66	1,885
H8421-2	CP471-5 X 1110-10 (BA10)	8.0	1	0.66	1,885
H8421-3	CP471-5 X 1110-10 (BA10)	8.5	1	0.83	2,372
H8421-4	CP471-5 X 1110-10 (BA10)	7.0	1	0.72	2,058
H8421-5	CP471-5 X 1110-10 (BA10)	7.7	1	0.93	2,658
H8411-3	CP467-1 X (1109-7 X CP387-17)-1-3	7.7	4	0.63	1,800
H8411-5	CP467-1 X (1109-7 X CP387-17)-1-3	8.3	4	0.66	1,886
H8431-6	CP474-1 X 1518-2 (S333)	7.3	4	0.79	2,257
H8517-3	CMP386-2 X (C1109 -7 X CP387-17)-1-3	7.3	4	0.57	1,635
H8429-4	CP474-1 X 1120-35 (X321)	7.7	4	0.84	2,401
H8396-6	CP382-14 X (C1109-7 X CP387)-1-3	8.3	4	0.58	1,663
H8414-2	CP467-1 X 1133-2 (Harar)	7.3	4	0.75	2,143
H8414-3	CP467-1 X 1133-2 (Harar)	6.7	4	0.89	2,549
H8126-6	C1107-4-1 (BA21) X CP474-4	8.0	1	0.59	1,608
H8427-3	CP474-1 X 1110-4 (BA10)	7.0	4	0.73	2,080
H8420-6	CP471-5 X 1110-1-1 (BA10)	6.0	1	0.84	2,407
H8518-5	CMP386-2-4-9 x 1110-1-1 (BA10)	6.0	1	0.75	2,146
	Controls				
CP474-7	Acaiá IAC 474-7	6.7	4	0.62	1,772
H8216-2	1120-16 X 1125-3	4.7	4	0.53	1,513

[1]C1109-7 BA8; 1110 = BA10; CP474-1; -4, -7 = cv. Acaiá; CP 471-5; CP 467-1; CMP 386-2; CP 382-14; and CP387-17 = cv. Mundo Novo.

[2]Leaf turgency index (IT): 1 = plants with wilted leaves; 10 = coffee with turgid leaves.
[3]Rust resistance: 1 = resistant; 4 = susceptible

Analyzing table 1 it turns out, it was possible to select some F1 plants with drought tolerance, rust resistance and high yield. The highlights were the trees of the hybrids H8421 and H8187 derivatives by crossing BA10 and Mundo Novo IAC 471-5 and Acaiá IAC 474-7 cultivars, respectively.

The selected hybrids H8421 had leaf turgency index (IT) ranging from 7.0-8.5 points and average yields from 1,885 to 2,658 kg of green coffee/ha/year. All selected coffee plants were highly resistant to rust. The coffee F1 H8421-5 got 7.7 points of IT and average yield of 2,658

kg of green coffee per hectare and per year. The two coffee plants selected of H8187 were highly resistant to rust. The H8187-6 coffee tree got 8.0 points of IT and 3,000 kg of green coffee/ha/year. The best plant used as control Acaiá IAC 474-7 C280 had 6.7 points of IT and an average yield of 1,772 kg of green coffee/ha/year and another control H8216-2 gave 4.7 points of IT and an average yield of 1,513 kg of green coffee/ha/year. The F1 hybrid H8187-6 showed heterosis of 69.3% compared to more productive parent (cv. Acaiá IAC 474-7) and the F1 H8421-5 got 50.0%. In the final selection, two plants, H8421-5 and H8187-6, were selected. So generally you can select coffee tree F1 hybrids from *C. arabica* with drought tolerance, rust resistance and high yield.

CONCLUSION

1. The F1 H8421-5 coffee tree, with high size, derived from the cross of cultivar Mundo Novo IAC 471-5 with BA10, showed excellent tolerance to drought, high rust resistance and excellent yield (2,658 kg of green coffee/ha/year).
2. The F1 H8187-6 coffee tree, with high size, derived from the cross of cultivar Acaiá IAC 474-7 with BA10, showed good tolerance to drought, high rust resistance and optimum yield (3,000 kg of green coffee/ha/year).

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Evaluation and Selection of *Coffea arabica* Progenies Resistant to Coffee Leaf Rust in Mococa, SP, Brazil

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SUMMARY

The aim of this study was to evaluate 86 progenies of *Coffea arabica* from the Breeding Program Coffee at Campinas Institute Agronomic (IAC). The agronomic and technological characteristics of 80 progenies to coffee leaf rust resistant and six controls cultivars were evaluated at Polo Nordeste Paulista (APTA Regional) in Mococa-SP. The experiment was installed in 2006, following the design randomized blocks with three replicates end seven plants per plot, planted at a spacing of 3.50 x 1.00 m. Five harvests were included in the experiment from 2008 to 2013, except 2011. The genetic materials were classified in groups by the statistical analysis of yields in kilograms of green coffee/ha/year. In addition to the visual assessment of strength and maturity of the fruits, seed characteristics were also studied (percentage of grains flat, peaberry and elephant bean types, 100 seeds weight and average sieve size). The lines 56, 30, 78, 21, 65, 66, 23, 36, 43 and 74 were the ten more productive treatments. The treatment 21 (IAC 5158-2) showed high yield and also the best seed characteristics.

INTRODUCTION

The breeding program aim to obtain superior cultivars of *C. arabica* with high yield and also incorporate resistance to the major diseases of the crop, including the coffee leaf rust caused by the fungus *Hemileia vastatrix* Berk. et Br. [1]. Studies conducted by CIFC identified more than 45 physiological races of rust, of which 20 races with virulence spectrum in coffee derivatives were characterized Timor Hybrid [1]. In IAC, over 64 years of research, numerous progenies with rust resistance have been developed using different genetic materials derived from intra and interspecific crosses [2,3]. The objective of this study was to evaluate the yield, vigor, fruit ripening, resistance to coffee leaf rust and others agronomic and technological characteristics of these materials with potential for commercial planting and are also leveraged to obtain new hybrids with complex genetic and durable resistance to rust.

MATERIALS AND METHODS

Eighty six treatments were evaluated (80 progenies derived from crosses of arabica coffee) in which at least one of the parents showed resistance to rust and six control cultivars, two resistant (Obatã IAC 1669-20) and four susceptible (Catuaí Vermelho IAC-144 and Catuaí Amarelo IAC-62). The progenies with rust resistance are derived from Catimor, Sarchimor, Sarchimor X Catuaí, Catuaí X BA10 and Icatu X Catuaí. For example 12 progenies and yours parents are related:

The experiment was established in 2006 at the Polo Regional do Nordeste Paulista – APTA, in Mococa, SP, Brazil. The experimental design employed was a randomized block with three

replications and seven plants per plot, spacing of 3.50 m between rows and 1.00 m between plants. The yield was obtained with the manual harvest of all plants of the experiment in the years 2008 to 2013(except 2011) which was later analyzed in kg/ha/year. Evaluations of the index of vigor (IAV vigor) plants (notes 1 = low vigor, 10 = highly vigorous) and fruit ripening (P = early, MP = medium to early, M = medium, MT = average for delayed and T = late) before harvesting was also carried out. For studies of beans in 2010 samples of 1.5 kg of coffee cherries of all plots of the experiment was collected for obtaining mean values of percentage of types of beans flat, peaberry and elephant beans, weight of 100 dry beans and average sieve size of each treatment. The yield data were submitted to statistical analysis, using the Scott-Knott test at 5% to compare the treatment means.

RESULTS AND DISCUSSION

In table 1 are presented the data of the average yields of five harvests of green coffee in kg/ha/year and the statistical analysis of 86 treatments of arabica coffee. The average yield of green coffee of the progenies and cultivars analyzed in five years were 1,992 kg of green coffee/ha/year. Yield mean data analyses by Scott-Knott at 5% test indicated that there are significant differences between treatments. The ten best progenies were 56, 30, 78, 21, 65, 66, 23, 36, 43 and 74 with 3,108; 3,030; 2,808; 2,796; 2,754; 2,730; 2,688; 2,610; 2,592 and 2,562 kg of green coffee/ha/year. The data of agronomic and technological characteristics are found in table 2. All treatments analyzed proved to be vigorous, ranging from 6.5 to 8.1 points and 6.5 points of control. The ripening period of fruits in treatments ranged from medium to late. The weight of 100 dry beans ranged from 12.0 to 18.5g, evidencing great variability. The treatment 21 (IAC 5158-2) is noteworthy since exhibit the best results of all data analyzed after five years of harvests. It is a vigorous progeny (IAV vigor = 7.3), medium to late ripening, with qualities of excellent beans of flat, peaberry and elephant beans types (87%, 10% and 3%), respectively, weight of 100 dry beans with value extremely high for arabica (18.5g) and high sieve size average (19.1) standing out from other progenies. Importantly, all progeny exhibited short stature and resistance to coffee leaf rust.

Table 1. Average yields of five harvests in kilograms of green coffee/ha/year and statistical analysis of 86 treatments (progenies) of arabica coffee of the experiment in Mococa, SP, Brazil.

Treatments	Genotypes (progenies)	Yields	Treatment	Genotypes (progenies)	Yields
56	IAC 4520 EP506	3,108 a	45	IAC 4722 L99	1,992 b
30	Obatã IAC 1669-20	3,030 a	55	IAC 4722 L99	1,974 b
78	IAC H13439-4 EP506	2,808 a	5	IAC 5161-23	1,968 b
21	IAC 5158-2	2,796 a	22	IAC 5158-6	1,956 b
65	IAC 4553 EP506	2,754 a	20	IAC 5157-10	1,932 b
66	IAC 4553 EP506	2,730 a	19	IAC 5157-8	1,926 b
23	IAC 5159-1	2,688 a	6	IAC 5161-25	1,920b
36	IAC 4722 L99	2,610 a	14	IAC 5162-8	1,914 b
43	IAC 4722 L99	2,592 a	67	IAC 4555 EP506	1,896 b
74	IAC 1669-31-8 EP506	2,562 a	54	IAC 4722 L99	1,896 b
68	Obatã IAC 1669-20	2,526 a	64	Catuaí SH3 EP506	1,884 b
31	IAC4836	2,520 a	10	IAC 5162-14	1,878 b
72	IAC 1669-31-1 EP506	2,514 a	81	Catuaí SH3 EP506	1,878 b
47	IAC 4722 L99	2,460 a	60	IAC 4520 EP506	1,860 b
58	IAC 4520 EP506	2,448 a	40	IAC 4722 L99	1,848 b
34	IAC 4721 L99	2,382 a	44	IAC 4722 L99	1,842 b
35	IAC 4722 L99	2,370 a	33	IAC 4721 L99	1,830 b
16	IAC 5156-7	2,358 a	18	IAC 5157-5	1,800 b
79	Catuaí SH3 EP506	2,346 a	27	IAC 5160-5	1,788 b
82	IAC 125 RN	2,292 a	86	Catuaí Amarelo IAC 62	1,740 b
57	IAC 4520 EP506	2,268 a	61	Catuaí SH3 EP506	1,734 b
69	Obatã IAC 1669-20	2,256 a	15	IAC 5156-1	1,716 b
41	IAC 4722 L99	2,178 a	77	IAC H13439-8 EP506	1,692 b
39	IAC 4722 L99	2,178 a	1	IAC 5161-3	1,692 b
37	IAC 4722 L99	2,178 a	29	IAC 5160-10	1,602 b
73	IAC 3311-3-2 EP506	2,172 a	46	IAC 4722 L99	1,566 b
76	IAC 1971-1-11 EP506	2,160 a	70	IAC 1669-31 EP506	1,566 b
42	IAC 4722 L99	2,142 a	3	IAC 5161-10	1,530 b
49	IAC 4722 L99	2,136 a	85	Catuaí Amarelo IAC 62	1,512 b
51	IAC 4722 L99	2,136 a	26	IAC 5160-4	1,512 b
53	IAC 4722 L99	2,130 a	52	IAC 4722 L99	1,500 b
59	IAC 4520 EP506	2,112 a	12	IAC 5162-24B	1,476 b
80	Catuaí SH3 EP506	2,112 a	28	IAC 5160-6	1,446 b
11	IAC 5162-28	2,100 a	2	IAC 5161-5	1,404 b
9	IAC 5162-20	2,082 a	83	Catuaí Vermelho IAC 144	1,392 b
32	IAC4835	2,052 b	24	IAC 5160-1	1,392 b
75	IAC 3487-6 EP506	2,046 b	84	Catuaí Vermelho IAC 144	1,356 b
38	IAC 4722 L99	2,022 b	63	Catuaí SH3 EP506	1,338 b
71	IAC 1669-31 EP506	2,016 b	8	IAC 5163	1,302 b
17	IAC 5156-8	2,010 b	62	Catuaí SH3 EP506	1,266 b
48	IAC 4722 L99	2,010 b	4	IAC 5161-14	1,230 b
13	IAC 5162-9	2,010 b	7	IAC 5164	1,212 b
50	IAC 4722 L99	2,010 b	25	IAC 5160-3	942 b

To compare the means used the Scot-Knott test 5%.

[1]IAV vigor = vigor on a scale of 1 to 10, where 1 = low vigor and 10 = high vigor;

[2]Fruit maturation: M = medium; MT = medium to late; T = late;

Table 2. Average data of IAV vigor, fruit maturation and the characteristics values of beans of 10 best treatments (progenies) of arabica coffee, obtained in 2010.

Treatments	Genotypes/progenies	Parents
56	IAC 4520 EP506	Icatu X Catuaí
30	Obatã IAC 1669-20	(Villa. Sarchi. X HT832) X Catuaí Vermelho
78	IAC H13439-4 EP506	Catuaí Vermelho X (Catuaí Vermelho X HT832/1)
21	IAC 5158-2	Villa Sarchi X HT 832/2
65	IAC 4553 EP506	Icatu X Catuaí
23	IAC 5159-1	Villa Sarchí X HT 832/2
36	IAC 4722 L99	Caturra Vermelho X HT 832/1
74	IAC 1669-31-8 EP506	Villa Sarchí X HT 832/2
31	IAC4836	[(Villa Sarchí X HT 832/2 Catuaí Vermelho .] X Catuaí Am.
72	IAC 1669-31-1 EP506	Villa Sarchí X HT 832/2
79	Catuaí SH3 EP506	Catuaí Vermelho X 1110/8
82	IAC 125 RN	Villa Sarchí X HT 832/2

CONCLUSION

1 - The ten best progenies were 56, 30, 78, 21, 65, 66, 23, 36, 43 and 74 with 3,108; 3,030; 2,808; 2,796; 2,754; 2,730; 2,688; 2,610; 2,592 and 2,562 kg of green coffee/hectare/year.
2 - The progeny 21 (IAC 5158-2) presented high yield, superior bean characteristics, with high percentage of beans of the flat type and larger grains with 19.1 of sieve size average.

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Evaluation of Clones Conilon Coffee (*Coffea canephora*) in Cafelândia, SP, Brazil.

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SUMMARY

Clones of Conilon coffee being planted on a large scale in Brazil, mainly in the states of Espírito Santo, Bahia and Rondônia. In the State of São Paulo there is an extensive area provides the planting Conilon coffee. Thus, it is very important to evaluate clones of Conilon and also Robusta coffee in this state. The aim of this study was to assess in Cafelândia, SP , Brazil, the average yield 19 clones of Conilon coffee and two clones of Robusta coffee (clones Ipiranga 501 and Ipiranga 502), used as controls. The experiment was maintained with irrigation, was established in September 2009 at a spacing of 3.80 X 1.00 m for clones Conilon and 3.80 X 1.50 m for the two clones Robusta type. The plots were formed of 40 plants, with two replications. Coffee plants were harvested in the period 2012 to 2013, in kilograms of coffee cherries and the data were converted in kilograms of green coffee/hectare/year using an outturn of 25% for the ratio of green coffee and fresh coffee berries. The six most productive clones of Conilon were EMCAPA 11, Conilon Vitória 3V, EMCAPA 75, EMCAPA 139, Conilon Vitória 2V and Conilon Vitória 13V, with average yields of 3,791; 3,112; 2,898; 2,483; 2,378 and 2,374kg of green coffee per hectare and per year, respectively. The clones Ipiranga 501 and Ipiranga 502 used as controls yielded 2,069 and 2,580 kg of green coffee per hectare and per year, respectively. Data from this study show the possibility of planting successfully clones of Conilon and Robusta coffee in São Paulo, since the trees are kept under irrigation.

INTRODUCTION

Clones of Conilon coffee being planted on a large scale in Brazil, mainly in the states of Espírito Santo, Bahia and Rondônia. In the State of São Paulo there is an extensive area provides the planting Conilon coffee. Thus, it is very important to evaluate clones of Conilon and also Robusta coffee in this state. The aim of this study was to assess in Cafelândia, SP , Brazil, the average yield of 19 clones Conilon coffee and two clones of Robusta coffee (clones Ipiranga 501 and Ipiranga 502), used as controls.

MATERIALS AND METHODS

The experiment was maintained with irrigation, was established in September 2009 at a spacing of 3.80 X 1.00 m for 19 clones Conilon and 3.80 X 1.50 m for the two clones Robusta type. The plots were formed of 40 plants, with two replications. Coffee plants were harvested in the period 2012 to 2013, in kilograms of coffee cherries and the data were converted into kilograms of green coffee per hectare per year using an outturn of 25.0% for the ratio of green coffee and fresh coffee berries.

RESULTS AND DISCUSSION

The data obtained for the 21 clones in relation the average yield of green coffee, (2012 and 2013) in kg/ha/year and the agronomic characteristics in 2013 are found in Table 1.

Table 1. Average yield of two harvests (2012 and 2013) in kilograms of green coffee/hectare/year and agronomic characteristics in 2013 of 19 clones Conilon coffee and two Robusta clones coffee in the experiment in Cafelândia SP, Brazil.

Clones *C. canephora*	Average yield of green coffee kg/ha/year	Agronomic characteristics in 2013				
		Height plants (m)	Diameter canopy (m)	IAV[1] vigor	IAV[2] production	Maturation[3]
Conilon						
1V	1,604	2.0	1.8	7	7	P
6V	1,357	2.0	2.2	9	9	P
8V	579	2.2	2.4	8	3	P
11V	1,920	1.9	2.1	7	3	P
12V	1,415	1.9	1.9	7	7	P
03	708	2.0	2.5	9	2	P
26	1,139	2.0	2.1	9	4	P
4V	1,826	2.0	2.0	7	8	M
7V	1,675	2.0	2.0	9	8	M
10V	2,069	1.9	2.2	8	3	M
11	3,791	1.9	2.3	7	6	M
16	1,920	2.1	2.0	8	8	M
3V	3,112	2.3	2.6	10	10	MT
5V	1,541	2.2	3.0	8	2	MT
13V	2,374	2.1	1.9	8	6	MT
120	2,076	2.1	2.3	7	7	MT
139	2,483	2.1	3.0	6	3	MT
75	2,898	2.2	2.6	8	8	MT
2V	2,378	2.0	2.0	8	7	MT
Robusta						
Ipiranga 501	2,069	2.5	2.3	10	7	T
Ipiranga 502	2,580	2.4	2.9	9	9	MT

1. IAV Vigor: 1 = low vigor; 10 = high vigor;
2. IAV Production: 1 = low production; 10 = high production
3. Fruit Maturation: P = early; M = medium; MT = medium lo late; T = late;

Seven clones presented early maturation, five medium, eight medium to late and one late maturation. All the clones were vigorous. The six most productive clones were EMCAPA 11, Conilon Vitória 3V, EMCAPA 75, EMCAPA 139, Conilon Vitória 2V and Conilon Vitória13V, with average yields of 3,791; 3,112; 2,898; 2,483; 2,378 and 2,374 kg of green coffee per hectare and per year, respectively. The clones Ipiranga 501 and Ipiranga 502 used as controls yielded 2,069 and 2,580 kg of green coffee per hectare and per year, respectively.

CONCLUSION

1. The six most productive clones were EMCAPA 11, Conilon Vitória 3V, EMCAPA 75, EMCAPA 139, Conilon Vitória 2V and Conilon Vitória13V, with average yields of 3,791; 3,112; 2,898; 2,483; 2,378 and 2,374 kg of green coffee per hectare and per year, respectively.
2. The clones Ipiranga 501 and Ipiranga 502 used as controls yielded 2,069 and 2,580 kg of green coffee per hectare and per year, respectively.
3. The data obtained from this study show the possibility of planting successfully clones of Conilon and Robusta coffee in São Paulo, since the trees are kept under irrigation.

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Where on Earth Is Coffee Grown? Spatial Disaggregation of Harvested Area Statistics Using Suitability Data

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SUMMARY

To date, knowledge on the physical distribution of global coffee production is limited. Publicly available datasets of coffee production are incomplete or of limited value to coffee research. Spatially explicit data on the physical distribution of coffee production would help to understand and design policies that address challenges to the industry that result from global change processes and resource limitations.

Here, we demonstrate a method to spatially disaggregate global harvested area production statistics from FAO using point location based suitability maps. Machine learning methods are applied to distinguish climate in coffee production countries from the climate at geo-referenced locations of coffee production to derive spatially explicit probabilities of presence of coffee production sub-nationally. Using a cross entropy approach harvested area statistics for *C. arabica* and *C. canephora* are allocated to areas with suitable climate. Finally, we compare our dataset with other available datasets and demonstrate that our approach best represents the existing knowledge of the global distribution of area dedicated to coffee production.

The resulting dataset may serve to more adequately represent coffee production in research that is interested in the spatially explicit analysis of land use, land use change and resource management than before possible.

INTRODUCTION

Policies that address the challenges from stresses to coffee production systems are in high demand. Their evaluation requires an integrated systemic view of economic and physical spatial relationships. Despite the significance of coffee production in the coffee regions little is known about their exact global distribution. Available spatially explicit datasets of crop distribution are unsatisfactory in their representation of coffee production, e.g. Monfreda et al. (2008) and You and Wood (2006) aggregate data in a generic "green coffee" category.
As a step towards a better understanding of the spatial dynamics of coffee production associated land and resource use we here describe a method to spatially disaggregate coffee production data based on coffee production statistics, subnational land use statistics, satellite data of land cover, and climatic suitability information.

METHODS

First, a database of harvested area statistics is disaggregated into productions systems. Then, spatially explicit prior probabilities that a production system is present in a pixel cell are derived. Finally, the harvested area statistics database is spatially disaggregated using the

prior probability and an entropy approach. For evaluation independent data from subnational surveys is used as reference distributions.

For total area harvested of both main coffee species we use data from the Food and Agriculture Organization of the United Nations of the "green coffee" category averaged over the years '98 – '02 (FAO 2012). In order to separate the harvested area statistic into area for Arabica and area for Robusta production shares are calculated from data provided by the United States Department of Agriculture for the same time period (USDA 2012). To disaggregate according to production systems we rely on data provided in a recent publication by Jha et al (2014). The result is a dataset of harvested area in 4 categories: Arabica- shaded, Arabica-unshaded, Robusta - shaded, Robusta - unshaded.

A prior probability of presence is calculated for each system based on three principal data sources for known subnational distributions, suitability and land cover: Known subnational distributions of "green coffee" harvested area are downloaded from AgroMaps (FAO 2014). The suitability maps are based on different machine learning classification algorithms and various plausible parameter combinations (Bunn et al. 2014).The disaggregation should avoid assigning area to regions where current land use makes coffee cultivation unfeasible, e.g. urban areas, water bodies, or forest. The GLC2000 global land cover database offers a globally coherent classification of land cover for the year 2000 (European Commision 2003).
The prior probability p in pixel i for each species j and system l is then given by: p_{ijl}=$agromaps_i$*$suitability_{ij}$*$landcover_{il}$

Disaggregation of harvested area by species and system is then done by minimizing equation (1) in GAMS 23.8.2.

$$\min \sum_i \sum_j \sum_l \left(\frac{Area_{ijl}}{\sum_{Country} Area_{jl}} - \frac{p_{ijl}}{\sum_{Country} p_{jl}} \right)^2 \forall country \quad (1)$$

s.t. $$\sum_i Area_{ijl} = Area_{Country} \forall j \forall l \quad (2)$$

$$\sum_j \sum_l Area_{ijl} \leq Available\ Area_i \forall i \quad (3)$$

Conditions are that all area for each country by species and system equals is disaggregated to grid cells (equation 2), and that the area within a grid cell does not exceed available area in a grid cell (equation 3). The maximum area is estimated as 1715ha (20% of total grid cell area) under sun and 857ha (10% of total grid cell area) under shade.

We compare the resulting maps for Arabica and Robusta production under shade and sun with subnational data not included in AgroMaps, and the Monfreda and MapSpam "green coffee" data.

RESULTS

For each coffee production system a map of the distribution of area on 5" is derived. Due to space limitations only the Arabica under biodiverse shade map is shown in Figure 1.
Visual comparison of our model with other efforts shows the differences between the results of outcomes. Due to space limitations only the Ethiopia data is shown. Ethiopia does not produce Robusta style coffee. Most production is Arabica under biodiverse shade and no subnational data is available from AgroMaps. Disaggregation results differ for this country. Compared to the survey data from IFPRI 2006 ((Tadesse 2006) adapted from Ruegsegger

2008) the Monfreda land cover based effort appears to place coffee outside of actual coffee regions. MapSpam reflects the actual pattern a little closer. Our effort describes major coffee regions somewhat better but nevertheless overpredicts area in the Southern part of Ethiopia (Figure 2).

Compared to the other efforts our approach reflects actual distributions with greater certainty than previous efforts. Especially where no subnational data is available or production is characterized by biodiverse shade this effort is less likely to misplace area to grid cells that are unfeasible. Nevertheless, some area is underestimated, though most error seems to be of the commission type.

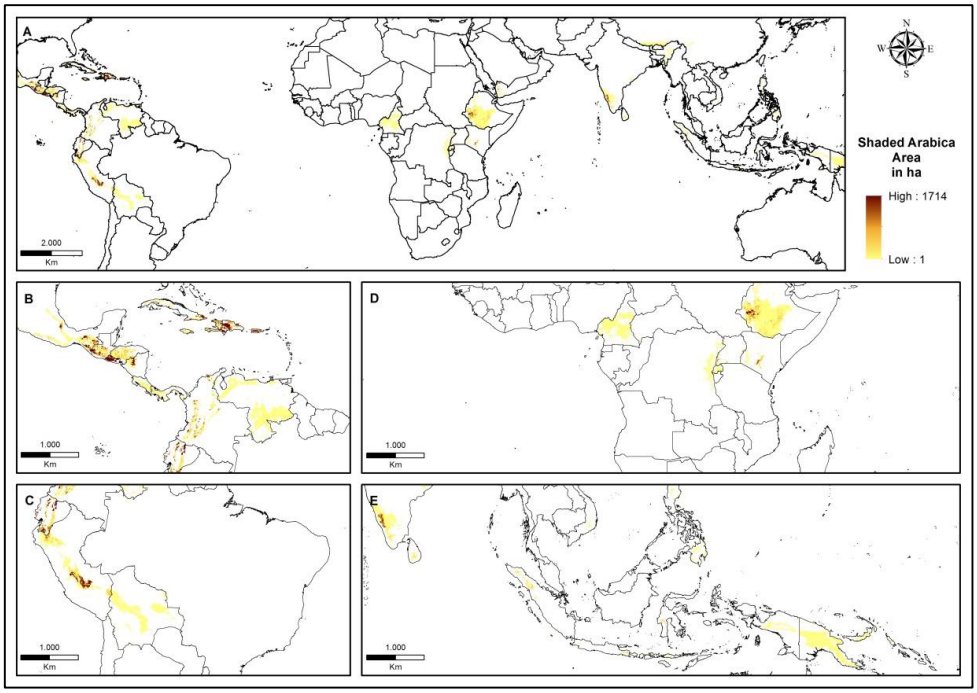

Figure 1. Distribution of Arabica area under biodiverse shade in ha. A) Global; B) Central America; C) Brazil; D) Africa; E) South Asia.

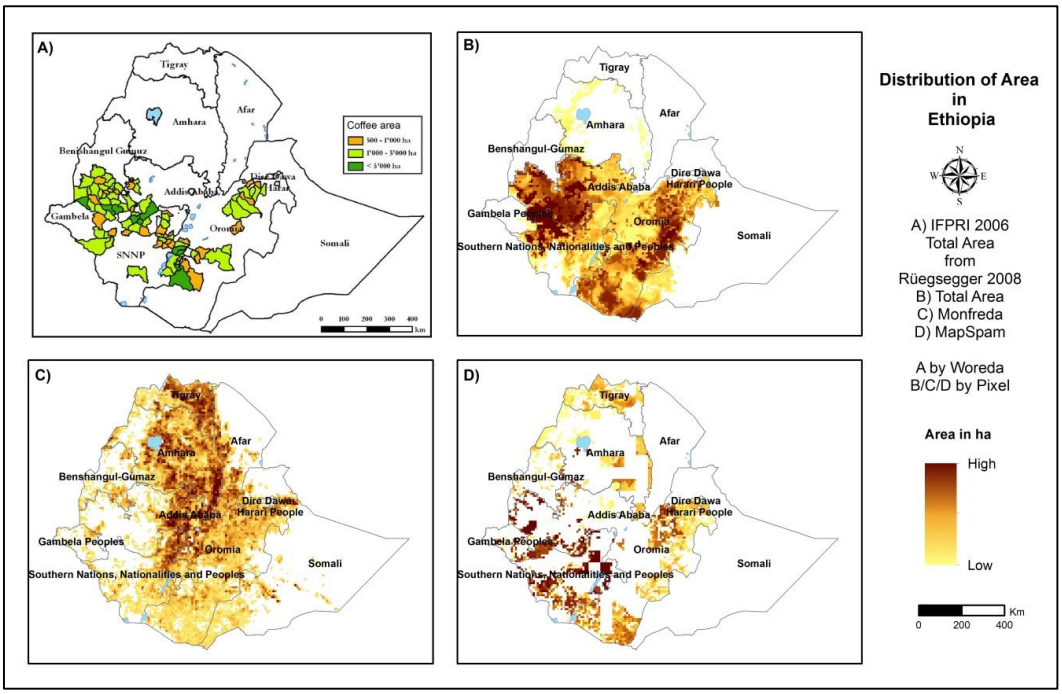

Figure 2. Comparison of area distribution of different data sources: A) IFPRI 2006 survey data (adapted from Ruegsegger 2008); B) Total area aggregated over Arabica and Robusta; C) Monfreda; D) MapSpam.

CONCLUSION

Previous efforts to disaggregate harvested area statistics to grid cell level are unspecific to coffee despite its high importance in some regions. Here, we addressed this issue by combining input data from several sources.

Results are a database and maps of coffee production area for each coffee producing country by the coffee production systems “arabica under sun”, “arabica under biodiverse shade”, “robusta under sun”, and “robusta under biodiverse shade”. No reference data for these systems is available so that for comparison with subnational production data the spatially explicit data had to be aggregated by species or a generic “coffee” category. The model results show reasonable alignment with survey data. Major production regions are well reflected but minor regions are often underestimated.

We conclude that despite its shortcomings this disaggregation effort represents an improvement over previous efforts even when aggregating into a generic green coffee category. In addition we present the first globally coherent spatially explicit dataset of a coffee specific disaggregation into production systems. These systems differ fundamentally in their resource requirements, the climatic and socio-economic conditions under which they strive, and also in their typical outputs. This effort therefore is an important step towards an improved spatial analysis of coffee production globally. Furthermore, the methodology presented here has the potential to be applicable to similar crops that are equally underrepresented in subnational datasets such as cocoa, banana or rubber trees.

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Effect of Shade on the Severity of Coffee Leaf Rust

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SUMMARY

Coffee Leaf Rust (CLR) caused by the fungi *Hemileia vastatrix* Berkeley and Broome has been causing leaf losses of up to 70% in Arabica coffee. The aim of this study was to determine the effect of shade on the progression and severity of Coffee Leaf Rust in Murang'a County in Kenya. Thirty eight (38) small scale coffee farms were randomly selected. On each farm, four (4) coffee bushes of which two were in tree shade and two were in open sun were selected and marked. Four branches were marked from the top, middle and lower canopies of the bush. Ten leaves were then marked on each branch clearly showing the oldest and the youngest leaves. Scoring was done on the number of lesions per leaf, level of severity and the period between infection and leaf fall. ANOVA showed that coffee bushes under shade retained the infected leaves for significantly longer periods than bushes in open sun while the disease severity was significantly higher in the trees in shade than open sun. The results indicate that shade can be used to manage CLR.

INTRODUCTION

Since its introduction in 1893, coffee has remained among the most important cash crops in Kenya. The coffee industry has seen tremendous growth in terms of innovations and research. However, it has also been through challenges such as low world prices, preference for other enterprises, effects of climate change and upsurge of insect pests and diseases. One of the major diseases that constrain coffee production in Kenya is Coffee Leaf Rust (CLR) that is caused by the fungus *Hemileia vastatrix* Berkeley and Broome. CLR was first reported in cultivated coffee in Kenya around 1912 (King'ori & Masaba, 1994). It is a major disease of Arabica coffee causing significant economic losses and has been reported from over fifty (50) coffee growing countries (Silva et al., 2006; Daivasikamani and Rajanaika, 2009). The disease mainly attacks leaves forming yellow pustules resulting to defoliation. Severe rust incidence may cause 50% defoliation and 70% berry loss (Daivasikamani & Rajanaika, 2009).

The key breakthrough in management of CLR was development of resistant coffee varieties. However, most farmers still maintain the susceptible varieties hence the need for other disease management strategies. Moreover, *H. vastatrix* has been shown to develop physiological races that are able to break resistance in coffee varieties (Silva et al., 2008; Gichuru et al., 2012). The roles of cultural practices such shade in the management of CLR cannot be overlooked as studies have shown that shade influences coffee pests and diseases (Adejumo, 2005). The aim of this study was to ascertain the effects of shade on the severity of CLR in terms of defoliation within the main coffee growing region in Kenya.

MATERIALS AND METHODS

Study site

The study was carried out in Murang'a County in Central Kenya. The county has two rain seasons and temperatures of 21-35^{0}C. The average altitude is 1298metres above sea level. The soils are volcanic loam. The average rainfall is 1200mm to 1600mm biannually. The long rains are received between March and August while the short rains usually fall between October and December. As a result of the varying altitudes, Murang'a can get quite cold from May to Mid-August and may experience hail.

Experimental design

Thirty eight (38) small scale farms were selected at random for a disease survey from lists of growers in each co- operative society in Murang'a County. The selection criterion was based on farms that have not been sprayed with fungicides for a period of not less than 5 years and have more than 50 bearing bushes either under shade or in full sun. Under each level of shade, two trees were selected and marked in the 8 farms. On each tree, four branches were selected such that they represented the top, middle and lower canopy levels on the four directions of the campus. On each of the branches, 10 leaves were marked with one (1) being the oldest leaf and ten (10) the youngest leaf.

Disease assessment

The number of pustules per leaf, period between infection and leaf drop and level of severity were recorded monthly. Severity was scored using the scale by Capucho et al., (2011) with slight modifications to capture low disease levels. The farms were surveyed from December 2013 to July 2014.

Results and discussion

The trees under shade had a significantly ($P < 0.05$) higher number of leaves per branch over time than the trees in full sun as shown in Figure 1. The results show that the trees under shade retained the infected leaves for longer time than the trees in full sun. This supports the findings reported by Craves (2011) that shade increases the life of leaves and their size, so rust spores have more leaf area to colonize and time to be dispersed. The severity of CLR was significantly ($P<0.05$) higher in the shaded coffee than in the unshaded coffee (Figure 3). This supports the results of López-Bravo et al., (2012) that with standard fruit load, the intensity of the coffee rust epidemic was greater in the shaded subplot, with a 21.5% increase in incidence and a 22.4% increase in severity. However, regression analysis of the infection and the number of leaves showed there is correlation between the number of leaves and the percent infection (Figure 2). This explains the high disease level in the shaded coffee as compared to the coffee under full sun. This shows that in order to determine the effect of shade on the severity of CLR, more factors need to be considered, other than the number of pustules per leaf and the infected leaves as has been the case in many studies.

CONCLUSION

This study indicated that CLR can be effectively managed using shade. However, all disease influencing factors and pests present should be taken into account in order to find the right level of shade, type of shade trees, period of shading where positive ecological control mechanisms are enhanced and negative effects are reduced (Alwora & Gichuru 2014).

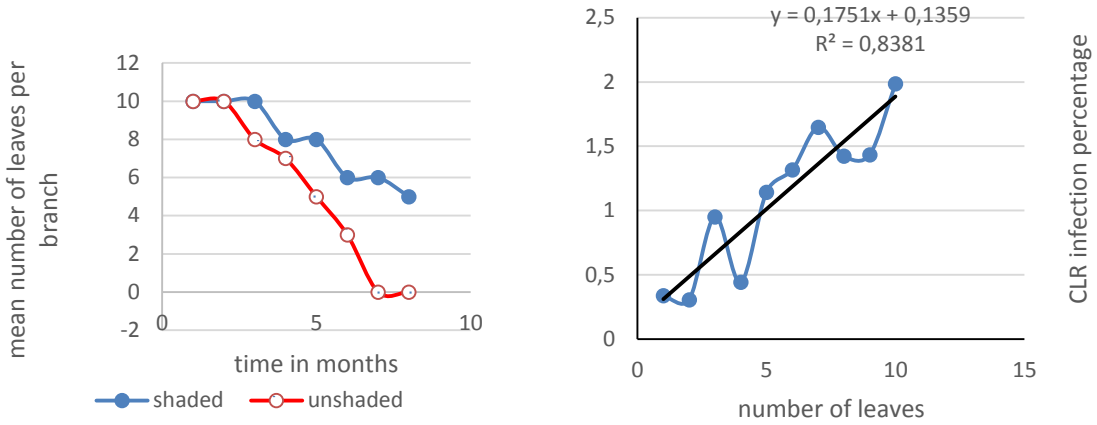

Figure 1. (left) Average number of leaves per branch on shaded and unshaded coffee trees after infection. Figure 2. (right) Correlation of percent CLR infection and the number of Leaves.

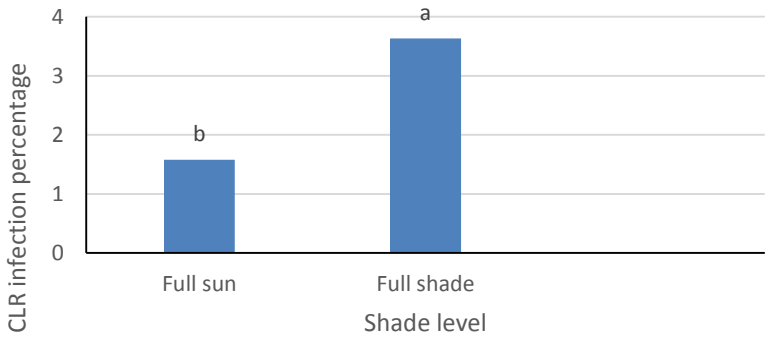

Figure 3. Percent CLR infection on shaded and unshaded coffee trees.

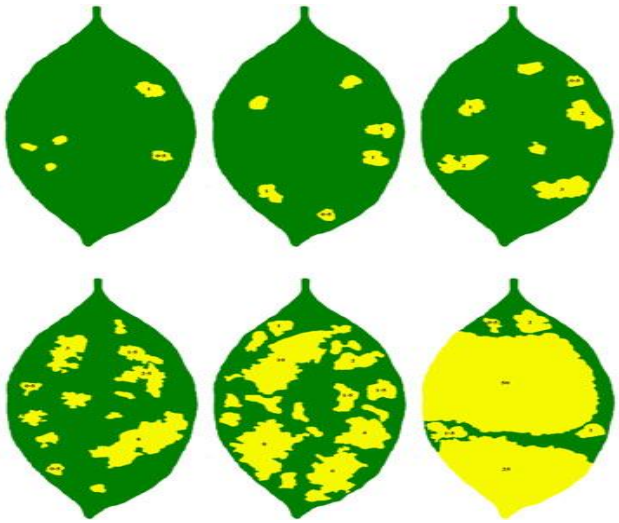

Figure 4: The standard area diagrams of coffee leaf rust severity on (Coffea spp.). The data are expressed as the percentage (%) of the foliar area displaying symptoms of the disease. 2.5,5,10,20,40 and 80% levels respectively (Capucho et al., 2011).

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Modeling the Berry Distribution in AmostraCafe3D Software for Coffee Arabica Reconstructions in 3D

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SUMMARY

Coffee mock-ups are reconstructed after plant coding using multi-scale trees graph (MTGs). AmostraCafe3D software is being developed to generate the MTGs of whole coffee plants by data inclusions at metamer scale in partially coded axes. The aim of this work was to implement the equations of metamer/berry distributions and the probability of berry occurrences on AmostraCafe3D. The implemented linear regressions of phyllochron ratio between orthotropic and plagiotropic axes helped to better distribute the metamer number and the metamer length and the Gaussian model with logical rules modeled the berry distribution along the 2nd order axes. The future development intends to allow the software for user data and models entrance, extracted from their own experiments.

INTRODUCTION

The analysis of plant architecture aims to understand the topological patterns of plant construction and the geometric component localization and their interactions with environmental factors. Considering the whole plant structure, from germination to death, the architectural analysis has one global, multi-scale and plant structure/development dynamics approach. One of the most frequently used mathematical methods for the plant topology and geometry representation is multi-scale trees graph (MTG). MTGs are hierarchical objects that contain the topological elements represented by vertex connections in graphs and eventually, the geometric attributes.

Coffee mock-ups are reconstructed after plant coding using MTGs with three scales of description - plant, shoot and metamer. In the protocol of coffee plants coding the orthotropic axes are always described at metamer scale by a number of variables: length of each internode, length/width, elevation angle/ and cardinal orientation of leaves, and position/orientation/total length of borne plagiotropic branches. In partially coding coffee plants, four 2nd order plagiotropic branches are sampled (each oriented to one of cardinal point) and described in details, to represent each 30 to 40cm-thick layers along the vertical tree profile. All other 2nd order plagiotropic axes are described by their position along the orthotropic axis, their total length, total berry number and the cardinal orientation. The 3D reconstructions of the coffee plant mock-ups are performed under the VPlants software.

AmostraCafe3D, Python-based software, is being developed to generate the MTGs of whole coffee plants with augmented data by data inclusions at metamer scale in partially coded axes, considering the probability of branching/leaf presence in a cardinal orientation. AmostraCafe3D treats the leaf and branch number (3rd to 5th order axes) based on estimated probability of respective leaf and branching distributions on sequences of 2nd order plagiotropic axes, computed for the correspondent orientation/layer/observation period. For

this purpose, the randomized algorithm is run. The proposed modelling methodology in AmostraCafe3D results in a slightly overestimation of the leaf area and the berry distribution should be explored. The aim of this work was to implement the equations of metamer/berry distributions and the probability of berry occurrences in an improved version of AmostraCafe3D, called AmostraCafe3D-v2.

MATERIALS AND METHODS

The experimental data were collected at IAPAR, Londrina (23°18'S and 51°17'W), Brazil. Two densities (6,000 and 10,000 plants ha^{-1}) combined with two planting patterns (square – Q and rectangular – R), resulted in four treatments (Q_{10}, R_{10}, Q_6 and R_6) of coffee plant management. Plants were codified as multi-scale tree graphs – MTG, considering complete or partial measurement at metamer scale. Two periods were considered, each occurred few days before the berry harvests (1st production year - June 2010 and 2nd production year - May 2011. The MTG data extraction was performed using the AMAPstudio - XPlo software, a free computational tool aimed to explore the plant architecture at different scales.

SOFTWARE DEVELOPMENT

The equations for AmostraCafe3D-v2 were obtained by manipulation of experimental data sets extracted from MTGs, which were crossed and combined to logical rules referent to the coffee Arabica architectural patterns. The extracted results from AMAPstudio - XPlo were used to generate linear regressions (established between the orthotropic bearer and metamer number / length of plagiotropic branches) and berry distribution models.

The berry distribution was implemented to follow the general curves found in experimental datasets (Figure 1). Dependent on period and data availability, the differentiation among mature – *mb* (Figure 1A) and immature – *ib* (Figure 1B) berries was performed. The curves were adjusted to the berry zone modifications (by rules for zone definition dependent on plant management and plant development) respecting the orthotropic and plagiotropic indexes (branching order 1st – 3rd).

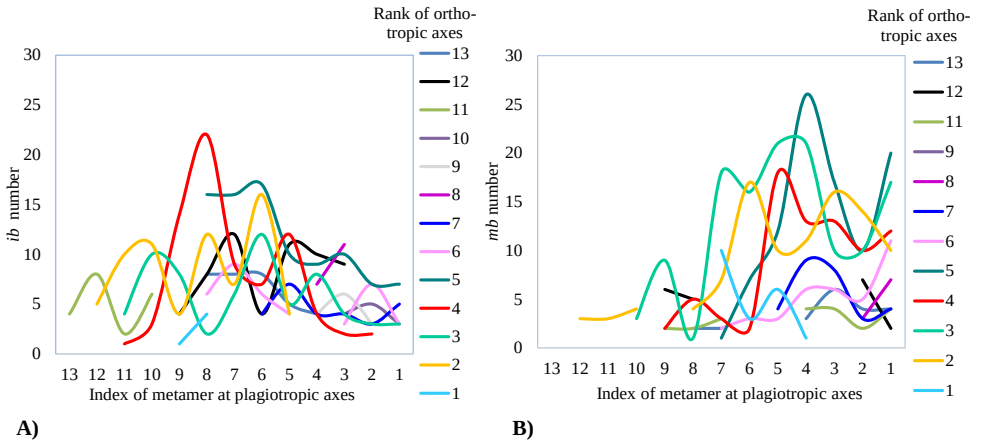

Figure 1. Examples of berry distribution datasets referent to 1st year production and Q_{10} plant management treatment. A) Distribution of immature (*ib*) and B) mature berries (*mb*).

AmostraCafe3D-v2 was focused on berry distribution and a diagram of steps performed on the software, reeling vegetative and reproductive attributes, is shown at Figure 2. Following the AmostraCafe3D-v2 diagram, the first step consisted on the definition of restriction rules

that were relied to the spatial limits of the berry bearing element positions in 3D (indexes of metamers along the orthotropic and plagiotropic axes). Posteriorly, the vectors of completely measured axes were coded (0, 1) to define presence (1) or absence (0) of berries at metamer scale (Figure 2). For partially measured axes, the probability of fruit presence/absence was computed by random algorithm. The third step consisted on the exploration of distribution curves (Figures 1), using the mathematical models.

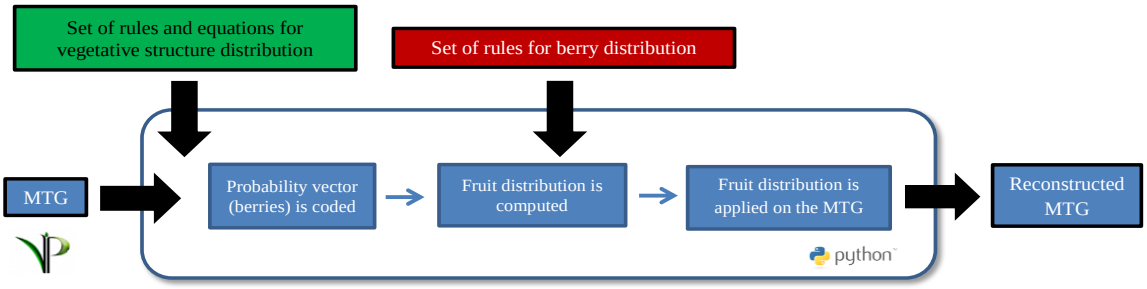

Figure 2. The simplified diagram of steps performed on the AmostraCafe3D-v2 software.

In modelling the berry distribution along the plagiotropic axes, a Gaussian model was included being the most reliable [eq.1]:

$$f(x) = a.e^{-\frac{(x-b)^2}{2c^2}} \quad (1)$$

where *a* is a curve height, defined as the maximum number of berries (*mg* or *ig*); *b* is a position of curve peak center, defined as the local of highest incidence of berries; *c* controls the length of the sinus and *x* is the index of a plagiotropic internode. The modification of curve center allowed the curve deformation and asymmetry along the plagiotropic axes to reproduce the most adequate berry distribution. The fourth step resulted in computed berry distribution based on computed metamer number, computed sequences of berry presence/absence and measured total berry number per axes.

CONCLUSION

To validate the structural model, the slope inclination of linear regressions of number of phytomers of plagiotropic axis by the orthotropic rank was calculated from the MTGs of partially measured plants reconstructed with AmostraCafe3D-v1 (Fig. 3A) and AmostraCafe3D-v2 (Fig. 3B). The implemented linear regressions (the phyllochron ratio between orthotropic and plagiotropic axes) in AmostraCafe3D-v2 helped to better distribute the metamer number and the metamer length (Fig. 3B).

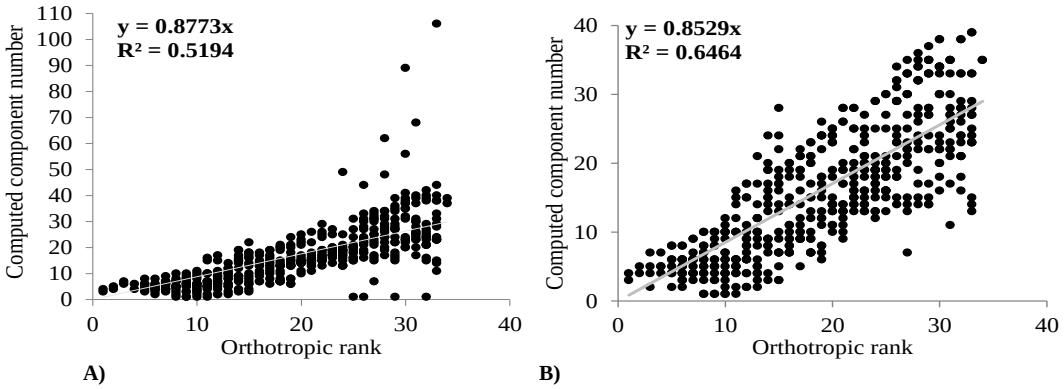

Figure 3. The linear regressions between the phytomer number on plagiotropic axes (coded from top to the bottom) and ranks on orthotropic trunk - example of Q_{10} in a 2nd year of production - 2011. A) AmostraCafe3D-v1 and B) AmostraCafe3D-v2.

Additionally, the method was validated by reduction of MTGs of completely coded coffee plants (1st year of production, 2010) in according to the sampling protocol and the berry distribution modeling. The obtained curves of computed berry distribution (Fig. 4) along the 2nd order axes of various orthotropic ranks reproduce well the original ones (Fig. 1).

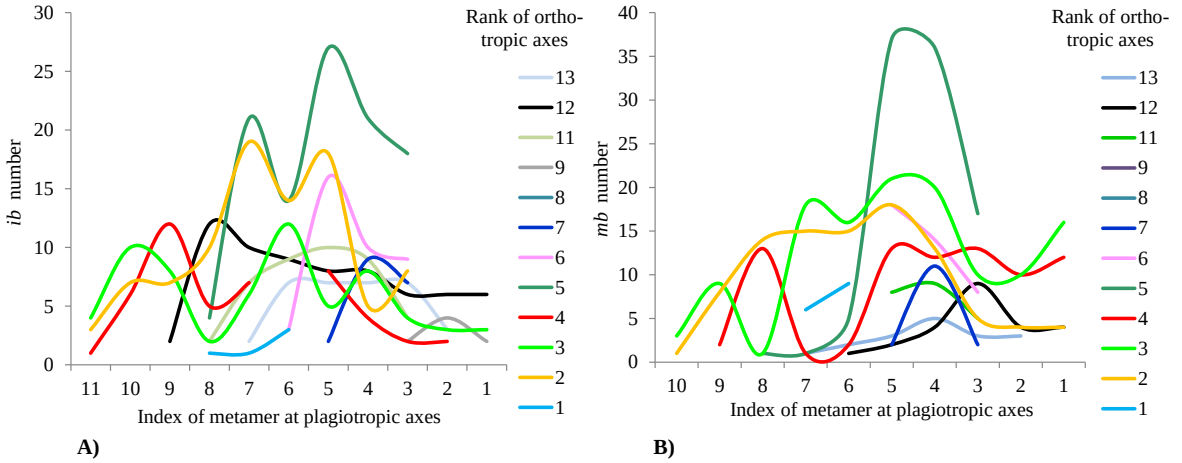

Figure 4. The example of dataset of berry distribution referent to 1st year production and Q_{10} plant management treatment. A) Distribution of computed immature (*ib*) and B) mature berries (*mb*) on reconstructed partially coded plants at metamer scale.

AmostraCafe3D-v2 explores the modelling techniques and experimental data, by integration of the knowledge about the coffee plant architecture at different scales – metamers, axes, plant and datasets from planting plots. This integration occurs in two directions, from the finest scale (metamers) to the largest one (production plots), and in *vice-versa*. The future development of AmostraCafe3D-v2 intends to implement the commodities for larger pallet of users, where the distributions and final berry production would be possible to be computed, based on user data, or recomputed based on bibliography available in software.

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The Potential of Arabusta to Sustain the Indonesian Arabica Production: Comparative Studies of Cup Qualities

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SUMMARY

The Arabusta coffee is originally coming from interspecific cross between Arabica and tetraploid Robusta. One of the famous Arabusta in the world was natural cross coming from Timor island famously namely *Hibrido de Timor* (HdT) which become the parent of many Catimor varieties. During the beginning of 1980's, the HdT seeds were spread largely to almost all Arabica production areas in Indonesia. This genotype in the recent decade, however, becomes the farmer's choice in many areas as practical Arabica varieties due to its superior agronomic performance compared to major type of Indonesian Arabica presently namely Typica, S line, and Catimor, although cup taste qualities of this genotype is often questioned. Facing of this situation, the Indonesian Coffee and Cocoa Research Institute (ICCRI) has been investigated the Arabusta cup qualities from several production areas of Arabica (Aceh, East Java and Bali). This investigation showed that Arabusta is not consistently having lower cup qualities mainly to Catimor varieties in each studied location, means that Arabusta could also have better cup quality. However, next Arabusta crossing generation with existing varieties were showing even high cup quality compared to Typica. Therefore, this study suggests that Arabusta is one of the promising alternatives to sustain Arabica production in Indonesia, especially if we could select the genotypes having good cup quality. In other side, this genotype is mainly better to used as parent in the breeding programs.

INTRODUCTION

Indonesia is one of important producer countries of Arabica (*Coffea arabica*) coffee even only contributed to 2 % of world production. The importance of Indonesian Arabica coffee is due to their high qualities which lead to almost all production areas known for their specialty grade such as Gayo, Mandhaeling, Linthong, Java and Toraja. Furthermore, some new comers already recognized in the world market during the last decade namely Bali-Kintamani, Flores-Bajawa, Java Ijen-Raung and Java-Preanger.

Behind the popularity of Indonesian Arabica coffee, several problems in the plantations are become more seriously threatening the beans production such as Coffee Berry Borer, Leaf Rust and climatologic changes of increasing temperature and erratic precipitations. Facing of those conditions, the recent Arabica varieties in Indonesia are seems become failure to produce optimum yield, as well as the quality. In other side, the Arabusta trees are still keep it good performance of yield and resistance to the many pest and disease, mainly for leaf rust (*Hemileia vastatrix*), and having good adaptation to various environment conditions. The good yield performance of Arabusta is also supported by the big size of beans which is an important agronomic trait for mostly coffee farmers. Those contradictive conditions have been finally lead the Arabusta as variety of choice in many production areas even the cup taste quality of this genotype is still questioned.

Indonesian Arabusta is actually progenies of natural cross between Arabica and Robusta coffee introduced in 1980's directly from Timor island and distributed largely to many Arabica area. This genotype, famously called *Hibrido de Timor* (HdT), has been cultivated separately or mainly mixed with many others varieties. In the recent conditions, this Arabusta is still found in their original genetic constituent, but in some places have been crossed by chance with previously existing varieties of Catimor, S-line and Typica. Some of the crosses become popular and spread widely in certain area.

This research is aimed to evaluate the potential of Arabusta to sustain the Indonesian Arabica production, mainly for the cup qualities which is the most important characteristic for specialty grade.

MATERIALS AND METHODS

Samples were collected from three production areas of specialty coffee namely Aceh, East-Java and Bali. Those samples were processed in the same method for each area namely full wet-dry hulling process for East-Java and Bali, and full wet-wet hulling for Aceh. Samples were then grouped in the similar altitude for each area before cup test conducted. Samples coming from East-Java were grouped into two ranges of altitude namely 1300 m to 1400 m asl (Group A) and 1400 to 1500 m asl (Group B), whereas samples from Aceh were grouped in the altitude of 900 m to 1000 m asl (Group C) and 1300 m to 1400 m asl (Group D). Samples from Bali were only collected from one similar altitude of 1200 m to 1300 m asl (Group E). Finally, each group of A to E was consisted of eleven, ten, six, six and four samples, respectively.

All bean samples were medium roasted and cup tested by three panelists following the method of Specialty Coffee Association of America (SCAA). Results of observations were performing using simple statistic profile.

RESULTS AND DISCUSSION

Results of cup taste assessment for all groups are presented in Figure 1, covering six main attributes. According to the compared result from all groups, the Arabusta was performing generally lower cup quality rather than others varieties especially in group D. However, even has generally lower cup quality than other varieties as showed in Table 1, total score of some Arabusta could reach > 80 which means considered as specialty grade. These conditions perhaps due to farmers have been accidentally selected the better cup taste tree as source for propagation. The possible Arabusta selection with specialty grade has also been done by Gimase et al. [4]. In other side, Arabusta cup taste showed relatively equal quality especially with existed Catimor varieties, and even has better taste as showed in Group D and E.

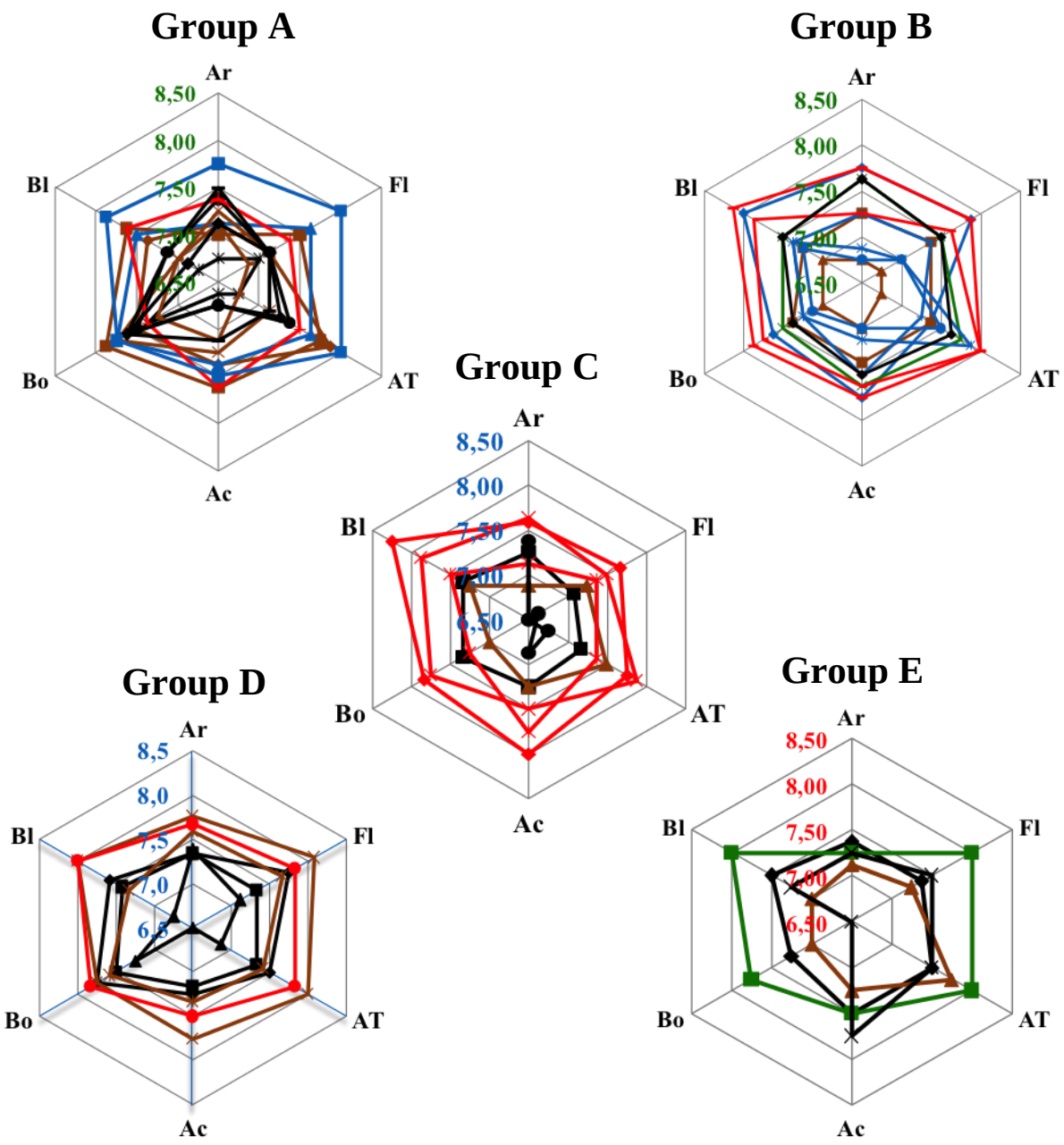

Figure 1. Result of cup tasting from five groups samples based on their origin and altitude. Note: Ar= Aroma, Fl = Flavor, AT = After taste, Ac = Acidity, Bo = Body, Bl = Balance, black line = pure HdT, brown line = Catimor, green line = S-line, red line = Hybrid HdT, blue line = Typica.

Table 1. Total score of cup taste from all locations.

Genotype*	Total Score				
	Group A	Group B	Group C	Group D	Group E
Pure HdT1	78,25	82,75	78,08	82,83	82,00
Pure HdT2	80,63	---	76,88	79,42	81,25
Pure HdT3	80,75	---	---	78,75	---
Pure HdT4	79,63	---	---	---	---
Hybrid HdT1	82,38	84,00	84,50	83,17	---
Hybrid HdT2	---	85,75	84,00	---	---
Hybrid HdT3	---	---	81,00	---	---
Catimor1	81,88	81,50	79,38	85,50	80,38
Catimor2	83,13	78,13	---	82,42	---
Catimor3	80,50	---	---	---	---
Catimor4	79,00	---	---	---	---
Maragogype1	---	80,00	---	---	---
Maragogype2	---	81,50	---	---	---
Typica1	82,50	84,63	---	---	---
Typica2	84,88	---	---	---	---
Mocca	---	80,88	---	---	---
S-line	---	83,13	---	---	84,50

**The same genotype names within groups is only showing numbers of genotypes type found in each location (group), not truly means the same varieties.*

The most interesting result from this study was actually the cup taste of Arabusta hybrid which performs high quality in all groups. Not only identified has better cup quality rather than pure Arabusta, cup taste of those Arabusta hybrid were also relatively equal to the famous Typica. This perhaps due to those Arabusta hybrids were emerged from naturally crossing of pure Timor hybrid with existed Arabica varieties mainly Typica, Catimor or S 795, similarly result with Bertrand et al. [5] who have found that cup quality of Arabusta hybrids were equivalent to the traditional varieties of Arabica lines.

Therefore, this research suggests that the use of Arabusta to sustain Arabica production is promising. However, the use of Arabusta as parental for next generation breeding by crossing with especially pure Arabica lines is the most recommended ones since this genotypes performs better cup qualities and relatively equal to the pure Arabica such as Typica. In other side, the performance of this hybrid in the lower altitude (< 1000 m asl) of equatorial producer countries, as like as Indonesia, still also gives better cup taste rather than pure Arabusta and relatively equal to Catimor as showed in Group C. Moreover, good agronomic traits of pure Arabusta namely the resistance to leaf rust, high yield and large bean are still conserved in the Arabusta hybrids of our studies.

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Performance of *Coffea arabica* Genotypes Grown at High Altitude in São Sebastião Da Grama, SP, Brazil.

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SUMMARY

The present study aimed at evaluating agronomic and technological traits of a group of 24 genotypes of *Coffea arabica* grown in a region of high altitude in São Sebastião da Grama, SP, Brazil. A variety trial was established in 2008 using a randomized block design with three replications and 10 plants per plot. During three good cropping seasons yield, outturn (% green coffee in relation to dried berries) and technological bean traits were evaluated. For the three cropping seasons, average annual yield of the genotypes varied from 908 to 2,184 kg/ha/year. The 3-year yield data were submitted to analysis of variance, using the F and Duncan tests at 5% probability. Significant variation was observed for average yield of the 24 genotypes (F=0.008). The five most productive genotypes were Catuaí Vermelho IAC 144, Mundo Novo IAC 502/9 and Bourbon Amarelo, Bourbon Amarelo LC J 9 and Bourbon Amarelo J 9 with average yields of 2,184; 2,097; 2,038; 1,910 and 1,882 kg/ha/year, respectively. The data obtained for the outturn showed no statistically significant differences. Regarding the technological characteristics of the beans the genotype Bourbon Vermelho showed the highest percentage of flat beans and the lowest percentage of peaberries. The average sieve rating was high for all genotypes, ranging from 17.6 to 18.3.

INTRODUCTION

The Brazilian coffee has highly productive cultivars of arabica coffee, results of decades of research developed by IAC since 1932. Advances in productivity or profitability for the producer may come with the development of other cultivars possessing characteristics such as resistance to pests and diseases, adaptation to unfavorable environmental conditions and the different systems of cultivation and improvement of product quality.

In recent years, were recorded different cultivars in RNC, and productive and resistant to rust and with good quality drinking. The selection of other genotypes in breeding programs is of utmost importance to coffee, as this may result in new cultivars, more productive and superior technological features in the use. This work aimed to evaluate yield, agronomic and technological characteristics of various origins of the Bourbon Amarelo, Bourbon Vermelho and Caturra Vermelho cultivars and other selections of *Coffea arabica*.

MATERIALS AND METHODS

For this study an experiment was installed in March 2006 at the Fazenda Recreio located in São Sebastião da Grama, SP - Brazil. The experimentes is located in longitude - 46º82 "latitude - 21º71" and altitude of 1300 meters. The climatic features of this locality are precipitation annual average of 1560 mm and a mean annual temperature of 20° C on average ranging from 16° C to 22° C in the coldest month on average in the warmest month. The experimental design was a randomized block design with three replications with plots

containing 10 plants each. The spacing used was 3.0 x 0.8 m. Were evaluated 17 genotypes of Bourbon Amarelo, one of Icatu Precoce IAC 3282, one of Mundo Novo Vermelho, two of Mundo Novo Amarelo, one of Catuaí Vermelho, Caturra Amarelo and Caturra Vermelho. The relationship of these genetic materials is found in table1. The measured technological characteristics as yield, outturn and beans characteristics (percentage of grains flat, peaberry and elephant beans types and medium sieve size). The production of the cultivars in the three harvest years (2009, 2011 and 2012), was obtained by weighing the fruits berries harvested from each plot. A sample of fruits of each plot was also removed, and the samples were dried, weighed (weight of coffee beans) and processed with the aim to transform the weight of the fruits coffee in kilograms per hectare. From these samples, we also evaluated the types of grains (flat and peaberry) and the average sieve size obtained in percentage. The data were submitted to analysis of variance, using the F test was employed Duncan, the level of 5%, to compare the treatment means test. Statistical analyzes were performed using SAS 9.0 software.

Table 1. Identification and origin of the seeds of treatments / genotypes of *Coffea arabica* evaluated at São Sebastião da Grama - SP, in the years 2009, 2011 and 2012.

Treatments	Genotypes	Provenance (origin of the seeds)
1	Bourbon Amarelo IAC J 9	Epamig – Machado/MG
2	Mundo Novo IAC 502/9	Epamig – Machado/MG
3	Catuaí Vermelho IAC 144	Epamig – Machado/MG
4	Bourbon Vermelho PV	Procafé – Varginha/MG
5	Icatu Precoce IAC 3282	Procafé – Varginha/MG
6	Bourbon Amarelo	Procafé – Varginha/MG
7	Bourbon Amarelo	Faz Bom Jardim – Santo Antonio do Amparo/MG
8	Bourbon Vermelho	Faz. São João Batista – Campos Altos/MG
9	Bourbon Amarelo	Faz. Betânia – Santo Antonio do Amparo/MG
10	Bourbon Amarelo	Faz. Daterra – Patrocínio/MG
11	Bourbon Amarelo IAC LCJ 9	Chebabi – Monte Mor/SP
12	Bourbon Amarelo	Faz Toriba – São Sebastião do Paraíso
13	Bourbon Amarelo IAC J 10	Faz São Paulo – Santo Antônio do Amparo
14	Bourbon Amarelo	Faz. Castro – Carmo de Minas/MG
15	Bourbon Amarelo	Faz. Nogueira – Carmo de Minas/MG
16	Bourbon Amarelo	Faz. Paixão – Carmo de Minas/MG
17	Bourbon Italiano	Faz. Monte Alegre – Alfenas/MG
18	Bourbon Trigo	Faz. Monte Alegre – Alfenas/MG
19	Bourbon Limoeiro	Faz. Monte Alegre – Alfenas/MG
20	Bourbon Amarelo	Faz. Samambaia – Santo Antonio do Amparo/MG
21	Mundo Novo Amarelo	Faz. Monte Deste/SP Coleção IAC
22	Mundo Novo Amarelo IAC 4266	IAC – Campinas/SP
23	Caturra Amarelo IAC 476	IAC – Campinas/SP
24	Caturra Vermelho IAC 477	IAC – Campinas/SP

RESULTS AND DISCUSSION

The average yield in quilograms of green coffee per hectare per year, the outturn, characteristics of beans in percentage and average sieve size are shown in table 2. There was

no significant difference between the yields of the progenies. Nevertheless, the five progenies with higher yields were Catuaí Vermelho IAC 144, Mundo Novo IAC 502/9, Bourbon Amarelo, Bourbon Amarelo LCJ 9 and Bourbon Amarelo J 9 with average yields of 2,184; 2,097; 2,038; 1,910 and 1,882 kg/ha/year, respectively. There was a significant difference for the beans of the flat type, highlighting the treatment 8 Bourbon Vermelho with 85.9%, differing only from the treatments 18 and 22 with 74.0 and 74.9%, respectively. There was no significant difference for the beans peaberry type, ranging from 11.1 to 16.9%. The treatments did not differ significantly for percentage of average sieve size, ranging from 17.6 to 18.3. Probably the reason why these treatments have high values in bean size is related mainly to the altitude of the location of the experiment.

Table 2. Average yield of kilograms per hectare per year, outturn in percentage and beans characteristics (percentage of grains flat, peaberry and elephant beans types) and medium sieve size, assessed in 2009, 2011 and 2012, of 24 genotypes (treatments) of *C. arabica* in an experiment set in São Sebastião da Grama, SP, Brazil.

Treatment	Yield kg/year/ha	Outturn (%)	Grains types		Average sieve size
			Flat (%)	Peaberry (%)	
1	1,882	52.6	78.9 a-c	14. 8	17.8
2	2,097	57.4	79.7 a-c	16. 3	17.7
3	2,184	52.6	78.9 a-c	16. 4	17.9
4	1,444	55.2	81.9 a-c	15. 3	17.6
5	1,661	49.3	81.7 a-c	16. 4	17.7
6	1,448	51.6	81.4 a-c	15. 8	17.8
7	1,798	50.1	83.7 ab	13.8	17.9
8	1,829	46.8	85.9 a	11.1	17.7
9	2,038	49.2	83.8 ab	12.2	17.7
10	1,830	49.3	83.3 ab	13.0	17.6
11	1,910	48.3	82.8 a-c	13.0	17.6
12	1,490	48.2	81.7 a-c	14.7	17.6
13	908	46.8	82.4 a-c	15.2	17.6
14	1,256	47.6	79.7 a-c	16.9	17.7
15	1,670	47.4	81.6 a-c	14.8	18.2
16	1,558	45.6	82.9 a-c	13.0	18.3
17	1,342	47.9	78.7 a-c	16.1	17.7
18	1,320	47.7	74.0 c	16.6	17.9
19	1,589	51.9	77.1 a-c	15.4	17.9
20	1,340	49.9	82.2 a-c	14.0	17.8
21	1,765	53.6	82.9 a-c	13.0	17.7
22	1,762	43.7	74.9 bc	13.7	17.6
23	1,791	44.9	79.3 a-c	13.3	18.1
24	1,792	49.1	81.4 a-c	14.0	18.1
Average	1,654	51.5	80.9	14.1	17.8
F. Genotypes	2.07^{**}	0.99^{ns}	4.50^{**}	1.69^{*}	2.28^{*}
F. Year	68.82^{**}	2.39^{ns}	33.77^{**}	37.73^{**}	325.90^{**}
F. Genotypes * Year	2.43^{**}	1.11^{ns}	0.75^{ns}	0.90^{ns}	0.96^{ns}
CV (%)	37.54	59.38	4.93	24.29	1.97

**, * *Significant, respectively, at 5 and 1% probability by F test; ns = not significant. To compare means, was used the Duncan test at 5%*

CONCLUSION

The five most productive genotypes were Catuaí Vermelho IAC 144 (3), Mundo Novo IAC 502/9 (2) Boubon Amarelo (9), Bourbon Amarelo LCJ 9 (11) and Bourbon Amarelo J 9 (1) with average yields of 2,184; 2,097; 2,038; 1,910 and 1,882 kg/ha/year, respectively.

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An Overview of Protein Changes in *Coffea arabica* Leaves upon Treatment with Inducers of Resistance to Coffee Rust

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SUMMARY

The coffee leaf rust (*Hemileia vastatrix*) disease causes serious losses in coffee production and quality, with a huge depreciation of marketing values. The reduction of availability of effective approved fungicides, due to health and environmental concerns, makes it necessary to intensify research for the development of novel, effective and sustainable disease control solutions, such as the resistance inducers. With the aim of understanding the mechanisms that are behind the disease control capacity of the resistance inducers "Greenforce Cuca" and Bion® we made a proteomic analysis of the *C. arabica* leaf responses upon the treatments. The leaf proteins were extracted with trichloroacetic acid in acetone and separated by two-dimensional gel electrophoresis followed by colloidal Coomassie blue staining and image analysis. About thirty five spots showed a statistical significant variation in abundance due to the treatments. The proteins identified by high-performance liquid chromatography-mass spectrometry (LC-MS/MS) followed by homology search in ESTs coffee databases, are mostly involved in the photosynthetic process (photochemical and carbon reactions) and its regulation and responses to stimulus/stress. The functional characterization of these proteins is ongoing and the metabolic processes involved in coffee induced resistance against the fungus will be further analysed.

INTRODUCTION

Coffee (*Coffea arabica* L.) is a valuable crop in Brazil, Minas Gerais being responsible for more than 50% of the national coffee production. Coffee leaf rust caused by the biotrophic fungus *Hemileia vastatrix* Berk. & Br. is the most widespread disease of *Coffea arabica* L. and may cause 10-40% of crop losses, if control measures are not applied. For a sustainable coffee production there are increasing societal expectations to reduce pesticide treatments and make use of alternative strategies of plant protection, such as the application of resistance inducers. Promising results have been obtained with resistance inducers of the benzothiadiazole (BTH) group, like the acibenzolar-*S*-methyl (ASM). After treating young coffee plants with ASM, a reduction of 52% in the incidence of *H. vastatrix*, when compared with the inoculated control, was observed. BTH treated leaves over-express genes involved in the pathogenesis-related protein synthesis, oxidative burst, and cell wall strengthening

processes, what suggests a general shift in metabolism from housekeeping to defense. In a recent work the effect of formulations based on by-products of coffee and citrus industries (like the "Greenforce CuCa" formulation) for the control of coffee rust in coffee seedlings was evaluated. This formulation showed an efficiency of 67.5% when compared to a standard fungicide, what is considered an effective alternative for the management of coffee rust. The presented work has the objective of obtaining a comprehensive overview of the protein changes occurring in the *C. arabica* leaves upon treatment with the resistance inducers "Greenforce Cuca" and Bion®, in order to elucidate the alterations that take place at the cellular metabolic level.

MATERIALS AND METHODS

Biological material and treatments: Young fully expanded leaves of *Coffea arabica* cv. Mundo Novo (susceptible genotype) were sprayed with Bion® (acibenzolar-S-methyl-ASM) and "GreenForce Cuca" (formulation based on a by-product of coffee and citrus industries, developed in Universidade Federal de Lavras - UFLA). Leaves treated with water were used as control. Samples were collected at 3 and 5 days after treatments. Three biological replicates were prepared for each treatment.

Protein extraction: Proteins of coffee leaves were extracted using the trichloroacetic acid (TCA) precipitation method. Precipitated proteins were recovered in 2% SDS sample buffer and following purification with the 2D clean-up kit (GE Healthcare) the proteins were ressuspended in sample buffer with 30mM Tris-HCl pH 8.5, 7M urea, 2M thiourea and 4% CHAPS. Protein content was measured using a modified Bradford assay.

Proteomic analysis: Protein samples (300 µg per sample) were run in 18 cm long IPG strips, pH 4–7 L (GE Healthcare). IEF was performed using the Ettan IPGphor (GE Healthcare) for a total of 32000 Vh at 20 ºC and a maximum current setting of 50 µA per strip. After IEF, the SDS-PAGE (12.5%T) was run using the 2-D Ettan Dalt II Gel apparatus (GE Healthcare) at 10 mA for 15 min and then at 20 mA at 25 ºC until the bromophenol blue dye front had run off the gel (around 20h). After electrophoresis, gels were stained by colloidal Coomassie blue G-250 and the protein profiles were scanned (ImageScanner II Amersham Biosciences) and analyzed with the Progenesis SameSpot 2D software *v.s. 4.5* (GE Healthcare). The spots with differential abundance (p-value <0.05) and with fold change ≥ 1.5 were excised from the gel and the proteins identified by high-performance liquid chromatography-mass spectrometry (LC-MS/MS), followed by homology search in several NCBI and ESTs Coffee databases. Proteins were subsequently analysed using Blast2GO software.

CONCLUSION

Statistical analysis of the polypeptide spots volumes of control, "GreenForce Cuca" and Bion® samples revealed that about 35 spots changed in abundance (Fig.1A). The functional annotation of the identified proteins was performed by Blast2GO software, but some of the GO classes were merged in order to simplify the classification. Concerning the biological process, most of the proteins were found to be involved in the cellular processes (35%), metabolic processes (25%), response to stimulus/stress (15%) and biological regulation/signaling (7%) (Fig.1B). Two categories dominate the molecular function annotation: catalytic activity (45%) and binding activity (45%) (Fig.1C).

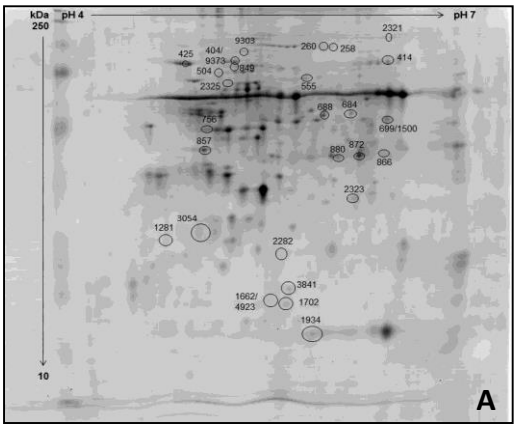

Figure 1A. 2DE representative gel of the coffee leaves. Numbers indicated on the image are polypeptide spots that changed in abundance after treatments with the resistance inducers. These spots were excised from the gel and subsequently identify by LC-MS/MS.

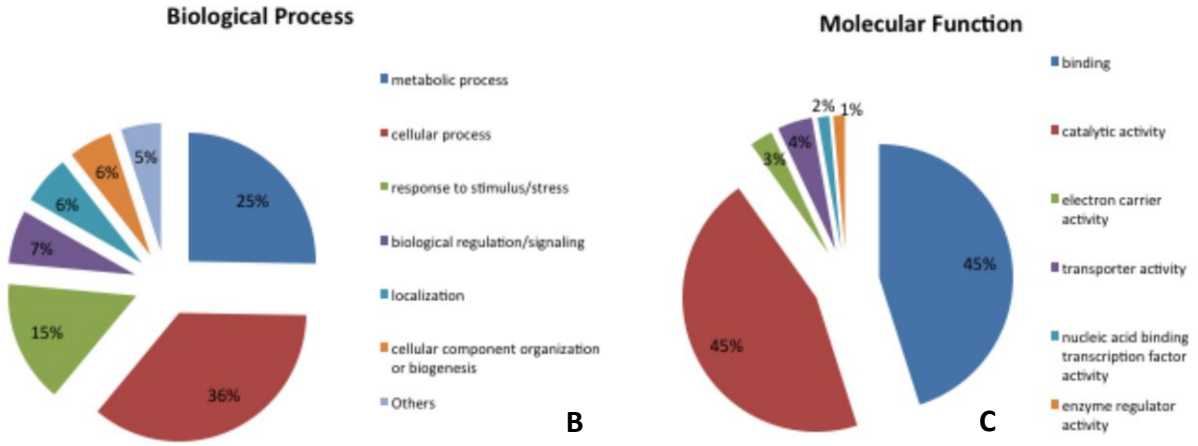

Figure 1B-1C. Functional annotation of the identified proteins in biological process (B) and molecular function (C) using Blast2GO.

Nearly 50% of the spots have increased in abundance for at least one of the treatments "GreenForce Cuca" and/or Bion®, when compared to the control. These proteins are mainly involved in photosynthesis and its regulation and in response to stimulus/stress. Concerning photosynthesis, we have identified proteins from the Calvin cycle, the photochemical reactions, and the pentose phosphate pathway, *e.g*., Rubisco large subunit-binding protein, chlorophyll a-b binding protein, oxygen-evolving enhancer protein chloroplastic-like, transketolases and sedoheptulose-bisphosphate. Concerning photosynthesis regulation and response to stimulus/stress, we have identified proteins involved in oxidoreductase activity, proteolysis, protein folding and stability and PR-proteins, *e.g.*, Rubisco activase, thioredoxin, heat-shock protein 70 (HSP70), ATP-dependent zinc metalloprotease and chitinase.

Thioredoxins are known to be involved in the regulation of the redox status of target proteins through thiol-disulfide exchange reactions in a variety of cellular processes. Sedoheptulose-bisphosphate, rubisco activase, transketolases, triose phosphate isomerases have been described as some of the possible target proteins of thioredoxins. Further, Tada *et al*. (2008) proposed that thioredoxins (TRX-5h) are positive regulators of salicylic acid-induced defense response in plants, probably by denitrosylation. HSP70 and chitinases have long been described as induced stress/defense related proteins in many plant species. HSP70 can protect

cells from thermal or oxidative stress, acting as shields in the protection of other proteins against ROS damage. Endochitinase, belongs to one of the firsts and most prominent family of PR-proteins, acting directly against fungi activity and growth. In conclusion, coffee leaves treated with the resistance inducers imposed metabolic adjustments at cellular level, mainly on photosynthesis and its regulation. This metabolic reorganization suggests a shift from common substrates and energy for growth processes to defense responses. On-going analysis of the proteomic changes of *H. vastratrix* inoculated coffee leaves previously treated with "Greenforce Cuca" and Bion® will provide further insights on the mechanisms of the resistance induction.

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Evaluation of Coffee Progenies (Timor Hybrid X Catuaí) for Yield and Reaction to Diseases

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INTRODUCTION

Most of the coffee plantations of *Coffea arabica* L. cultivated in Brazil is still constituted constituted by the cultivars Mundo Novo and Catuaí susceptible to coffee leaf rust caused by the fungus *Hemileia vastatrix* Berk. et Br. Currently this disease is considered a major disease problem in coffee, being found in almost all crops grown in Brazil, resulting in a reduction of up to 50% of production in regions with climatic conditions favorable to disease and in the absence of control measures. Another important disease in the crop is gray leaf spot, caused by the fungus *Cercospora coffeicola* Berk et Cook, which is a problem since the coffee seedlings in the nursery until the plants in full production.

Although the control method most used worldwide in the management of these diseases is accomplished by fungicide treatments, has long been seeking new cultivars with resistance to these pathogens, eliminating whole or in part the application of fungicides. Therefore, the resistance gene is the optimum control method in the management of disease, since the continuous use of chemicals increases production costs and the likelihood of resistance of phytopathogenic fungi, and environmental impact.

The Timor Hybrid, derived progenies and their junction with other cultivars have been studied in various coffee regions in the world, as a source of resistance to *H. vastatrix* L.. The Timor Hybrid is probably a natural hybrid between *C. arabica* and *C. canephora*. This has been valuable germplasm for breeding programs aimed at achieving durable rust resistance, and exhibit resistance to other diseases and pests of coffee.

The breeding program developed by Agricultural Research Company of Minas Gerais (EPAMIG) in partnership with other institutions has been successful with crossing the Timor Hybrid cultivars directly with the Catuaí group. The progeny resulting from these crosses has shown promising productivity, coupled with multiple resistance to various pathogens.

On the exposed, the aim of this work was to evaluate the behavior of progenies derived from the cross of Timor Hybrid with Catuaí about the characteristics of productivity and incidence and severity of rust and gray leaf spot.

MATERIALS AND METHODS

The experiment was installed in December 2000 at Ouro Verde Farm, private property, located in the municipality of Campos Altos, in the Paranaíba Minas Gerais region at 19 ° 41'47 "south latitude, 46 ° 10'17" longitude and altitude of 1.230m. The average annual temperature is 17.6 ° C, with average rainfall of 1830 mm. The soil type is "*Oxisol*" with clayey and relief plan.

The material used in the experiment comprises 23 progenies with potential for resistance to coffee rust, and seven commercial cultivars used as control. The progenies refer to generation $F_{3:4}$ Hybrid Timor cross between Catuaí and were obtained from the breeding of the coffee in Minas Gerais led program, coordinated by the Agricultural Research Company of Minas Gerais (EPAMIG) and with participation of Federal University of Viçosa (UFV) and Federal University of Lavras (UFLA).

The randomized block design was used with four replications, totaling 120 plots, each plot consisted of eight plants. The spacing used was 4.0 x 0.8 m in rows and between plants, respectively, corresponding to a total area of 3.072 m^2. The deployment and driving were made according to the technical recommendations for coffee plantations in the region. The plant management was done preventively or curatively through chemicals following the seasonal occurrence of pests and diseases, except for the chemical control of Cercospora and rust, not performed, aiming at the identification and selection of pathogens resistant to these progenies. The following characteristics were evaluated in yield and 2011/2012 2012/2013: (Bags of 60 kg of processed coffee ha-1) productivity, assessed annually, fruit production in liters of "farm coffee 'per plot Since harvest is held in July each year, assuming an average yield of 480 liters of "farm coffee" for each bag of 60 kg of coffee benefited; incidence of Cercospora leaf spot on leaves, assessed by sampling at random 10 leaves of 3rd pair per plant in the upper third, totaling 60 sheets per working portion. Samples were collected monthly from January through August of each year. Incidence was determined as a percentage by counting the number of sheets of coffee with Cercospora leaf collected in 60. The percentage of disease incidence were transformed into area under the curve of progress incidence of Cercospora leaf spot (AUCPIC) according to criteria established by and incidence and severity of rust, held monthly in the months from January to August each year by collecting 10 leaves the 3rd or 4th pair of leaves per plant, branches located in the middle third, totaling 60 leaves per plot. The incidence was determined as a percentage by counting the number of coffee leaves with sporulating pustules on 60 leaves collected. Disease severity was evaluated by diagrammatic adapted by, assigning notes as an arbitrary 5-point scale, with 1 given to the note sheet with less area occupied by lesions (<3%) and note 5, the leaves more area occupied by lesions (25 to 50%). The percentage of disease incidence were transformed into area under the curve of progress of the incidence and severity of rust (AUCPIR, AUCPSR) according to criteria established by. All variables in the analysis of variance of plot arrangement in time, the plots were the progeny and the subplots for the years of assessment were submitted. We adopted a 5% significance probability for the F test, and detecting significant differences, means were grouped by the Scott-Knott test at 5% probability. Analyses were performed using the computer program SISVAR.

RESULTS AND DISCUSSION

Significant effects ($P \leq 0.05$) for progeny, for years and years x progeny interaction for all traits (Table 1). For AUCPIC feature observed a progressive increase in the incidence of Cercospora leaf spot for all 23 progenies and the seven cultivars used as witnesses, highlighting the progenies 514-7-14-C73, C101-514-5-2, 516-8-2-C109, C182-518-2-6, 514-7-16-C211, C593-518-2-4 and to cultivate icatu Red IAC in 2942 that showed the lower the curve of progress and incidence of cercospora leaf spot in both years of evaluation. to mineral nutrition is related to lower disease progress, for it favors the accumulation of inhibitory compounds around the site of infection and / or mechanical barriers to penetration and infection by pathogens. Thus, these progenies that had a lower AUCPIC may have been influenced by higher nutrient and water efficiency or even resistance / tolerance gene.

Table 1. Average Productivity of green coffee in bags of 60 kg. ha-1, Area Under Curve Progress Incidence of Cercospora leaf spot (AUCPIC), Area Under Curve Progress Incidence of Rust (AUCPIF) and Area Under Curve Progress Severity Rust (AUCPSF) of 23 progenies and 7 cultivars evaluated in Campos Altos, Minas Gerais, in 2011/2012 and 2012/2013 seasons.

Progenies	AUCPIC		AUCPIR		AUCPSR		Productivity		
	2011	2012	2011	2012	2011	2012	2011	2012	Biennium
514-5-4-C25	4508 d	3653 c	435 a	225 a	191 c	75 b	54,1 a	7,9 b	31,0 c
436-1-4-C26	3818 c	3405 b	120 a	165 a	36 a	38 a	59,8 a	13,6 b	36,7 b
518-7-6-C71	2880 b	3135 b	0 a	60 a	0 a	15 a	41,7 b	22,9 a	32,3 b
514-7-14-C73	1673 a	1680 a	0 a	45 a	0 a	15 a	53,6 a	19,2 a	36,4 b
514-5-2-C101	2093 a	1913 a	45 a	75 a	23 a	23 a	47,5 b	19,4 a	33,5 b
516-8-2-C109	1733 a	1680 a	0 a	15 a	0 a	8 a	40,3 b	16,3 b	28,3 c
504-5-6-C117	3833 c	4455 c	45 a	540 a	34 a	98 b	45,4 b	25,0 a	35,2 b
514-5-4-C121	5018 d	4103 c	405 a	210 a	116 b	105 b	35,6 c	16,1 b	25,8 c
514-7-4-C130	2723 b	2925 b	0 a	120 a	0 a	26 a	57,9 a	23,6 a	40,7 a
493-1-2-C134	2798 b	2910 b	0 a	210 a	0 a	62 a	69,6 a	23,6 a	46,6 a
505-9-2-C171	3420 c	3405 b	0 a	150 a	0 a	34 a	50,1 a	14,9 b	32,5 b
518-2-6-C182	2235 a	2573 a	0 a	60 a	0 a	30 a	46,3 b	19,5 a	32,9 b
514-7-16-C208	2625 b	2243 a	0 a	15 a	0 a	8 a	55,1 a	18,5 a	36,8 b
514-7-16-C211	2108 a	2018 a	0 a	60 a	0 a	15 a	53,7 a	16,0 b	34,9 b
493-1-2-C218	3690 c	3368 b	0 a	135 a	0 a	26 a	44,6 b	20,6 a	32,6 b
438-7-2-C233	2775 b	2745 b	0 a	60 a	0 a	23 a	38,7 c	13,4 b	26,1 c
514-7-16-C359	2408 b	2550 a	0 a	45 a	0 a	23 a	55,9 a	17,9 a	36,9 b
514-7-8-C364	2895 b	3000 b	0 a	135 a	0 a	38 a	57,9 a	21,4 a	39,6 a
518-2-10-C408	2190 a	2948 b	0 a	45 a	0 a	19 a	57,9 a	24,3 a	41,1 a
514-5-2-C494	2715 b	2970 b	45 a	105 a	15 a	23 a	45,1 b	27,8 a	36,4 b
518-2-4-C593	1800 a	2258 a	0,a	270 a	0 a	47 a	44,6 b	22,6 a	33,6 b
516-8-2-C568	2715 b	3180 b	45 a	195 a	34 a	41 a	48,7 b	24,2 a	36,4 b
518-2-6-C685	2453 b	2588 a	2130 b	180 a	200 c	55 a	31,5 c	17,5 a	24,5 c
Catuaí Vermelho IAC 99	3653 c	3023 b	6255 f	1140 b	260 d	146 b	43,9 b	13,4 b	28,7 c
Catuaí Amarelo IAC 62	3308 c	3023 b	5970 f	1275 b	276 d	125 b	43,9 b	17,9 a	30,9 c
Topázio MG 1190	3885 c	2768 b	6180 f	450 a	280 d	104 b	45,2 b	13,4 b	29,3 c
Rubi MG 1192	4110 c	3218 b	4965 e	540 a	237 c	94 b	38,0 c	22,3 a	30,1 c
Acaia Cerrado MG 1474	3840 c	2145 a	7920 g	435 a	290 d	94 b	33,6 c	3,3 b	18,4 d
Icatu Precoce IAC 3282	3458 c	2588 a	4275 d	240 a	298 d	83 b	30,9 c	4,9 b	17,9 d
Icatu Vermelho IAC 2942	2243 a	2108 a	3405 c	360 a	235 c	68 b	41,8 b	14,0 b	27,9 c
Mean	2987 A	2819 A	1408 B	272 A	84 B	52 A	47,1 A	17,8 B	32,5
CV(%)	**18,87**		**57,78**		**51,49**		**26,32**		

Means followed by the same letter in the column and capital on the line, do not differ by Skott-Knott test at 5% significance.

In relation to the mean area under the curve for incidence (AUCPIR) and disease severity (AUCPSR) it appears that the cultivars used as witnesses confirmed its high susceptibility to the pathogen presenting high incidence and severity (Table 1). As the progenies except 514-5-4-C25, C117-504-5-6, 514-5-4 and 518-2-6-C121-C685, all exhibited lower values AUCPIR AUCPSR and two years of evaluation, behaving as resistant / tolerant to the rust pathogen. It is worth noting that no progeny showed immunity to rust, however, according to low / intermediate incidence of progeny is important, considering that you can not select progenies with horizontal resistance in the progenies that have no impact, because these probably have specific or vertical resistance of the kind that covers the horizontal resistance.

When we analyze the agricultural years, one realizes that the harvest 2011/2012 higher incidence and severity of rust on the average of all materials when compared with the 2012/2013 harvest. This fact can be explained by nutritional imbalances in plants, since the crop 2011/2012 was a year of high productivity (average of 47.1 bags ha^{-1}) and the 2012/2013 season of low productivity (average of 17. 8 bags. ha^{-1}). Mentions that there is a positive correlation between grain yield and the incidence of coffee rust and disease incidence is higher in years of high production due probably to a change in plant resistance by nutritional imbalance.

Productivity is considered by many authors as the main criterion for selection of coffee. In Table 1, we note that there was formation of three and two groups for this variable in 2011/2012 and 2012/2013 seasons, respectively. The coffee crop, a significant factor that affects the variation of its production is a biennial alternation. This factor is commonly attributed to the diminishing reserves of plants in years of crop with high yields, which makes production the following year is very low due to the lower growth of primary branches. Thus, the grouping of samples in biennia has been indicated to reduce this effect, thus increasing the experimental precision. Thus, when considering the biennium (average of two seasons), we observe the formation of four groups, highlighting the progenies 514-7-4-C130, C134-493-1-2, 514-7-8 and 518-2-10-C364-C408 showed that the best yield averages. These results agree with who claim that materials resulting from crosses of Timor Hybrid x Catuaí has been performing and promising equal or greater yields the best cultivars Catuaí, combined with high resistance to rust and with potential to be released as cultivars.

CONCLUSION

The progenies 514-7-14 1-C73, C101-514-5-2, 516-8-2-C109, C182-518-2-6, 514-7-16-C211, 518-2-4- C593 and the Red Icatu cultivar IAC 2942 show were tolerant to cercospora leaf spot of coffee;

Most progeny behaved as resistant / tolerant to the rust pathogen;

The progenies 514-7-4-C130, 493-1-2-C134, 514-7-8-C364 and 518-2-10-C408 stood out with high productivity coupled with resistance to coffee rust, showing great promise for release as cultivars.

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Genetically Low Caffeine Coffee Genotypes: Agronomic Evaluations of Plants in Process of Selection

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SUMMARY

Cultivars of coffee with beans containing reduced caffeine may constitute a new cultivation option for producers, since the value added to the product already comes from the field, resulting from their genetic constitution. In addition, the product of a cultivar with this profile would provide a new option for the group of consumers who appreciate the drink but present some degree of sensitivity to the alkaloid. This study aimed to evaluate and select plants among F_2 and F_1BC_1 generations from crosses between the low caffeine mutant AC_1 and elite coffee cultivars. In addition to the characteristic reduced caffeine content, an attempt was made to aggregate adequate levels of productivity resulting from good agronomic characteristics such as: vegetative vigor, plant height, crown diameter and resistance to diseases. Genotypes in selection were planted in the field along with the controls: clones mutant AC and elite cultivars. The data presented are evaluations average made in 2011/2012 and 2012/2013 yields. It was found that the progenies with better behavior in relation to the average production were originated from backcrosses with cultivars IAC Obatã and IAC 81. Although it has been identified several plants with low caffeine content in F_2 progenies generation, the higher frequency of plants with this trait was found among progenies from backcrosses to the mutant AC. Data production ranges of progenies showed great variability both between and within progenies, occurring since unproductive plants to plants with productions overcoming that of controls. In data indicated the possibility of selection among progenies showing individual plants with low caffeine content and most productive genotypes. Data on plant height and crown diameter showed up as expected, according to the elite cultivar used in obtaining the F_1 generation, i.e. cultivars with high or low stature.

INTRODUCTION

Arabica coffee (*Coffea arabica*) is the most consumed species and cultivated in the world, due its superior beverage. The popularity of coffee as a beverage is due in large part to the stimulating effect of caffeine. However there are people who have a higher sensitivity to this alkaloid and that even so, enjoy the drink. To attend these consumers the industrially decaffeinated coffee was developed. Beyond caffeine a decaffeinated coffee can lost important substances for development of aroma and flavor, as they are water soluble, needing to be recovered at the end of the process [1, 2]. Cultivars of arabica coffee containing low caffeine in beans may constitute a new cultivation option for producers, since the value added to the product already comes from the field, resulting from their genetic constitution. In addition, the product of a cultivar with this profile would provide a new option for the group of consumers who appreciate the drink but present some degree of sensitivity to the alkaloid. This study aimed to evaluate and select plants among F_2 and F_1BC_1 generations from crosses between the low caffeine mutant AC_1 [3] and elite coffee cultivars. In addition to the characteristic reduced caffeine content, an attempt was made to aggregate adequate levels of

productivity resulting from good agronomic characteristics such as: vegetative vigor, plant height, crow diameter and resistance to diseases.

MATERIAL AND METHODS

Genotypes in selection were formed by approximately 400 plants of F_2 and F_1BC_1 generations derived from crosses involving AC1 mutant with low caffeine content and elite cultivars.They were planted in the field along with the controls: clone mutant AC and elite cultivars The data presented are evaluations average made in 2012 and 2013 yields in Experimental Center of Agronomic Institute, Center of Coffee 'Alcides Carvalho', in Campinas-SP, Brazil. The evaluations were made on agronomic features of individual harvest on cherry state during two years. The individual yield was weighted and the average yield of each progeny was calculated. For the determination of caffeine % (db) were collected samples of 30-50 ripe fruits from each plant, which after drying in an oven were peeled and the grains grinded in a mill and submitted to a methanolic extraction of caffeine. After centrifugation and filtration of extracts the separation and quantification of the compounds was done using a Shimadzu HPLC by means of a reverse phase C18 column and an UV detector at 272 nm wavelength [1]. The caffeine concentration of samples in the study were calculated using calibration curves obtained from the injection of solutions of known caffeine concentration standards.

CONCLUSION

Table 1 presents progenies that showed plants with low caffeine, the range of production of these progenies and the average yield in 2012 and 2013 crops. Among F_2 progenies derived from the cross between MN and AC the best progenies were 9 and 22. Regarding among F_2 progenies derived from crosses between short stature cultivars and AC, the best progenies were 61 and 65 from Obatã and IAC 81, respectively. Progenie number 42 from the backcross (obx1)x1 presented the best average yield. Althoug it has been identified several plants in F_2 progenies showing low caffeine content, the higher frequency of plants with this trait was found among backcrosses progenies to the mutant AC. The results showed the possibility to select individual plants combining low caffeine content and adequate levels of productivity.

Table 1. Numbers of plants per progenie, plants with reduced caffeine content, average yield and production range in F_2 and F_1BC_1 progenies of coffee (*Coffea arabica*) evaluated in 2012 and 2013 crop years – Campinas-SP-Brazil.

Treatment (progênies)	Identification	Generation	Nº plants/ progenies	Nº plants with reduced caffeine content	Plants with reduced caffeine content	
					Average yield	Production range
					(g)	(g)
9	(MNx1)	F_2	15	2	1428,8	(0-3570)
10	(Mnx1)	F_2	19	1	403,0	(0-806)
13	(Mnx1)	F_2	20	1	604,0	(280-928)
15	(Mnx1)	F_2	20	1	100,0	(0-200)
22	(Mnx1)'	F_2	10	1	2040,5	(1403-2678)
17	(1xMn)	F_2	15	1	779,5	(589-970)
2	(Mnx1)x1	F_1BC_1	10	2	685,3	(0-1335)
6	(Mnx1)x1	F_1BC_1	10	3	404,2	(0-1030)
1	1x(MNx1)	F_1BC_1	30	8	788,3	(0-2155)
18	(Mnx3)	F_2	5	1	751,0	(592-910)

29	(obx1)'	F_2	10	2	2478,3	(671-5945)
47	(Ovx1)	F_2	6	1	2454,0	(1008-3900)
60	(81x1)	F_2	10	1	2217,0	(200-4234)
61	(obx1)	F_2	25	3	2730,0	(789-6407)
62	(obx1)	F_2	15	2	1921,5	(137-3925)
63	(144x1)	F_2	15	1	3018,5	(2545-3492)
64	(81x1)	F_2	10	1	2386,5	(953-3820)
65	(81x1)'	F_2	20	1	3470,0	(595-6345)
66	(144x1)'	F_2	15	1	118,0	(110-126)
42	(obx1)x1	F_1BC_1	5	1	4130,5	(3049-5212)
43	(81x1)x81	F_1BC_1	9	3	1520,2	(314-2375)
44	(81x1)x1	F_1BC_1	5	2	1795,8	(300-3699)
39	AC1clone		6	6	877,3	(0-1620)
G1	AC1		44	44	342,1	(0-1605)
MN	Mundo Novo	control	44	0	785,8	(0-5910)
69	IAC Obatã	control	10	0	2592,8	(190-6125)

'open pollination

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Reproductive Development Phases of Coffee Tree in Full Sunshine and Intercropped with Olive Tree

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SUMMARY

The aim of this study was to evaluate the phenological changes of coffee trees intercropped with olive trees compared to coffee trees in full sun. This experiment was carried out at the EPAMIG Experimental Farm in São Sebastião do Paraíso, MG, Brazil. The development of the phenological stages was evaluated through marking of five branches in the middle third of the coffee tree in the full sun and intercropped plots. A scale with images of each phase of reproductive development of the coffee tree, which goes from dormant buds to dry fruit, was used, with assignment of the following numerical values: 0 (dormant bud); 1 (swollen bud); 2 (bud development); 3 (blossoming); 4 (post-blossoming); 5 (small green fruit); 6 (fruit expansion); 7 (green fruit); 8 (green-yellow fruit); 9 (cherry); 10 (dried cherry); and 11 (dried fruit). Based on this scale, the phases of phenological development of the coffee tree were recorded in monthly intervals in percentages in the period from October 2013 to July 2014. The shading effect of the olive tree led to changes in the phases of reproductive development of the coffee tree, exhibiting slower maturation/grain filling. In full sun, a small percent of cherry fruits was observed.

INTRODUCTION

This study was carried out to evaluate the reproductive development phases of the coffee tree in association with cultivation of the olive tree, compared to coffee grown in full sunshine. In this context, the development of the phenological stages of the coffee tree in sunshine and shaded by the olive tree was evaluated.

Coffee (*Coffea arabica* L.) growing in Brazil developed mainly in the full sun environment, exposing the crop to climate risks such as frost, high temperatures, and excessive winds. Given these characteristics, some regions may have problems with low yield and sustainability of the coffee crop. The return of competitive coffee growing to these regions requires new technologies with the use of more productive and sustainable systems.

Intercropping of trees in coffee fields, in addition to changing the microclimate and exhibiting beneficial effects on the soil, also influences the physiological processes of the coffee tree. Although there is the possibility of a decrease in coffee production, this may be offset by an increase in the beverage quality of the coffee intercropped with trees. The improvement in coffee quality is due to the delay and synchronizing of fruit ripening, bringing about the proper accumulation of sugars, which makes the fruits larger and softer. Coffee plants subjected to intercropped tree systems have better beverage quality, adding value to the product.

A species with potential for intercropping with coffee is the olive tree (*Olea europaea* L.); it is one of the earliest fruit species grown, along with the grapevine. Currently, the commercial importance of this species is mainly related to preparation of olive oil. The potential for expansion of this activity in Brazil can be estimated by the volume of imports of these products observed in recent years. Currently, to meet domestic demand for these products, it is estimated that 62,000 hectares of olive trees would be needed, which could generate foreign exchange currency of approximately R$ 1.4 billion.

MATERIALS AND METHODS

The study was carried out at the EPAMIG Experimental Farm in São Sebastião do Paraíso, MG, Brazil. Both the coffee trees in full sun and the intercropped coffee and olive trees were planted in January 2008. The coffee tree cultivar Red Catuaí IAC – 99 was planted at a spacing of 7.0 x 0.7 m, and the olive trees at 7.0 x 4.0 m, such that every 3.5 m had alternating rows of coffee trees and olive trees. The cultivars of coffee trees in full sunshine were planted at 3.5 x 0.7 m.

The development of phenological stages was evaluated through the marking of five branches in the middle third of the coffee trees in the full sun and intercropped plots. A scale with images of each phase of reproductive development of the coffee tree, which goes from dormant buds to dry fruit, was used, with assignment of the following numerical values: 0 (dormant bud), 1 (swollen bud), 2 (bud development), 3 (blossoming), 4 (post-blossoming), 5 (small green fruit), 6 (fruit expansion), 7 (green fruit), 8 (green-yellow fruit), 9 (cherry), 10 (dried cherry), and 11 (dried fruit). Based on this scale, the numerical values representing the phases of phenological development of the coffee tree were recorded in monthly intervals in percentages in the period from October 2013 to July 2014.

Figure 1. Coffee trees intercropped with olive trees.

CONCLUSION

The beginning of evaluations in October 2013 coincided with the post-blossoming phase (Fig. 2), with more than 90% of the plants in this phase in the plots. According to Livramento (2010), under water deficit conditions during the dormant phase, coffee trees usually present exhibit three blossoming periods. The first generally occurs in August, and the second at the end of September and beginning of October, which is responsible for more than 90% of production. In this study, blossoming occurred in September because in October the predominance of post-blossoming was registered. The third blossoming may occur in the

middle of November. These uneven blossomings lead to fruit in different stages of maturity during harvest, and this may affect coffee quality.

In the following month, in November, the small green fruit phase appeared for more than 90% in coffee trees in full sunshine, and 76% in intercropping with olive trees. In this phase, the stage of fruit development is characterized by defined phases of growth, accumulation of dry matter, and changes in chemical composition, as well as coloring. The first part of this stage is called the small green fruit phase, with minimal growth observed. It is noteworthy that blossoming lasts around 3 to 4 days, and as evaluations were performed in the first week of each month, the post-blossoming phase was not registered.

The fruit expansion stage arose in December 2013, with greater percentages being registered in the coffee trees under the effect of shading from olive trees. The fruit rapid-expansion phase is characterized by the accumulation of dry matter and is predominantly in December.

From January to April, the green fruit phase predominates (Fig. 2); this constitutes the grain filling phase of the fruit. As of March, it may be observed that coffee trees in full sun exhibit 10% cherry fruits, while the shading effect from olive trees led to slower grain filling. Coffee trees in full sunshine exhibited a small percentage of cherry fruits. Possibly the effect of shading led to a delay in fruit development.

In the following months, from May to July, there was continuity of reproductive behavior in coffee trees under the influence of the shade afforded by olive trees, in comparison to coffee trees in full sun. In Fig. 2, in the months of June and July 2014, slow fruit maturation as an effect of the olive trees may be seen, with greater grain uniformity and a higher percentage of cherry fruits compared to coffee trees in full sun. This also results in higher grain quality since the fruit reached its peak physiological maturity. Crop harvest took place after the last assessment in July.

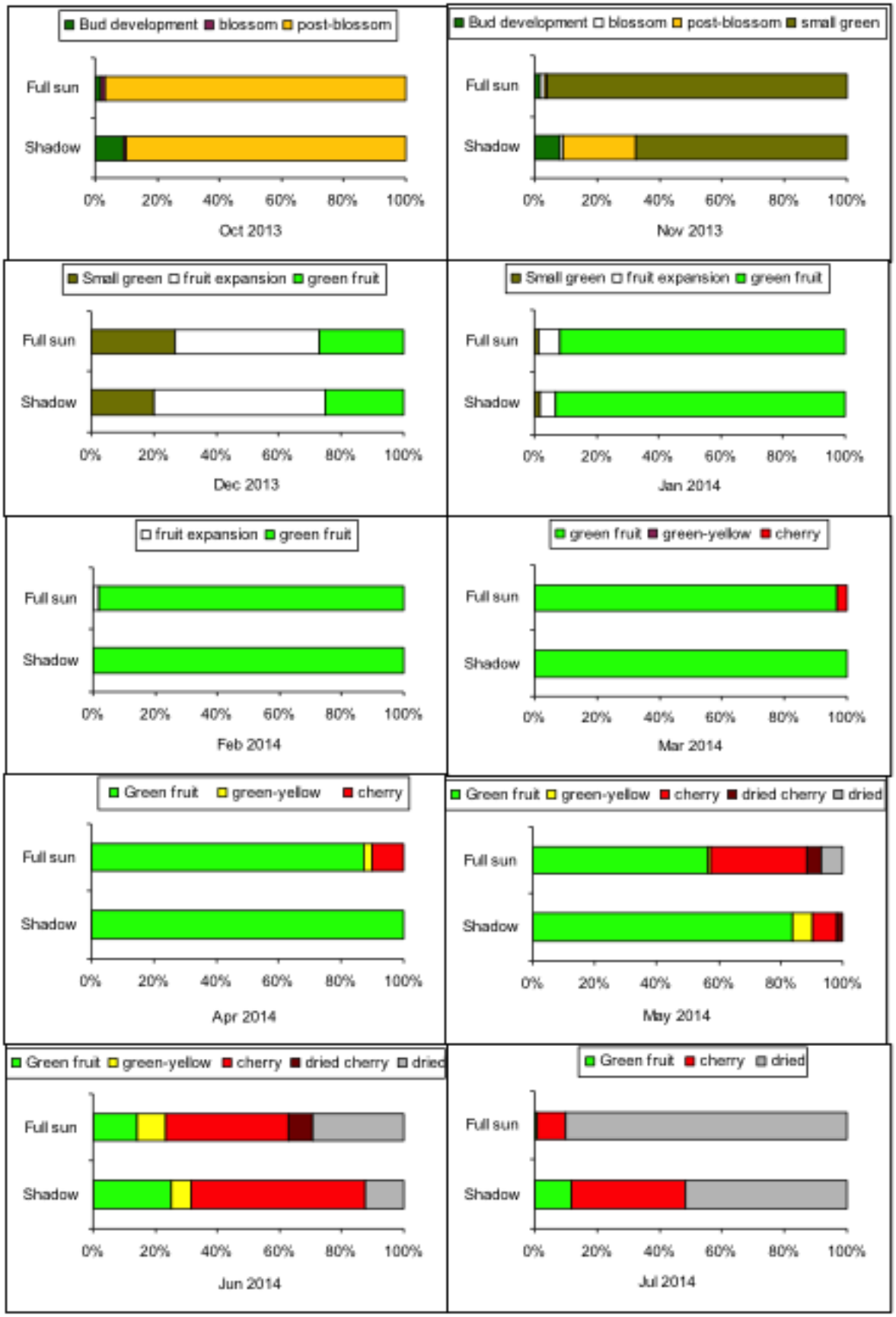

Figure 2. Graph representation of the stages of reproductive development of the coffee tree in full sun and under the influence of shade provided by the olive tree, from October 2013 to July 2014.

Under full sun, the average annual temperature greater than 23°C accelerates fruit development and maturation, increasing loss of beverage quality. Both solar radiation and excess temperature on coffee fields accelerates fruit ripening, preventing proper accumulation of sugars, which causes impairment in beverage quality. [2] Thus, with proper shading, coffee berries take longer to fully ripen, ensuring suitable maturation.

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Effect of Weed Control Methods in Coffee Inter-Rows on Coffee Yield

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SUMMARY

The coffee crop is very sensitive to competition from weeds for water, light, and nutrients. Weed control represents 30% of production cost. Because of that, several weed control methods were tested to find the best one for use in the inter-rows of the coffee crop. For that purpose, in 2006, an experiment was set up in a randomized block design with seven treatments in the inter-rows: a mower, disk harrow, rotary tiller, post-emergence herbicide (glyphosate) at 720g ai / ha, pre-emergence herbicide (oxyfluorfen) at 720 g ai / ha, hand weeding, and inter-rows with no control as a check, in three replications. The experimental area is a red Oxisol with an 8% slope and 3364 coffee plants of the cultivar Paraíso (MGH 419), planted with 4.0 m inter-rows and 0.7 m plant spacing at the EPAMIG Experimental Station of São Sebastião do Paraíso, MG, Brazil. Yields from 2008, 2009, 2010, 2011, 2012, and 2013 were analyzed. After six years, results show that the pre-emergence herbicide led to the highest yield. The inter-row without control had the lowest production. The use of the disk harrow, rotary hoe, manual weeding, and post-emergence herbicide showed intermediate yields because they are methods that depend on the timing and availability of weed control. Furthermore, use of the disk harrow contributed to infestation by bermudagrass [*Cynodon dactylon* (L.) Pers] due to the shearing of stolons into diverse single segments giving rise to many plants, and the rotary tiller led to the predominance of purple nutsedge (*Cyperus rotundus* L.) due to the release of several tubers, increasing and distributing their propagules throughout the agricultural area.

INTRODUCTION

Competition from weeds may cause reduction in coffee yield from 55 to 77% according to BLANCO, OLIVEIRA, and PUPO (1982). Due to the rising cost of weed control, several methods have been introduced and used in weed management in coffee inter-rows (SILVEIRA and KURACHI, 1981; MUZILLI, 1987; and SOUZA et al., 2006), but the consequences of each method of weed control in regard to many agricultural aspects have been poorly evaluated, especially their relationship to the environment (LAL, 1993).

However, AVATRAMANI (1974) expressed concern when he suggested integrated weed control in coffee with reduced cultivation and mulch formation looking toward environmental preservation. Weed control that maintains plant cover may favor all the other soil quality properties in a positive way by the effect of soil organic matter on soil physical, chemical, and biological parameters, as shown by STEVENSON (1986) DUXBURY et al. (1989), and

FERNANDES et al. (1997). Because of that, organic matter in the soil is regarded as the key to sustainability, and its deficiency in the soil directly contributes to degradation in quality and impoverishment of the soil (STEVENSON, 1986).

The effect of various methods of weed control in coffee on the chemical and physical soil parameters and on production has been demonstrated (ALCÂNTARA, 1997; ALCÂNTARA and FERREIRA, 2000a, 2000b; ALCÂNTARA, FERREIRA and MERCER, 2003; and NOBREGA, ALCÂNTARA, and FERREIRA, 2006). These studies showed that the continued use of pre-emergence herbicide increased soil density, resulting in soil surface crust formation. Rotary tiller use formed a hard-pan layer at the 20 cm depth (ALCÂNTARA, 1997). It was also observed that although some methods contributed to an increase in the organic matter content in the soil (ALCÂNTARA and FERREIRA, 2000a), they did not result in increased yield.

In contrast, it was shown that the use of pre-emergence herbicides for many years, despite decreasing organic matter content, increased coffee yield in a study carried out from 1977 to 2005 (ALCÂNTARA and FERREIRA 2007). Coffee growers have sought to minimize weed control cost with the introduction of cover crops like brachiaria (*Brachiaria decumbens* L.) planting it in the inter-rows, as well as the use of other brachiaria species. However, studies have shown that the presence of these plants in coffee fields leads to reductions in coffee dry biomass of the above ground parts. SOUZA, L. S. (2006) showed that brachiaria plants less than 100 cm from coffee plants cause interference. Moreover, RONCHI (2002) showed that brachiaria grass growing along with the coffee plant reduces the number of coffee leaves, plant height, stem diameter, and shoot dry matter weight.

Therefore, the objectives of this study were to ascertain the effects of different methods of weed control in coffee inter-rows and how they may affect coffee production.

MATERIALS AND METHODS

A new weed control study was set up in the EPAMIG Experimental Station in São Sebastião do Paraíso, Minas Gerais, Brazil using a rust resistant coffee cultivar, Paraíso MGH 419, with coffee planted at 4 m between rows and 0.7 m spacing between plants in the row in an red Oxisol in an area with an 8% slope. A randomized block design was used with seven weed control methods between rows. The treatments were use of a mower, rotary tiller, post-emergence herbicide (glyphosate at 720g a.i./ha), pre-emergence herbicide (oxyfluorfen at 720 a.i./ha), manual weeding, and the inter-row with no control, as a check, in three replications.

The plant rows were kept weed free by manual weeding or by herbicides. The number of operations per year to keep the inter-rows free of weeds is shown in Table 1.

Table 1. Number of annual operations for weed control in a coffee plantation. São Sebastião do Paraíso, MG, Brazil, 2013

Inter-row treatment	Number of operations/year
Mower	Five
Disk harrow	Three
Rotary tiller	Three
Post-emergence herbicide	Three
Pre-emergence herbicide	Two
Manual weeding	Five
No control (check)	---------

Yields were evaluated by harvesting the production of twenty plants per treatment, using 2 kg of coffee cherries, which were dried and the yields determined and transformed into processed bags/ ha. Yield data for each year were tabulated and analyzed using analysis of variance in a split plot arrangement per year.

RESULTS AND DISCUSSION

Brachiaria grass (*Brachiaria decumbens* Stapf) represents 70% of weed infestation among weeds that occur in the unweeded inter-row treatment. Other invasive plants occur in smaller proportions in the unweeded treatment during the rainy season: white hairy beggarticks (*Bidens pilosa* L), Siberian motherwort (*Leonurus sibiricus* L.), smallflower (*Galinsoga parviflora* Cav.), tall morningglory (*Ipomoea purpurea* (L.) Roth), mattress grass (*Digitaria horizontalis* Willd), purple nutsedge (*Cyperus rotundus* L.), and goosegrass (*Eleusine indica* (L.) Gaertn). In the decreased rainfall period, other weeds that occur are balsamapple, (*Mormodica charantia* L.), American black nightshade (*Solanum americanun* Mill), purslane (*Portulaca oleracea* L.), virginia pepperweed (*Lepidium virginicum* L.), wild poinsettia (*Euphorbia heterophylla*), pigweed (*Amaranthus viridis*), bermudagrass (*Cynodon dactylum* (L.), and purple nutsedge (*Cyperus rotundus*), which remain in growth throughout the year.
The disk harrow and rotary tiller treatments favor the dominance of bermudagrass and purple nutsedge due to the shearing of stolons into diverse single segments, giving rise to many plants, and the rotary hoe led to the predominance of purple nutsedge (*Cyperus rotundus* L.) by releasing a large amount of purple nutsedge tubers, which gives rise to and distributes its seedlings throughout the tilled area.

Production data show that controlling weeds through pre-emergence herbicide has advantages over all other methods for it avoids weed competition with coffee during the year. Moreover, in the other methods of weed control between rows in coffee, competition from weeds occurs as of the germination and growth of weeds through production and becomes more pronounced as the weeds grow, being eliminated only when the control operation is performed. Therefore, the data show a logical result when weeds are controlled by pre-emergence herbicides annually, consequently providing the highest mean yield in processed bags / ha (37.6) over a six-year period (Table 2).

When weed control was done by hand weeding, the mean yield was 31.5 bags / ha, i.e. 6.1 bags / ha lower than treatment with pre-emergence herbicide. Other methods, like the use of a mower, disk harrow, rotary tiller, and post-emergence herbicide, showed lower yields, ranging from 27.5 to 31.8 bags / ha, with no statistical difference in the treatments, due to the fact that weed control was always made after the appearance of weeds, and therefore after competition with the coffee.

Coffee with inter-rows without weed control had a mean yield of only 23.1 bags / ha after six years. In Table 2, note that this is the lowest yield observed among treatments since the first production in 2008 (with 4 bags / ha) and each year until 2012. The effects of weeding methods on coffee production have been shown in previous studies done in the period of 1978-2005, (ALCANTARA and FERREIRA, 2007) with results similar to this study in regard to production.

The negative effect of the presence of weeds on coffee has been shown by many researchers, such as TOLEDO et al. (1996), RONCHI (2002), and others, and some studies have shown that *B. decumbens* and *B. Brizantha* are good hosts for the nematode *Pratylenchus spp*, which, according to INOMOTO et al. (2007), enable its multiplication in the area.

Other studies attest to the aggressiveness of brachiaria when infestation occurs within the distance of 100 cm from the coffee plant (SOUZA, et al., 2006). In another study, RONCHI (2002) showed that the effect of brachiaria grass growing along with the coffee plant reduces the number of coffee leaves, height, stem diameter and dry matter by 42%, but a notable study was led by BLANCO, OLIVEIRA, and PUPO (1982), which showed a reduction in coffee production in the formation from 55.9% to 77.2%.

Despite this, many coffee growers have introduced *Brachiaria decumbens* L. as cover plant in coffee inter-rows.

In this study, the inter-rows without control had brachiaria grass as the main infesting plant, as may be observed in *Figure* 1.

CONCLUSION

The coffee with inter-rows without weed control showed the lowest yield. The coffee with inter-rows controlled by pre-emergence herbicide showed the greatest yield in processed coffee. Inter - rows controlled by mower, disk harrow, rotary tiller, hand weeding and post-emergence herbicides showed similar yield in the average of six years.

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Table 2. Number of 60 kg bags of processed coffee / ha in six years. São Sebastião do Paraíso, MG, Brazil.

Inter-row treatment	Years						
	2008	2009	2010	2011	2012	2013	2008-2013
Mower	8.0 bc	26.0ab	41.7 ab	27.0 a	45.0 ab	36.6 ab	30.7 ab
Disk harrow	11.7 bc	20.7 b	55.3 ab	20.0 a	30.0 b	45.4 a	28.9 b
Rotary hoe	17.0 bc	25.7ab	48.3 ab	28.0 a	46.0 ab	20.1 b	32.2 ab
Post-emergence herbicide.	14.4 bc	25.0ab	49.0 ab	28.0 a	42.3 ab	24.1 ab	29.9 b
Pre-emergence herbicide.	31.3 a	33.0a	61.0 a	31.7 a	48.3 a	16.6 b	37.6 a
Manual weeding	17.7 b	24.0ab	48.3 ab	36.7 a	42.3 ab	19.3 b	31.5 ab
No control (check)	4.0 c	18.7 b	37.3 b	25.3 a	30.0 b	37.0 ab	25.5 b
C. V.(%)	16.4	9.34	8.55	14.25	7.93	15.64	11.81

Mean values with the same letters are not significantly different by the Tukey test at the level of 0.05.

Figure 1. Inter-rows without weed control.

Population Dynamics Miner of Coffee in Relation to Temperature and Precipitation Changes in the Southeast Region of Brazil

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SUMMARY

The aim of this study was to evaluate the fluctuation of the coffee leaf miner in relation to changes in temperature and precipitation in Southeastern Brazil. The monitoring was conducted at Epamig Experimental Farm in Brazil. Samples were taken monthly from ten random plants in the period 2010 - 2013, and the leaves with injury was counted to determine the average infestation. Cumulative rainfall data and monthly temperature of region was plotted for each year. The highest levels of infestation occurred during the dry season, thus the behavior of the coffee leaf miner population ranged over the years due to rainfall not coincide in years evaluation. The temperature varied also influencing the level of pest infestation. Thus, the realization of monitoring of leaf miner should be used to determine the timing control in coffee plantations.

INTRODUCTION

Brazil is the largest producer and exporter of coffee, with estimated production for 2014 of 44.57 million bags of 60 kilograms of processed product. In the state of Minas Gerais the coffee culture occupies a highlight place with approximate production of 22 million bags of benefited coffee corresponding to about 52% of the country production.

The production of this crop is affected by many factors as pests, which each year cause large losses, decreasing crop yields. A pest of great importance in the cultivation of coffee is the coffee leaf miner *Leucoptera coffeella*, being a major pest of coffee in Brazil due it be widespread occurrence in the coffee plantations and the economic damage caused by this insect in relation to production coffee.

The population of the leaf miner of coffee varies depending on regions due to biotic and abiotic factors that act in the coffee [3], it shows a correlation with climate variables. The temperature is positively correlated, since rainfall and relative humidity are negatively correlated, requiring prolonged periods of drought to infestation outbreaks.

The monitoring could be useful to know the times of occurrence of the insect, the conditions favorable to their development and monitor the evolution of infestation in crops. Thus, the aim of this study was to evaluated the dynamics of the coffee leaf miner in relation to changes in temperature and precipitation in southeastern Brazil.

MATERIALS AND METHODS

The experiment was conducted at Experimental Farm of Agricultural Research Company of Minas Gerais in South of Minas Gerais, Brazil. The period of evaluation was in 2010-2013,

the coffee leaf miner was monitored by a demarcated area implanted with cultivar Catiguá MG1 in spacing of 3.0 x 0.70 m. This area was not used insecticide during the evaluation period to avoid interference in the population dynamics of the pest. Other cultural practices were usually held in the crop at appropriate times. Within this area 10 random plants and representative sample of which five leaves were collected from each plant in the third pair of leaves branch counted from the tip to the apex were chosen. Samplings were carried out monthly.

The meteorological data were collected in the same period at Meteorological Station located at Experimental Farm Epamig. The percentage of infestation data and meteorological data were plotted on graphs in order to compare the behavior of the coffee leaf miner with climatic data in the region during the study period.

RESULTS AND DISCUSSION

The infestation of coffee leaf miner is under direct influence of precipitation (Fig. 1). In the relevant region the onset of the rains usually occurs from October to March. Temperature also influenced the evolution of insects in the field.

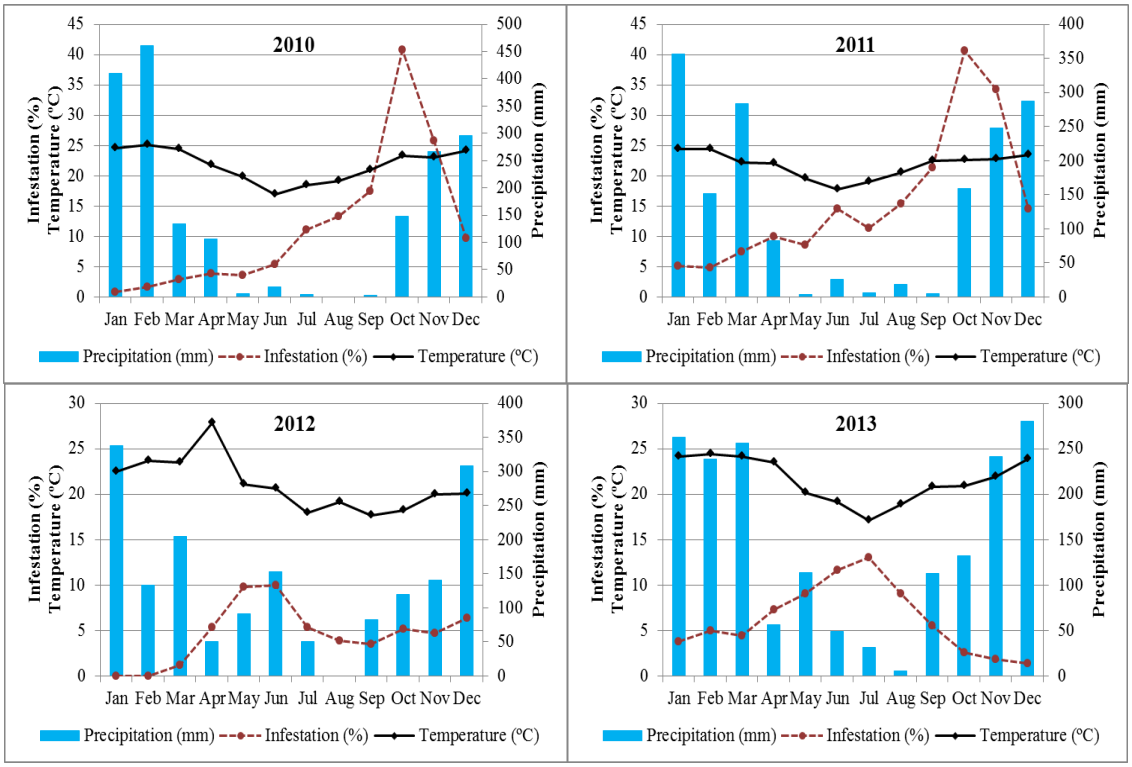

Figure 1. Infestation of the coffee leaf miner (%), cumulative precipitation (mm) and average temperature (° C) for the years 2010 to 2013 in Minas Gerais, Brazil.

In 2010, the precipitation was low in the first months, except to February, this fact led to the development of the coffee leaf miner in increasing levels over the months reaching a peak of infestation in October, probably by late rains. In 2011 there was also a high level of infestation of the coffee leaf miner, which also found that from November, before a high precipitation occurred, the level of infestation decreased in the rains. It was observed that the average temperature was above 20°C from the month of August for these two years increasing the infestation.

In the years 2010 and 2011 there was a similar behavior on the population dynamics of the coffee leaf miner (Figure 1). After July, the population increased with a peak in October, with levels of 41% in 2010 and 40.6% in 2011. These two years defoliation was intense as a result of pest attack. It should be noted that these levels of infestation are highly detrimental for the coffee, which should impair the production due to high defoliation. Furthermore, drastic defoliation between August and October in influencing and flowering of fruit formation also compromed the production.

In the years 2012 and 2013 the pest low rates were observed, probably due to precipitation that occurred in an atypical way to area until July. The highest levels occurred in June and it did not represent a problem in this year. Similar to this was done to infestation of the coffee leaf miner in the period from 1998 to 2001 evaluated in the Zona da Mata region of Minas Gerais State. The results obtained by cited before corroborate this work, where there was the occurrence of the coffee leaf miner in all months of the year, with peaks of ranging infestation, but concentrating between August and November.

Since the population dynamics of the coffee leaf miner is highly variable and that relates to the weather conditions, it becomes essential to the monitoring of evolution of the pest in the field.

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Population Dynamics of the Coffee Berry Borer in Relation to Temperature and Precipitation Changes in the Southeast Region of Brazil

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SUMMARY

The aim of this study was to evaluate the infestation of the coffee berry borer in Southeast Region of Brazil in relation to temperature and precipitation. For this, a coffee plantation with Acaiá MG1474 implanted in 2002 was used to evaluate the percentage of infested fruit. 20 fruits from each side of the plant 50 plants totaling 40 fruits / plant were sampled. The plants were in an experimental area of 1500 plants at the Experimental Farm Epamig. Samples were taken monthly from 50 random plants during the years 2010, 2011, 2012 and 2013 In addition to the data of percentage of infestation data accumulated precipitation and temperature at the assessment were plotted. Variations of infestation occurred during the four years sampled, being necessary to perform monitoring in coffee due to weather and local variations to determine the need and time to apply control measures.

INTRODUCTION

The Brazilian coffee is an activity of great importance for the country, as Brazil is the largest producer and exporter of coffee. Production in 2014 is estimated at 44.57 million 60-kg bags of processed coffee, which highlights the state of Minas Gerais with 22 million bags of processed coffee, representing over 50% of national production.

The infestation of the coffee berry borer is subject to several factors, among them stand out the weather. The temperature directly influences the duration of the cycle of the coffee berry borer higher, temperature smaller the length of the insect cycle resulting in increased population. Precipitation is a major factor for adults of coffee berry borer let the fruit where they were sheltered during the offseason and colonize the fruits of the new harvest, because humidity less than 90% inhibit the output of borers coffee fruits.

The monitoring of the coffee berry borer is an important tool because the infestation may vary from the region, management adopted in the crop year and weather conditions such as temperature and rainfall. And is the evaluation of these factors that define the best possible moment to take control, thus avoiding the unnecessary use of pesticides. The aim of this study was to evaluate the infestation of the coffee berry borer in Southeast Region of Brazil in relation to temperature and precipitation.

MATERIALS AND METHODS

The experiment was conducted at the Experimental Farm of Agricultural Research of Minas Gerais, located in the southern region of Minas Gerais, Brazil. To perform the monitoring a

stand with Acaiá MG1474 deployed in 2002 at a spacing of 3.20 x 0.70 m was used. Within this area 50 plants were randomly selected in a representative way. This area received no insecticide control during the monitoring period and all other cultural practices routinely performed normally.

Month 40 fruits per plant were collected, sampling is 20 fruit on each side of the plant, varying the harvest from the middle third to the lower third totaled 2000 fruit. After harvesting was performed the separation and counting of damaged fruits, subsequently making the determination of the percentage of damaged fruits. This procedure was performed monthly and began three months after the first flowering, starting in December and ending sampling at harvest.

The monitoring was carried out from 2010 to 2013 and climate data were obtained from the meteorological station located at the Experimental Farm. The data were plotted on graphs in order to compare the behavior of the coffee berry borer with the climatic data of the southern region of Minas Gerais in Brazil.

RESULTS AND DISCUSSION

In this work was verified along four consecutive years (2010, 2011, 2012 and 2013) that the incidence of coffee berry borer in the region was variable (Fig. 1). Was observed that there was a wide variation in the levels of infestation in the monitored period.

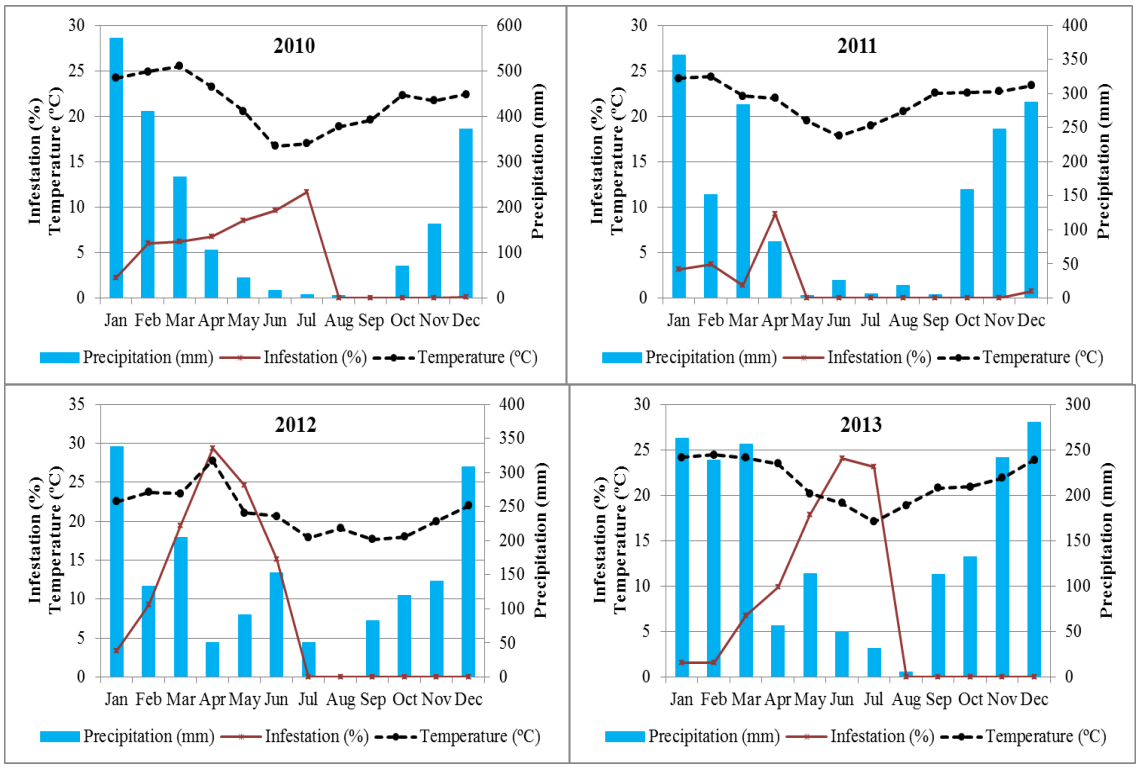

Figure 1. Infestation of the coffee berry borer (%), cumulative precipitation (mm) and average temperature (° C) for the years 2010 to 2013 in Minas Gerais, Brazil.

On the coffee crop in 2010 the coffee berry borer infestations in crops were high since February due to the occurrence of atypical rainfall on the coffee crop in 2009 that favored their survival and multiplication by higher humidity of the remaining nuts. Some studies

emphasize the importance of fruit on the ground in order to survive the coffee berry borer in the off season.

Already in the coffee crop harvested in 2011 due to offseason of 2010 was very dry, the coffee berry borer barely survived and multiplied in the remaining dried fruit, resulting in insignificant infestations.

In 2012, low levels of infestations were also observed since the beginning of the year, this probably occurred by the incidence of low rainfall levels in the off-season months of the previous year. The years 2012 and 2013 had high levels of infestation of the coffee berry borer, reaching 29% in April and 24% in June, respectively. These high levels of infestation besides affecting yield of grains, can damage the quality of the drink, the open gateway to other pathogens.

In this context, monitoring the coffee berry borer is necessary to know the behavior of the insect over the years thus enabling the application of control measures at the right time and in an appropriate manner since many times the infestation level does not reaches the level of control that is 3-5%.

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Histological, Histochemical and Phytochemical Characterization of *Coffea* spp. Resistance to the Root-Knot Nematode *Meloidogyne paranaensis*

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SUMMARY

Meloidogyne paranaensis is a root-knot nematode (RKN) affecting coffee production in Latin America. Simultaneous histological and histochemical studies were carried out on *Coffea arabica* cv Caturra (susceptible), a wild Ethiopian accession of *Coffea arabica* (resistant**)** and a rootstock *Coffea canephora* cv Nemaya (resistant) after inoculation with this RKN in order to characterize compounds accumulating after infection in resistant cultivars. No hypersensitive-like reaction was induced neither in *C. arabica*, nor in *C. canephora* resistant genotypes. Instead, these two resistant coffee genotypes develop the same original resistance mechanisms against *M. paranaensis i.e.* flavonoids accumulation into giant cells of the feeding sites induced by the RKN. Chlorogenic acids accumulating around infestation sites could act as flavonoid precursors.

INTRODUCTION

The root-knot nematode (RKN), *Meloidogyne paranaensis*, causes great losses to coffee crop in Latin America. RKN have developed a sophisticated interaction with host plants inducing the formation of feeding sites composed of 5-7 giant cells (GC).

Previous histological studies on roots of a resistant rootstock cultivar, *Coffea canephora* cv. Nemaya showed that unknown phenolic compounds accumulated in the feeding sites. A comparative study was carried out to localize and identify these compounds involved in defense mechanisms against this RKN on *Coffea arabica* and *C. canephora* since sources of resistance to *M. paranaensis* have been found and used in breeding programs in both species.

MATERIALS AND METHODS

Plant material

Three genotypes were used: *Coffea arabica* cv. Caturra (susceptible, **S**); *C. arabica* ET52, a wild Ethiopian accession, (resistant, **R**)*; C. canephora* cv. Nemaya rootstock (resistant, **R**). Seedlings were cultivated on vermiculite in a growth chamber at 24°C, 70% RH and 16 h of photoperiod.

Nematode inoculum

A population of *M. paranaensis* collected from coffee plants in Brazil was reared on tomato plants. Inoculum was extracted on a mist chamber. Each coffee seedling was inoculated at cotyledonary stage (1.5 month after germinating) with 3000 or 5000 2^{nd} stage juveniles for histological or histochemical studies, respectively.

Histological analysis

Roots of three coffee plants of each genotype, inoculated or not, were removed from the pots and carefully washed at 2, 3, 5, 8, 11, 18, 25, 32 and 45 days post inoculation (DPI). Roots segments (0.5 cm long) were immediately fixed with a Glutaraldehyde-Paraformaldehyde-Caffeine solution in sodium phosphate buffer, pH 7.2 during 48 h at 4°C. Dehydration was then processed in a graded series of ethanol (50, 70, 90, 100%, 1 h each) followed by ethanol-butanol (50/50) during 24 h and butanol during 10 days at 4°C. Samples were embedded in Technovit 7100 (Heraeus Kulzer, Germany) according to manufacturer's protocol. Mounted sections (3 µm) were stained with a toluidine blue [1% (w/v) in borax, pH 8.9] and examined with a light microscope (Leica DM6000B). Unstained sections were observed with the same microscope under UV light with 340-380 nm excitation and 425-800 nm emission to reveal aromatic compounds.

Histochemical analysis

Observations were realized 5, 12, 18, 30 and 45 DPI. Cross-sections (40 µm) were obtained using a vibratome Leica VT 1000 S then mounted in Neu's reagent (DPBA) [4] and observed using a Nikon Optiphot microscope with two sets of filters: UV filter: excitation at 365 nm - Blue filter: excitation 450 nm. In these conditions, chlorogenic acids (**CA**) are detected by their specific greenish-white fluorescence with UV filter while flavonoids have bright yellow fluorescence with Blue filter [5]. Slides were obtained with Nikon Coolpix 4500 camera.

Identification and quantification of phenolic compounds by HPLC analysis

After 90 days of culture in presence or not of nematodes, plants were harvested. Roots of each plant were isolated, washed with distilled water and plunged in liquid nitrogen to be lyophilized. Roots were then reduced in a fine powder (IKA 10) and 15 mg of were mixed (3h, 4°C, orbital shaker) in 4 ml of MeOH/H20 (80/20 v/v). After centrifugation (6 min, 3500 rpm), the supernatant was filtered (0.2 µm porosity, Millipore) and preserved at -20°C till analyzed using a HPLC system (Prominence LC 20, Shimadzu, Japan) equipped with a Eclipse XDB C18 column (3,5µm, 100mm x 4,6mm, Agilent) and a photodiode array detector (Shimadzu, SPD-M20A). The elution system (0.6 mL.min-1) involved H2O/Acetic acid (98:2) and H2O/MeOH/Acetic acid (5:90:5) as eluants. Compounds were identified comparing their retention time with standards.

RESULTS AND DISCUSSION

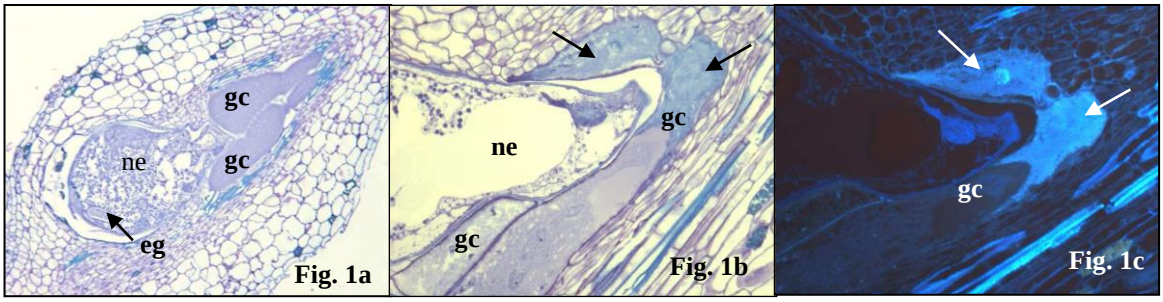

Figure 1. Longitudinal sections of infested coffee roots at 45DPI. a. Well-developed female nematode (ne) with initiation of egg formation (eg) and feeding site with giant cells (gc) on susceptible *C. arabica* cv. Caturra ; b. and c. Accumulation of phenolic compounds in giant cell cytoplasm (black and white arrows) on a resistant *C. canephora* cv. Nemaya closed to the female nematode (ne); b. Toluidine blue stained section; c. unstained section under UV light.

Histological observations

Currently, only sections of *C. arabica* Caturra (S) and *C. canephora* Nemaya (R) cultivars at 28, 35 and 45 DPI have been observed. No hypersensitive like reaction (necrotic reaction) was observed at any time on inoculated R cv. Nemaya. Instead, in R cv. Nemaya inoculated roots, at the three observation dates, azure cytoplasmic areas are observed in GC next to nematode heads (Fig. 1b). Under UV light, these areas present an intense blue autofluorescence revealing the presence of unknown compounds (Fig. 1c). This was not observed on inoculated S cv. Caturra. At 45 DPI, while females of *M. paranaensis* appear well developed with initial egg formation (Fig. 1a), nematodes on R cv. Nemaya appear smaller or damaged with no egg presence.

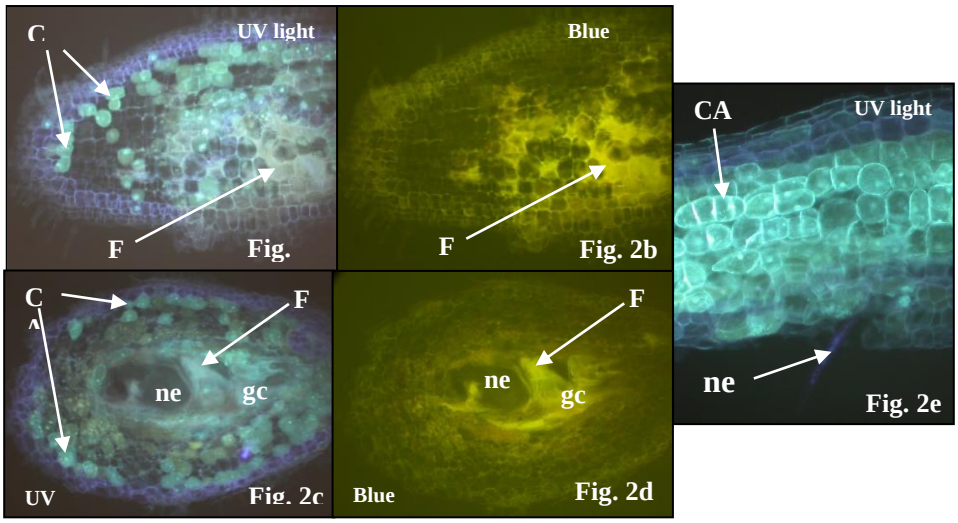

Figure 2. Sections of coffee seedling roots inoculated with *M. paranaensis* at 45 DPI mounted in Neu's reagent (DPBA). a, b. Resistant *C. arabica* ET52 accession; c, d. Resistant *C. canephora* cv. Nemaya; e. Susceptible *C. arabica* cv. Caturra; CA: caffeoyl quinic esters with greenish fluorescence under UV light and without fluorescence under blue light; F: Flavonoids with whitish and yellowish fluorescence under UV light and blue light, respectively (x100 magnification).

Histochemical characterization

In both R *C. canephora* cv. Nemaya and *C. arabica* ET52 accession (Fig 2a, 2c) as well as in S *C. arabica* cv. Caturra (Fig. 2e), numerous cells surrounding nematode feeding sites appear with a white greenish fluorescence under UV light which fade under blue light indicating the presence of caffeoyl quinic esters (CA). The observation of high levels of CA on S cv. Caturra (Fig. 2e) indicates that CA likely don't affect the nematode life cycle. In both R genotypes, whitish fluorescence areas under UV light turning yellow under blue light in giant cells and cell walls reveal that the accumulated phenolic compounds observed in histological sections are flavonoids (Fig.2b, 2d).

HPLC analysis

The flavonoids, naringenin and quercetin derivatives, accumulate in infested roots (90DPI) of R *C. canephora* cv. Nemaya and, at a lower level, of S *C. arabica* cv. Caturra (Fig. 3b, 3c). Other flavonoids, catechin and epicatechin derivatives, accumulate only in infested roots of R *C. canephora* cv. Nemaya (Fig. 3c). Caffeic acid and especially CA, in large amounts, are present in all studied roots with or without nematode inoculation (Fig. 3a, b, c).

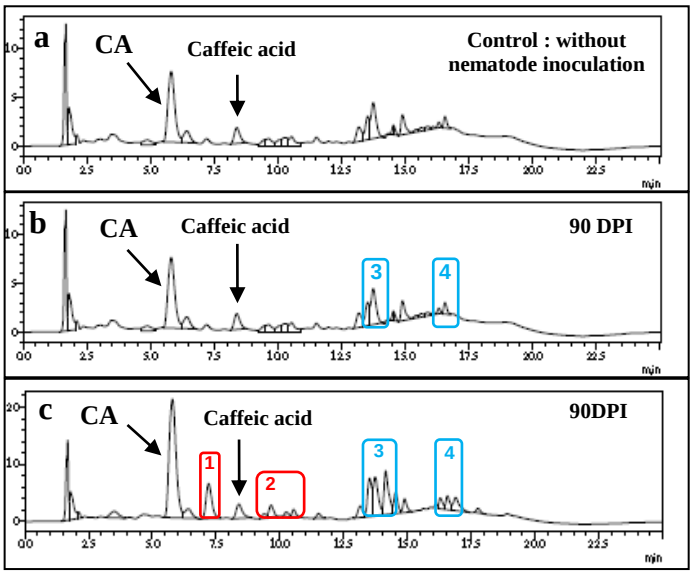

Figure 3. HPLC chromatograms of coffee seedling roots. a, b. Susceptible *C. arabica* cv. Caturra without inoculation and inoculated with *M. paranaensis* at 90 DPI, respectively; c. Resistant *C. canephora* cv. Nemaya inoculated with *M. paranaensis* at 90 DPI; CA: caffeoyl quinic esters; 1 & 2: Catechin and epicatechin derivatives; 3: Naringenin derivatives; 4: Quercetin derivatives

Both resistant coffee genotypes develop a same original resistance mechanism. No hypersensitive like reaction was observed (necrotic reactions). Instead, phenolic compounds accumulate *de novo* in GC of established feeding sites induced by *M. paranaensis*. These compounds are flavonoids, especially catechin and epicatechin derivatives. Flavonoids are known for various defense properties in plants and showed efficiency against *Meloidogyne spp. in vitro*. This *de novo* flavonoid accumulation seems to effectively prevent normal development and reproduction of *M. paranaensis*, conferring a quantitative resistance to this very pathogenic RKN. Caffeoyl quinic acids present with high amounts could act as flavonoid precursors.

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Thylakoid Lipids Changes May Account for Photosynthetic Acclimation Ability of Two *Coffea* Species Subjected to Heat

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SUMMARY

Coffee is one of the world's most traded agricultural products, and its production could be threatened by global warming. The aim of this work was to evaluate the effects of heat on photosynthetic activity and thylakoid membrane lipid dynamics, on genotypes of the two major coffee producing species. Potted plants from *C. arabica* L. cv. IPR108 and *C. canephora* Pierre Ex A. Froehner cv. Conilon Clone 153 were grown for 1 year under controlled conditions of temperature (25/20ºC, day/night), irradiance (650-800 μmol m^{-2} s^{-1}), RH (75%), photoperiod (12 h), and 380 μL CO_2 L^{-1}. Thereafter, temperature was gradually raised to 42/34ºC (0.5ºC/ day), with a 7 days stabilization step at 31, 37 and 42ºC. Studies focused modifications of thylakoid lipid composition and photosynthetic performance. In CL153 photosynthetic capacity (A_{max}) was not affected until 42ºC (40% reduction). In IPR108 it was reduced 35 and 57% at 37ºC at 42ºC, respectively. Thylakoid electron transport rate for photosystems (PS) I and II increased (*ca*. 10-25%) up to 37ºC in both genotypes. At 42ºC only IPR108 presented depressed activities on PSII (15%) and PSI (18%). Under 37 and 42ºC, CL153 plants presented digalactosyldiacylglycerol (DGDG) (*ca*. 42%) and monogalactosyldiacylglycerol (MGDG) (28-34%) increases, while IPR108 showed higher MGDG at all temperatures. In CL153 less unsaturated DGDG and phosphatidylglycerol (PG) along with stable or increased DGDG/MGDG ratio, may have contributed to sustain thylakoid electron flow at 37ºC and even 42ºC. IPR108 displayed a strong PG rise at all temperatures, in accordance with enhanced PSs activity.

INTRODUCTION

According to modeling studies, global warming resulting from climate change may affect coffee production, one of the world's most traded agricultural products. Ambient temperature fluctuations have a direct impact on photosynthesis, namely through its effects on the thermally sensitive biochemical and physiological processes that occur in membranes. Beyond a critical temperature, heat induces thylakoid membrane destabilization and photosynthesis inhibition. Lipoperoxidation may also occur leading to photosynthesis impairment and leaf senescence. Plants cannot regulate their own temperature (poikilotherm organisms). Yet, membrane lipids may undergo quantitative and qualitative modifications upon stress exposure, ensuring an adequate fluidity under changing environmental conditions. Thylakoid membranes are mostly constituted by monogalactosyldiacilglicerol (MGDG), digalactosyldiacylglycerol (DGDG) and phosphatidylglycerol (PG) that contribute to photosynthetic structures stability and functioning. The aim of this work was to assess heat effects on thylakoid lipids and photosynthetic activity of two coffee genotypes.

MATERIALS AND METHODS

Plant material and experimental design

Plants with *ca*. 1.5 years from *C. arabica* L. cv. IPR108 (IPR108) and *C. canephora* Pierre ex A. Froehner cv. Conilon Clone 153 (CL153), were transferred into walk-in growth chambers (EHHF 10000, ARALAB, Portugal) and grown in 28 L pots under controlled conditions of temperature (25/20ºC, day/night), irradiance (*ca*. 650-800 μmol m^{-2} s^{-1}), RH (75%), photoperiod (12 h) and 380 μL CO_2 L^{-1} for 1 year, without water, nutrient or root development restrictions. Temperature was then increased from 25/20ºC up to 42/34ºC, at a rate of 0.5ºC day^{-1}. After 7 days stabilization at 31/25, 37/30 and 42/34ºC., analyses were performed on newly matured leaves.

Photosynthetic capacity

The photosynthetic capacity (A_{max}), representing light (*ca*. 900 μmol m^{-2} s^{-1}) and CO_2 (*ca*. 7%) saturated rate of photosynthesis under optimal temperature (25ºC), was measured with a Clark-type O_2 electrode (LD2/2, Hansatech, UK) according to [7].

Thylakoid electron transport rates

To obtain sub-chloroplast fractions and determine the *in vivo* electron transport rates associated with both PSI ($DCPIPH_2$→MV) and PSII, including (H_2O→DCPIP) or excluding (DPC→DCPIP) the oxygen evolving complex (OEC), measured polarographically with an LW2 O_2 electrode (Hansatech, UK) at 25 °C, optimized methods for coffee were followed [7].

Chloroplast membrane lipids

Lipid extraction and analysis were performed according to [8]. Lipid classes were separated by thin layer chromatography using two solvent systems. Lipid bands were scraped off, saponified and methylated for individual FAs analysis by gas-liquid chromatography (Varian CP-3380, U.S.A.). Individual FAs and lipid classes were identified by comparison with known standards (Sigma, Supelco). Total fatty acids (TFAs) values are the sum of individual FAs. The double bond index (DBI) was calculated as DBI = [(% monoenes + 2 x % dienes + 3 x % trienes / (% saturated FAs)].

RESULTS AND DISCUSSION

In CL153 A_{max} remained unaltered until 37ºC and was reduced 40% only at 42ºC (Fig. 1), whereas IPR108 presented reductions already at 37ºC (35%), and also at 42ºC (57%).

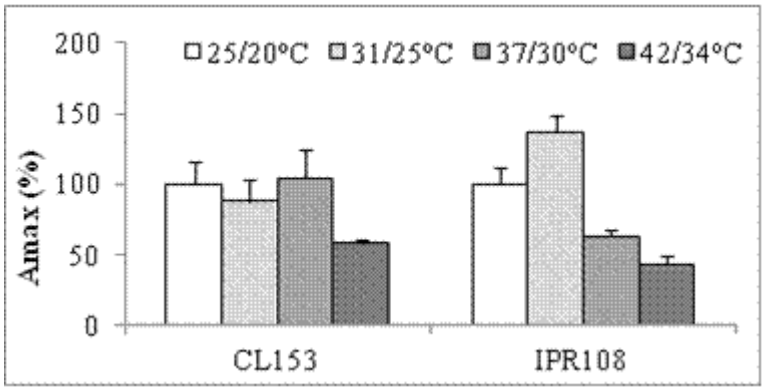

Figure 1. Changes (in % of µmol O_2 m^{-2} s^{-1}), within each genotype, relative to plants at 25/20ºC) for photosynthetic capacity (A_{max}) rates). Each value represents the mean ± SE (n=5-8).

As regards the thylakoid electron transport at photosystem (PS) I and II levels, increases at 37ºC (*ca*. 9-16%) were found in CL153 (Fig. 2). At 42ºC the values were similar to control. In IPR108 PSI and II activities strongly rised at 31ºC (34-40%) and 37ºC (20-26%), but at 42ºC fell 18% (PSI) and 15% (PSII). Oxygen evolving complex (OEC) did not contribute to changes in PSII activity (Fig. 2). Results denote a strong tolerance of such photosynthetic membrane bound events to high temperature in coffee. That agrees with the findings that light absorption, energy transfer and charge separation associated with PS II and I are quite insensitive to temperature in the biologically relevant range of 0-50ºC.

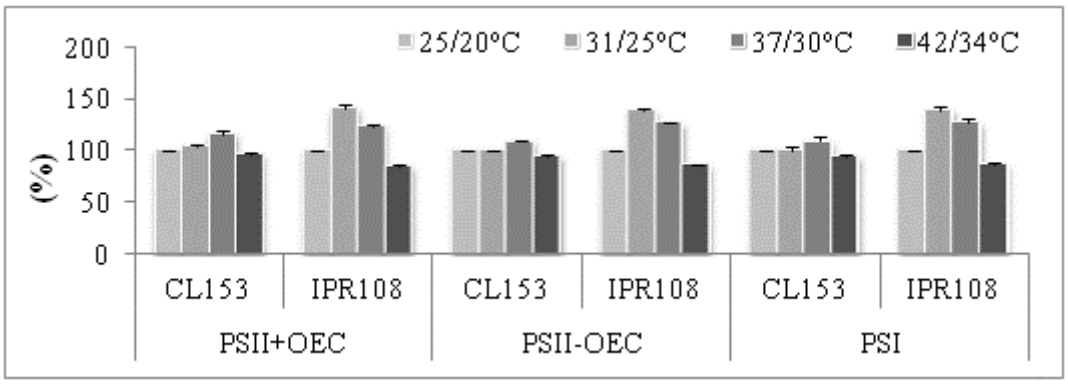

Figure 2. Changes (in %, from results expressed in µmolO_2 m^{-2} s^{-1}, within each genotype, relative to plants at 25/20ºC) for the *in vivo* electron transport rates associated with PSII, including (PSII+OEC) or excluding (PSII-OEC) the oxygen evolving complex (OEC), and PSI. Each value represents the mean ± SE (n=4-5).

Heat induces hyperfluidization of membranes, affecting lipid-protein interactions and causing various disturbances, such as, phase transitions of lipids and conformational changes, therefore altering their functions, namely in thylakoid PSII reactions. Such thylakoid

structural rearrangements are usually reversible when temperature returns to control values [10]. These short-term adaptive processes take place within minutes or hours and rapidly modify the thermal sensitivity of the photochemical apparatus of photosynthesis. Additionally, long-term acclimation may trigger other processes to high temperature that involves *de novo* protein and lipid synthesis. In CL153 chloroplast lipid, evaluated through total fatty acid (TFA) content, slightly decreased (12-16%) at 31 and 37ºC, while in IPR108 strong increases were found (Fig. 3), particularly at 37ºC (105% rise). At 42ºC the TFA contents approach control levels in both genotypes. Furthermore, the *de novo* synthesized lipids showed unaltered unsaturation (DBI) at 37ºC, but a decrease was found at 42ºC (Fig. 3).

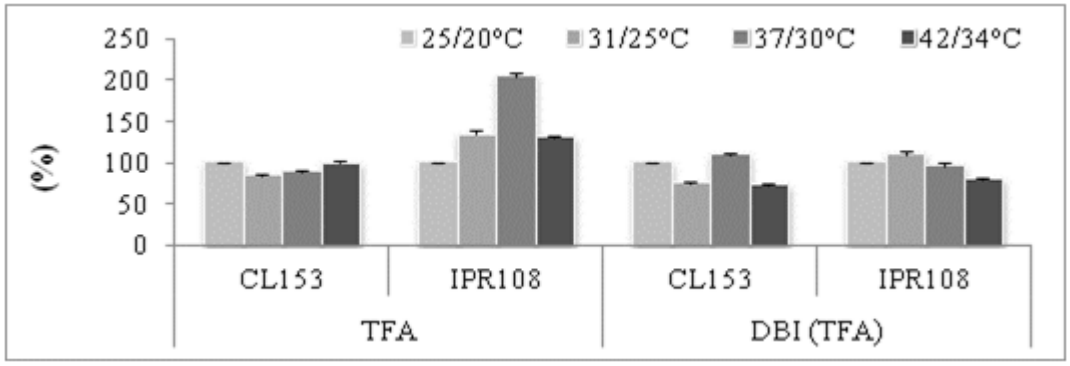

Figure 3: Changes (in %, within each genotype, relative to plants at 25/20ºC) for total fatty acids (TFA) and double bond index (DBI). Each value represents the mean ± SE (n=3-4).

Increased lipid content requires gross membrane biogenesis rather than modification of existing molecules, and may result in altered proportions of individual phosphoglycerides, which could indicate potential adaptive changes. As regards the galactolipids MGDG and DGDG (Fig. 4), CL153 plants subjected to high temperatures (37 and 42ºC) depicted significant DGDG (*ca*. 42%) and MGDG (28-34%) increases. In IPR108 DGDG decreased (32% at 37ºC), while MGDG increased at all temperatures (53, 12 and 39% at 31, 37 and 42ºC. Galactolipids ratio (DGDG/MGDG) was unaltered in CL153 (Fig. 4) except at 37ºC (10% increase). That ratio decreased in IPR108 with temperature (37% at 31 and 37ºC, and 31% at 42ºC), mainly due to a higher proportion of MGDG at all temperatures above control (Fig. 4). Synthesis of MGDG and DGDG may contribute for drought tolerance.

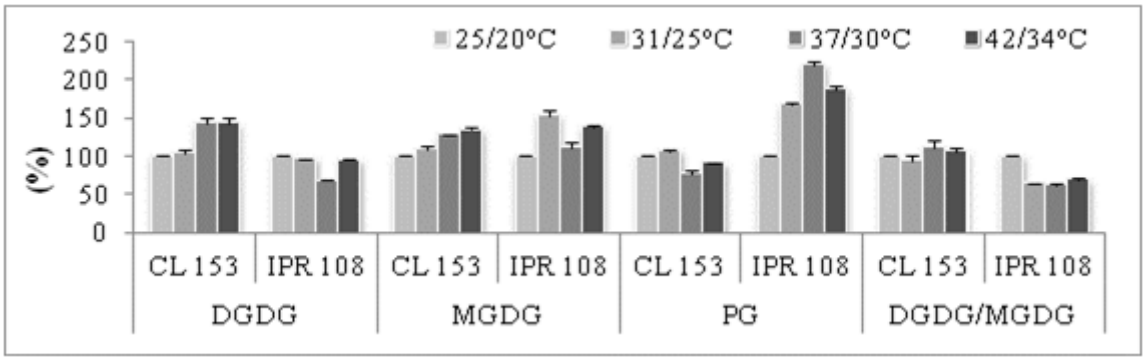

Figure 4: Changes (in %, within each genotype, relative to plants at 25/20ºC) for monogalactosyldiacylglicerol (MGDG), digalactosyldiacilglycerol (DGDG), phosphatidylglycerol (PG) and the ratio DGDG/MGDG. Each value represents the mean ± SE (n=3-4).

The packing arrangement of MGDG in the thylakoid outer monolayer is closely related to the rate of photo and dark-phosphorylation, being required for the functioning of the ATP synthase. Moreover, DGDG is a bilayer-forming lipid that helps to preserve thylakoid stability. Furthermore, variation in the proportion of galactolipids allows membrane structural and functional changes and the rise in DGDG/MGDG ratio due to enhanced DGDG synthesis was related to heat tolerance in other species, what agrees with the higher heat tolerance of *C. canephora* when compared to *C. arabica*.

In CL53 PG decreased at 37 and 42ºC (23 and 10%), while IPR showed a strong rise at all temperatures (67, 120 and 88% at 31, 37 and 42ºC) (Fig. 4). PG is a major chloroplast phospholipid, which plays an important role in photosynthetic structures stability and functioning [6]. In the inner monolayer PG and DGDG are needed for the formation, stability and functioning of photosynthetic complexes, namely of LHCII trimers.

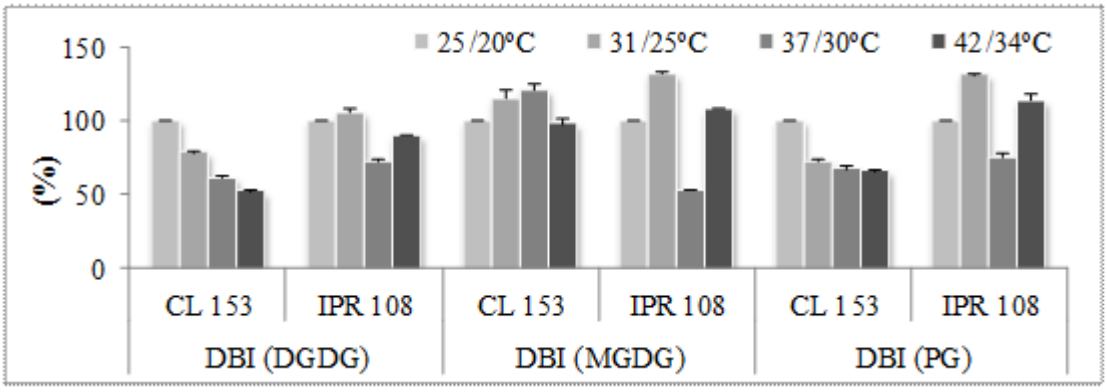

Figure 5: Changes (in %, within each genotype, relative to plants at 25/20ºC) for the double bond index (DBI) of monogalactosyldiacylglicerol (MGDG), digalactosyldiacilglycerol (DGDG) and phosphatidylglycerol (PG). Each value represents the mean ± SE (n=3-4).

Both genotypes tended to display higher TFA saturation (lower DBI) at 42ºC (Fig. 3). In CL153 this was due to less unsaturated DGDG and PG (Fig. 5). This feature, along with stable or increased DGDG/MGDG ratio, may have contributed to sustain thylakoid electron flow at 37ºC and even 42ºC (Fig. 2). IPR108 displayed a strong PG increase at all temperatures, which might be related to the enhanced thylakoid functioning and A_{max}. Yet, at 42ºC photosynthetic activity was strongly impaired. In fact, at both 37 and 42ºC, other limitations, linked, *e.g.*, to lower enzyme activity of RuBisCO, were likely involved in the reduction of photosynthetic performance.

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And if We Could Use Caffeine as a Tracer For Climate Change in the Coffee Production Regions of the Globe?

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SUMMARY

The application of stable isotopes at natural abundance levels has brought a new dimension to our understanding of plant physiology and ecology. Analyses of the relative natural abundances of stable isotopes of carbon, oxygen and deuterium have been used across a wide range of scales, from cellular to community and ecosystem level, contributing much to our understanding of the interactions between biosphere, pedosphere and atmosphere. Although isotope fractionation in seeds (*e.g.*, coffee beans) is yet poorly understood, previous work suggests that the interpretation of isotope abundance in the green coffee bean reflect a combination of environmental, climatic, and physiological processes. The $\delta^{18}O$ of green coffee beans and correspondent caffeine have been determined by isotope ratio mass spectrometry (IRMS) in a total of 122 coffees originating from 20 different coffee producing countries. The $\delta^{18}O$ of green coffee bean samples ranged from + 18.7 to + 37.4‰, which are values approximate to the $\delta^{18}O$ referred in literature for plant organic matter. Caffeine had $\delta^{18}O$ values varying from -5.5 to + 9.8‰, showing an isotopic signature closer to values 17 predicted for local precipitation. A correlation between the $\delta^{18}O$ values of caffeine and of coffee bean was obtained. Results show that $\delta^{18}O$ caffeine is a possible tool to study coffee plant's growth environment and physiological activity during the period of seed (coffee bean) development.

INTRODUCTION

Research based on isotope analysis has grown steadily in the past 60 years and it has become an important part of many studies in the fields of ecology, geochemistry, biochemistry and archaeology. The understanding of a wide set of processes responsible for the variations in isotope abundances in natural environments has also improved, allowing to know more on how environmental change is recorded. Isotope analysis of chemical elements of coffee beans or of specific organic compounds extracted from those seeds may help to understand how different ecological and physiological processes influence plant development and physiology during seed developmental period. Because this is intrinsically related with local climatic conditions (i.e., temperature, precipitation, air humidity), results generally potentiate the development of analytical tools towards traceability of coffee. The oxygen stable isotope ratio ($\delta^{18}O$) of plant water, of the organic molecules that make up plant tissues and of the gases

produced during plant metabolism, all record important aspects of the plant's physiological activity, growth and environmental conditions at various spatial and temporal scales. This isotopic signal is also modulated by morphological and other differences between species, including their responses to environmental variation. Stable isotope theory predicts that information on plant-environment interactions is retained on different time scales in the oxygen stable isotopes signature of different plant tissues, depending on the analyzed molecules and on their turn-over rates. Short-term information is provided by $\delta^{18}O$ of leaf and sap soluble sugars, whereas long-term information (seasonal or annual) is contained in cellulose or bulk tree rings. Other moieties, linked to well-defined phenological phases of the plant ontogeny, could assume an intermediate meaning, recording ecophysiological responses on a medium-term time scale. Such is the case of plant seeds. Caffeine in *Coffea* spp., as well as other alkaloids in many other plant species, is a candidate molecule in this sense. The pathway of caffeine synthesis during fruit development in *Coffea arabica* and *Coffea canephora* is similar, although the caffeine content in these two species is 0.6-1.5% and 2.2-2.7%. Caffeine is present in the seed (coffee bean) but it is neither degraded nor additionally formed during the development of cotyledons. As soon as leaflets develop and push apart the stipules, caffeine concentration sharply increases. The final amount and concentration of caffeine in the coffee bean is a result of (1) acquisition from the perisperm, (2) import from the pericarp, and (3) intrinsic biosynthesis. The first two depend on the allocation of chlorogenic acids in the plant. The intrinsic biosynthesis is higher during the time of endosperm expansion. As soon as the endosperm (coffee bean) is formed, caffeine biosynthesis in seeds and pericarp sharply decreases. Once the seed is totally formed, the fruits loose chlorophyll and accumulate anthocyanins and sugars, resulting in the characteristic coffee cherry. The fruiting phase lasts approximately 7 to 8 months and the caffeine synthesis and accumulation in the coffee bean is completed during the first 4 to 5 months. Accordingly to the caffeine biosynthesis pathway, a correlation between the $\delta^{18}O$ value of caffeine and that of the leaf water is expected. If this prediction is to be verified, then caffeine could be used as a tracer of environmental and agronomic conditions of the coffee plantations. It is important to bear in mind the fact that caffeine is biosynthesized during a period when the coffee plant is under steady-state conditions in what respects to the plant water relations, at least in most of the regions where it is cultivated. Once formed, this molecule remains highly stable in the coffee bean.

The aim of this work was to verify if caffeine's oxygen isotopic composition may be used as a proxy of relevant aspects concerning water relations during plant growth and that results in differences in micro-environmental conditions and physiological activity. The variations observed in the isotopic composition of caffeine molecule were interpreted in comparison to predicted values of local precipitation $\delta^{18}O$. Furthermore, the well-defined dynamic of caffeine synthesis on a time scale of a few months makes this specific compound an important tool for studies of water cycle processes from regional to global scales.

MATERIALS AND METHODS

Green Coffee Bean Samples

Samples of green coffee beans (n = 122) from 20 different countries were provided by Novadelta, Comércio e Indústria de Café, S.A. (Campo Maior, Portugal), by Coffea Consulting and the University of Hawai'i (Hawai'i, US), by University Federal of Lavras in Minas Gerais, Brazil. All samples were of Arabica coffee (*Coffea arabica*) except one sample from Angola of Robusta coffee (*Coffea canephora*). Each coffee sample consisted in 100 g of green coffee beans. Latitude, longitude and altitude data correspondent to each coffee sample were tracked with Google Earth software, version 5.0 (Google, UK). Annual and monthly

mean δO values of precipitation were acquired from the Online Isotopes in Precipitation Calculator (OIPC).

Caffeine Extraction

Caffeine was extracted according to [16] with some modifications in order to facilitate filtration steps. 50 g of finely ground beans of green coffee were boiled in Milli-Q isolated by filtration and recrystallised from methanol (Sigma-Aldrich, Germany). After filtrated with gauze. The solution was centrifuged at 5000 rpm for 10 minutes in order to remove remaining solid particles and mucilage. The supernatant was filtered with GFC 0.45 μm filters (Macherey-Nagel, Germany). The filtered solution was subjected to continuous liquid-liquid extraction (5h) with chloroform (100 mL). The organic phase was dried over anhydrous Na_2SO_4 (Panreac, Spain), filtered, and concentrated under reduced pressure to approximately 5 mL. The caffeine, precipitated by addition of 10 mL petroleum ether (Sigma-Aldrich, Germany), was HPLC. Extractions with synthetic caffeine as a surrogate were performed to check the extraction procedure for potential isotope discrimination.

Isotope Ratio Mass Spectrometry (IRMS)

Oxygen isotope ratios were determined on an Isoprime (Micromass, UK) isotope ratio mass spectrometer on continuous flow mode, coupled to an elemental analyzer (EuroVector, Italy) for were corrected against international standards (IAEA 601 and IAEA 602). Analytical packed with glassy carbon chips, inserted co-axially on a ceramic tube. The isotope ratio data of interest (*e.g.*, performance, stability and drift, were checked by inserting laboratory standards between samples. Corrections were made when necessary. Precision was 0.14‰.

Statistical analysis

Basic statistical analysis, Spearman's correlation, one-way ANOVA were performed with the Statistica software, version 10.0 (StatSoft, Inc., US).

RESULTS AND DISCUSSION

Previous work has shown that oxygen isotopes may enable the discrimination among small coffee producing regions and that this is related to oxygen isotopic composition ($\delta^{18}O$) of local precipitation. The same authors observed a global scale variation of the isotope composition of oxygen in the whole coffee bean. The global mean value was +28.4‰ (±3.8), approaching the global mean ^{18}O enrichment of +27‰ (± 4) of carbohydrates relative to the same source water [1-3, 6], with a global range of approximately 21‰. In the present study, the oxygen isotope composition of caffeine extracted from green coffee beans was determined. Accordingly to the previous reports, in this study we observed a positive correlation between $\delta^{18}O$ of the coffee bean and $\delta^{18}O_{caffeine}$. In Figure 1, it is possible to observe a highly significant correlation between these two parameters (R= 0.8). Moreover, both $\delta^{18}O_{caffeine}$ and $\delta^{18}O$ of the green coffee bean were correlated with $\delta^{18}O$ of local precipitation, at global scale (R = 0.5) (data not showed).

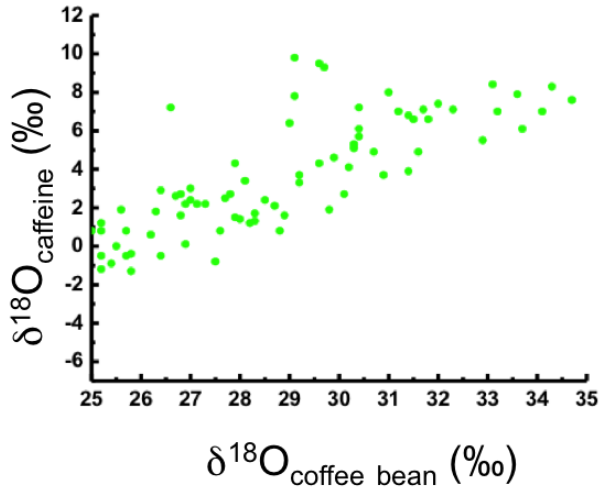

Figure 1. Relashionship between oxygen isotope composition ($\delta^{18}O$) of coffee bean and caffeine extracted from 29 producing countries from three different continents: Africa, Asia and America.

Figure 2 shows $\delta^{18}O_{caffeine}$ variation among the different geographic regions. It is possible to observe more enriched $\delta^{18}O_{caffeine}$ values for certain African countries (*e.g.* Kenya Zambia and Rwanda) comparing to other regions, *e.g.* in Asia (*e.g.* East Timor and Indonesia). The global mean of $\delta^{18}O_{caffeine}$ value was +3.8‰ (±3.6), with individual values ranging from -4.6 to +14‰ (Fig. 2), which corresponds to a global variation of 9.4‰. This global mean value is closer to the average of the annual mean value of $d^{18}O$ for precipitation (-4.9‰), for the locations included in this study. Moreover, the results show more enriched $\delta^{18}O_{caffeine}$ values for African countries such as Ethiopia, UR Tanzania, Kenya, Zambia and Rwanda (Fig. 2). In comparison, the more depleted values of $\delta^{18}O_{caffeine}$ were measured in caffeine molecules extracted from coffees produced in certain regions of Indonesia and Costa Rica.

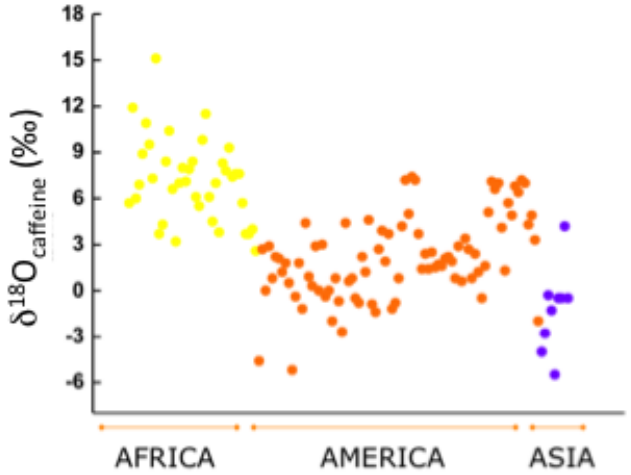

Figure 2. Variation of the oxygen isotope composition of caffeine ($\delta^{18}O_{caffeine}$) in 122 coffee origins from 22 different countries located in three different continents: Africa, America and Asia.

Higher differences between $\delta^{18}O_{caffeine}$ and $\delta^{18}O$ of rainfall could reflect environmental conditions leading to higher coffee plant evapotranspiration rates. Several factors influence the oxygen isotope ratio of plant organic material and of its specific compounds: variations in the $\delta^{18}O$ of water taken up by the plants, the leaf water enrichment in ^{18}O owing to transpiration and the fractionation in $\delta^{18}O$ of water in cells where organic material is synthesized (*e.g*. carbohydrates biosynthesis). Leaf water is sensitive to variations in humidity and stomatal conductance. In accordance to this, the measured $\delta^{18}O_{caffeine}$ values are expected to be the resultant of those various isotope fractionation events occurring during the coffee bean development period. In order to understand the magnitude of the difference between the $\delta^{18}O$ of coffee beans and that of caffeine, it is necessary to consider the caffeine biosynthetic pathway. One oxygen atom is introduced in the course of the incorporation of HCO_3^- into the purine nucleus, and the other by the addition of H_2O to the double bond of the purine nucleus to form xanthosine, the initial substrate of purine alkaloid synthesis. The biosynthetic pathway leading from primary metabolism to caffeine involves three methylation reactions and a de-ribosylation, which do not involve the oxygen atoms of the xanthosine purine nucleus. However, secondary partial exchange of oxygen atoms with water is possible. Thus, a correlation between the $d^{18}O$ value of caffeine and that of the leaf water is expected. If this prediction is verified, then caffeine could be used as a tracer of environmental and agronomic conditions of Results suggest that $\delta^{18}O_{caffeine}$ the may be affected by variations in $\delta^{18}O$ of both plant source water and enriched water in the leaves or in other transpiring organs, however, without reflecting additional metabolic isotope fractionations. Thus, $\delta^{18}O$caffeine may be used as a tracer for plant responses to climate at a global scale, since it integrates local precipitation, tempered by physiological transpiration. Thus, we may use in the near future, caffeine, as a tool to predict the physiological coffee plants response to precipitation and temperature under scenarios of climate change.

ACKNOWLEDGEMENTS

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Spatial Decision Support for Coffee Disease Management – Site-Specific Shade – Environment Interaction affecting American Leaf Spot Disease

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SUMMARY

Mycena citricolor, causal agent of the American Leaf Spot Disease (ALSD) is reported to be favored by cool and humid conditions and by excessive shade. However, there is little knowledge of shade effects in different environments. This study aims to prove that the effect of shade on ALSD severity depends on environmental and site-specific conditions and to predict these relations spatially. In two mayor coffee growing regions of Guatemala and Honduras, disease risk including shading was mapped at two different scales using CaNaSTA, a Bayesian statistics decision support tool that combines field survey data, expert knowledge and spatial climate data. Results indicate an interaction between shading and environmental factors, influencing disease intensity according to site specific conditions.

INTRODUCTION

Various studies analyzed the role of shade in coffee production, however its effects on diseases still remain controversial, particularly because shade interactions with environment are almost never taken into account. Only average effects are generally described. It is admitted that some pathogens benefit in average from shade trees, and yet others are negatively hindered. Furthermore, the effects of shade trees should also vary across environmental conditions, but in spite of the many studies conducted on shading in coffee production, there is little quantitative knowledge of field-level investigation on shade effects in different environments. Several studies concentrating on coffee quality and productivity concluded that optimum management should be site-specific and developed methods to determine the impact of specific environmental and agronomic management factors on coffee quality and to identify site-specific crop management practices, using statistical crop models revealing the relationship between variations in observed coffee quality and variations in certain growing conditions. This approach can also be applied to analyze the relation between coffee disease incidences and intensities, environmental parameters and agronomic factors and to demonstrate shade interactions with environment, in order to determine site-specific shading practices. The objective of the present study was to map *M. citricolor* distribution and to spatialize the relation between environment, shade practices and disease intensity at two different mesoscales (400 000 km² and 120 000 km²).

MATERIALS AND METHODS

Field data on ALSD severity and its relation to different disease driving factors were analyzed using logistic regression. Probability distribution of ALSD risk in dependence on shading was estimated as a function of categorized environmental predictors in Honduras (Marcala, La Paz) and Guatemala (south of Chimaltenango) using CaNaSTA. The Bayesian model combines field survey data, expert knowledge and spatial climate data to generate distribution maps by calculating the conditional probability for selected predictor variables against the response variable (synthetic index, calculated with disease severity data obtained in field) at each location. The created probability values are weighted to generate the score (a measure displaying the entire probability distribution in one map; a high score means a high probability of a high attack intensity) and certainty (a measure of confidence for the prediction, depending on the number of the observations). Thirteen predictors, explaining ALSD epidemics best were selected according to and empirical knowledge. Layer of topological predictors of the study area were generated with 90 m resolution from the digital elevation model (DEM) of the shuttle radar topography mission (SRTM). Climate layers were generated using the WorldClim climate database. Field data on shade cover percentage and ALSD severity were collected on 38 geo-referenced plots in Guatemala and 63 in Honduras in 2010. Half of the sample plots were managed under a high percentage (> 20 % for Guatemala, > 60 % for Honduras) of shade and the other half under little shade to full sun exposure (< 20 % for Guatemala, < 60 % for Honduras). Shade cover percentage was estimated with a spherical densitometer at two points in each plot. ALSD attack intensity was evaluated at the end of the rainy season, using a 0 to 3 disease severity rating scale. A synthetic index for each plot was calculated.

RESULTS AND DISCUSSION

A significant interaction between altitude and shade influencing ALSD severity jointly justified geospatial mapping (Tab. 1). Spatial score prediction for ALSD under high and low shade for the two study regions is shown in Figg. 1 and 2. The environmental predictors in the high scored areas obtained either with the low or the high shade model were compared in order to examine their influence on ALSD intensity in combination with different shade levels (Tab. 2). For low altitude areas of the spatially heterogeneous study region in Guatemala our mapping suggested a higher disease risk with high shade levels, whereas at high altitudes the model generally generated higher disease risk with low shade levels. The lower area is mainly characterized by suboptimal conditions for ALSD (Tab. 2), therefore shade effects such as buffering the above-optimal temperatures or interception of light, could make conditions for *M. citricolor* development more favorable and increase disease risk. In contrast, environmental conditions in the higher zones, conditions were generally more favorable, such that shade could negatively affect the pathogens' development. At high altitudes, fog and dew are very frequent and create conducible humidity conditions for ALSD. Shade trees could reduce this effect by intercepting dew and fog. Shade trees also intercept rainfall, which in the case of low rainfall intensities could result in a reduction of spore dispersal due to fewer drops falling on the coffee leaf surface.

Table 1. Statistical Analysis of field data of Guatemala.[1]

	DF	Deviance	Resid. DF	Resid. Deviance	Pr(<Chi)
Shade	1	2.4889	36	49.768	0.11465
Altitude	1	0.2293	35	49.539	0.63203
Slope	1	6.4353	34	43.106	0.01120*
aspect	1	4.2310	33	38.875	0.03969*
Shade*Altitude	1	2.8298	32	36.045	0.09253.

[1] *Logistic regression. Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '..' 0.1 '.' Chi-Square test: 0.284883*

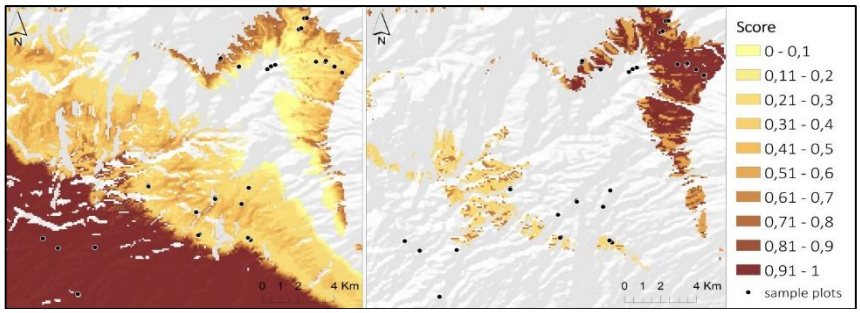

Figure 1. Probability distribution map for *M. citricolor* attack intensity with high (left) and low shade model (right) for Chimaltenango, Guatemala (Certainty ≥ 0.8).

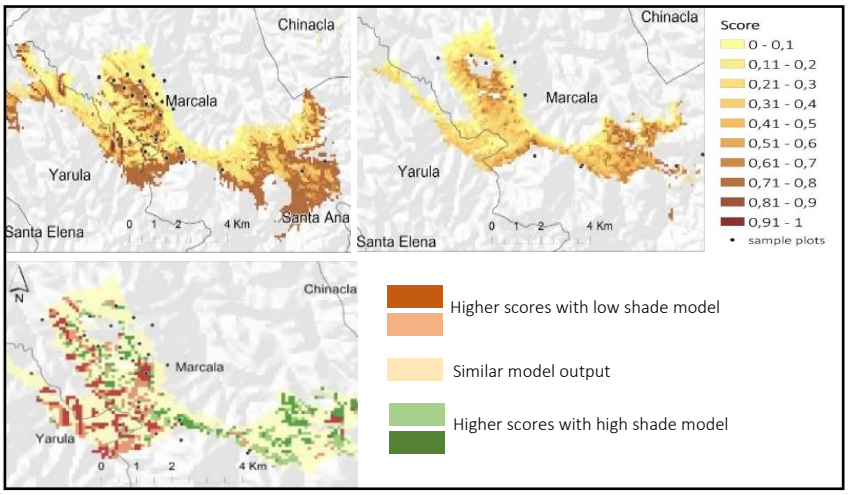

Figure 2. Probability distribution map for *M. citricolor* attack intensity with low (left) and high shade model (right) for La Paz, Honduras. Combined map of both model outputs (below) generated by subtraction of raster of low and high shade maps. (Certainty ≥ 0.8).

For the smaller study area in Honduras, where spatial heterogeneity of altitude and climatic patterns was low, the predictors slope aspect and solar radiation seemed to be critical factors. At eastern facing slopes, the model predicted a higher disease risk with high shade levels, while at western facing slopes a higher disease risk was associated with low shade levels. The exposition of slopes is a key factor in disease development – especially eastern facing slopes are more exposed to sunlight, leaf wetness duration and humidity is therefore reduced, which in turn reduces infection rates. Shade has the ability to intercept light and would therefore improve conditions for ALSD development if slopes are eastern faced. At western faced

slopes where humidity conditions are favourable for ALSD, shade trees may intercept wetness caused by dew and fog, which in turn would be disadvantageous for the fungus. Our results indicate an interaction between shading and environmental factors, influencing disease intensity according to site specific conditions. It was highlighted, that dependent on the environmental conditions, shade cover either had an increasing, decreasing or no effect on the disease severity. Therefore it can be claimed, that conventional assumptions about both, the relations between *M. citricolor* and shade cannot be generalized. Our findings suggest that under suboptimal conditions for ALSD (low altitudes, warm temperatures or eastern facing slopes), shade increases disease severity due to shifting disease driving factors closer to the tolerance range. Under optimal or more suitable conditions (higher altitudes, cool temperatures or western facing slopes), our model output indicated that a generally low percentage of shade could provide better conditions for the pathogens' development. This approach can be used to make better decisions in disease management and to improve site-specific shade practices, in order to better adapt crop health management of agroforestry systems.

Table 2. Comparison of predictor means in high scored zones obtained either with high or low shade model.[1]

	Guatemala		***Honduras***	
Predictor	**High shade model (> 20 %)**	**Low shade model (< 20 %)**	**High shade model (> 60 %)**	**Low shade model (< 60 %)**
Elevation	918 masl	1637 masl	1525 masl	1494 masl
Slope aspect [2]	6	6	4	6
Slope inclination	9 %	10 %	20 %	15 %
Solar radiation	23450 MJ/m²/day	23187 MJ/m²/day	36172 MJ/m²/day	23913 MJ/m²/day
Dew point	18.4 °C	13.3 °C	14.2 °C	14.2 °C
Precipitation Jan–Dec	3101 mm	1597 mm	1712 mm	1734 mm
Temperature Jan–Dec	23.5 °C	18.4 °C	18.6 °C	18.8 °C

[1]Mann – Whitney test resulted in significant differences ($p < 0.001$) in all predictor variables.
[2]Classes of orientation: 1 = North; 2 = Northeast; 3 = East; 4 = Southeast; 5 = South; 6 = Southwest; 7 = West; 8 = Northwest.

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Effect of Different Media on Rooting and Growth of *Coffea canephora* Cuttings

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SUMMARY

To optimize the propagation technology of *Coffea canephora*, a study was conducted to evaluate the effect of rooting media on optimum duration of root initiation and improvement of vegetative features of the coffee seedlings. The experiment was laid out in a randomized complete block design (RCBD) with three replications. One factor (single node) was exposed to three growth media; soil (soil + silt + manure in 1:1:1 ratio), coffee husks and saw dust (control). Data recorded includes: days to sprouting, number of leaves, leaf area and initiated root length. Data collected were analysed using GENSTAT statistical package. Results showed significant differences between growth media ($p \leq 0.05$), using least significance difference (LSD) test. Cuttings of *Coffea canephora* established in coffee husks were the least performed in days to root formation, sprouting and leaf development.

INTRODUCTION

Common methods that have been used for accelerated multiplication of improved coffee planting materials in Tanzania include use of clonal cuttings and grafting. Out of these methods, use of clonal propagation has been practised to produce true to type experimental materials since 1960s (Fernie, 1962). The method was perfected and is currently used commercially in accelerated multiplication of improved Arabica and Robusta in Tanzania (Nzallawahe *et al.*, 2004). For rooted cuttings to perform efficiently, it requires also appropriate rooting media. Most commonly rooting media used in Kagera for seedling multiplication is saw dust. But coffee growers have experienced limitations in using the media efficiently because of its readily available in almost coffee growing areas, and low moisture retention. This prompts for evaluation of alternative rooting media with a capacity for moisture retention and availability. This report present results of three rooting media evaluated for the propagation of *Coffea canephora* clonal cuttings with the objective to determine the most efficient.

MATERIALS AND METHODS

To study the effect of different growing media on the rooting and improvement of physiological features of *Coffea canephora* cuttings, a research study was carried out at clonal propagation Nursery at TaCRI Maruku substation in Bukoba District during the year 2013. The experiment was laid out in randomized complete block design (RCBD) with three replications. One factor (single node) was tested against three different growing media; soil, coffee husks and saw dust (control). A total of 179 cuttings per treatment were placed in the rooting boxes. Humidity and light are key factors for successful root formation and vegetative growth of the cuttings. Therefore the propagation boxes were covered with a clear polythene sheet to maintain high humidity and allow light penetration. The cuttings were regularly irrigated and misted for four months, from January to April 3013. Data recorded were on days to sprouting, number of leaves produced, leaf area, number of roots and root length. To count

number of formed roots and length measurements, the cuttings were uprooted and roots washed. Data were subjected to analysis of variance (ANOVA) and least significant difference (LSD) test using GENSTAT computer software.

RESULTS AND DISCUSSION

Data recorded the effects of different media on the rooting and growth of *Coffea canephora* cuttings are presented in Table 1. The analysis of variance (ANOVA) showed that planting media had a significant effect on days to sprouting and number of roots. Mean values for days to sprouting showed that soil media proved to be the earliest sprouting medium with 37 days, followed by sawdust with 42 days, and coffee husks 64 days. There were significant differences (p >) between saw dust and soil media, and coffee husks. Significant differences ($p > 0.05$) were also noted between leaf area of the soil media, and saw dust and coffee husks. The number of leaves and leaf area although not significantly different, had higher value scored from soil media than the rest. Lal and Dana (1984), reported that organic matter of the soils has effect on growth of the plants because of its richness in nutrients. Also soils with high contents of organic matter have higher soil moisture retention. It is therefore obvious that, clonal cuttings established in soil media sprouted earlier than in saw dust and coffee husks. Similar findings were reported by Fernie (1962), when studied rooting media appropriate to raise clonal seedlings of *Coffea arabica*.

Table 1. The effects of different media on the rooting and growth of *Coffea canephora* cuttings

Media	Days to sprouting	Number of leaves	Leaf area (cm2)	Number of roots	Root length (cm)
Control (saw dust)	42**a**	5**a**	18**a**	7**a**	8.50**ab**
Soil	37**a**	7**a**	34**ab**	9**b**	7.60**a**
Coffee husks	64**b**	5**a**	12**a**	4**c**	5.30**a**
Significance	**	*	*	*	*
LSD	**22**	**2.2**	**12.8**	**1.97**	**2.30**

Values followed by different letters are significantly different at $P > 0.05$ levels, according to least significance difference (LSD) test.

CONCLUSION

On the basis of results obtained from this study, it is recommended that sawdust can be used as a media to develop clonal seedlings of *Coffea canephora* where it is readily available. But soil media can be used widely in raising clonal cuttings for Robusta coffee. Use of coffee husks in raising clonal seedlings must be investigated further.

ACKNOWLEDGEMENTS

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A booster for Commercial Propagation of *Coffea arabica* F1 Hybrids: Somatic Embryo-derived Plantlets Can Be Efficiently Propagated in Nursery *via* Rooted Cuttings

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SUMMARY

This work was addressed to assess and optimize the rooting capacity of rejuvenated Coffea arabica F1 plants, derived from somatic embryogenesis (SE). We observed that very young plants can be propagated via rooted cuttings method with very high efficiency (up to 90% of rooting). We strongly believe that the possibility of multiplying somatic embryo-derived plants, through conventional vegetative horticultural mini-cuttings, would offer new perspectives to guarantee the profitability of the high scale micro-propagation business and hence will bring the interest of private sector.

INTRODUCTION

Somatic embryogenesis (SE) is used to multiply *C. arabica* F1 hybrids. This technology is now well mastered at the industrial scale since several millions plants have been regenerated through this technology but technical bottlenecks still limit SE profitability. For example this is the case of low embryo-to-plantlet conversion (approx. 50% in average) and significant losses occurring in nursery at each step of the acclimatization and hardening process. Consequently, SE-derived plant's production cost is still too high to guarantee the profitability of the high scale micro-propagation business and the interest of private sector.

In vitro cuttings of the *C. arabica* species are reputed to be highly recalcitrant to root but recently, we observed that very young *C. arabica* F1 hybrid plants derived from SE (more than four million plants were regenerated by this method), were able to root during the first month of nursery acclimatization process with a very high success rate (> 95% rooting) before losing progressively this capacity.

The combination of *in vitro* SE and *ex vitro* mini-cuttings methods could allow to decrease significantly the production cost per plant by multiplying the number of plants propagated from one expensive initial SE-derived plant produced in the laboratory (Fig. 1).

To boost our F1 Arabica coffee hybrids industrial propagation process and its profitability, we imagined to combine the two SE and micro-cuttings procedures by using the rejuvenated plants derived from SE as an initial pool for a further horticultural micropropagation step.

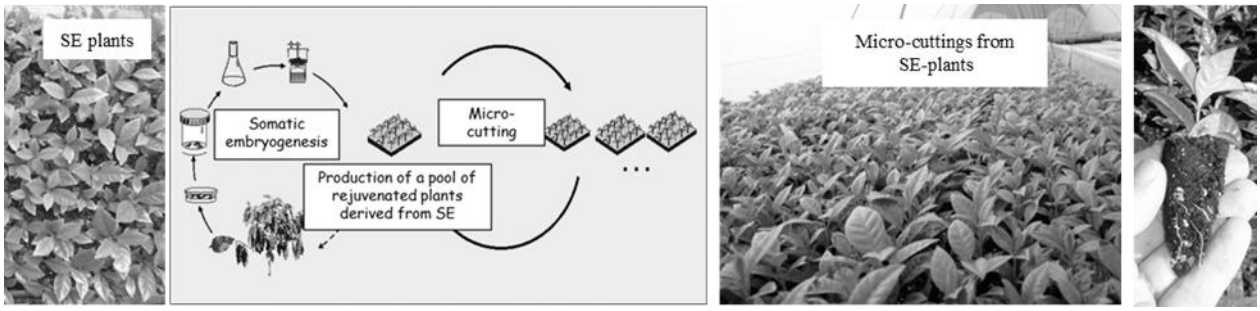

Figure 1. Scheme of cuttings-derived plants production from an initial batch of rejuvenated plants derived from somatic embryogenesis.

MATERIALS AND METHODS

In this experiment, 200.000 rejuvenated plants derived from SE were submitted to rooting conditions.

Approximately twenty weeks after the somatic embryo planting in *ex vitro* conditions, 3 to 5 cm-long stems are harvested that include the shoot apex and an average number of 2 entire leaf pairs.

The cuttings are immediately planted in trays filled with commercial Peatmoss substrate with Vermiculte. The trays are then placed in a greenhouse at high humidity (up to 95%) and 30-35°C day / 20-25°C night temperature average, for 6-8 weeks with 50% shade.

Fongicide preventive treatments are performed every week with Mancozeb (2.5g/l), Phyton (2cc/l) and Amistar (0.5g/l).

At the end of the rooting stage, plantlets were transfered from high humidity conditions to less humidity (50%) and more lightened conditions.

After weaning, the plants were directly transfered to traditional bag nursery under 20 % shade for a period of approximately 3-4 months.

The following four intraspecific *Coffea arabica* F1 hybrids between american varieties and ethiopian wild trees were tested for their ability to support a propagation by cuttings: H1, H3, H5 and H10. Considering the high number of plants, the rooting efficiency was evaluated by observing the presence of the roots at the bottom of each plant and calculating the rate between the number of cuttings initialy planted in trays and the number of rooted plants tranfered in bags in traditional nurseries after 8 weeks of rooting process.

As regard the kinetic of the rooting ability of cuttings, three repetitions of 50 cuttings were realised with H1 hybrid for each period of time (10, 15, 20, 25, 30, 35 and 40 weeks after sowing the plant in *ex vitro* conditions). The rooting response was assessed 8 weeks after planting by noting the presence/absence of roots and by measuring the lenght of the main roots. Plants with up to 3 cm lenght roots were considered to be rooted.

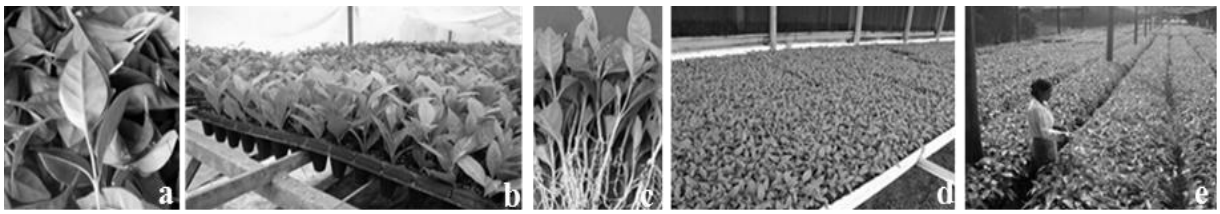

Figure 2. Rooting and acclimatization process. a: cuttings; b: rooted cuttings in trays; c: nude rooted cuttings; d: weaning stage in greenhouse; e: acclimatization of the rooted cuttings in traditional bag nurseries.

RESULTS AND DISCUSSION

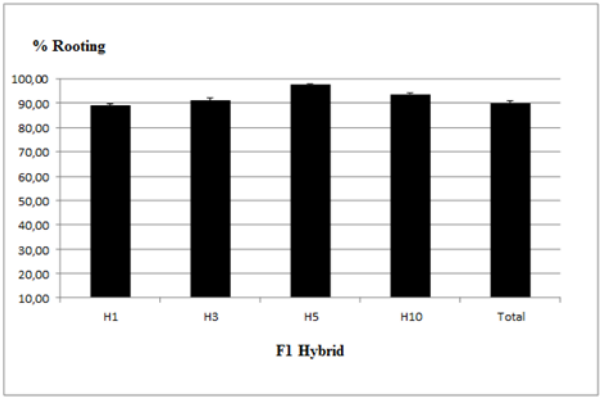

Figure 3. Rooting abaility for of 200.000 cuttings from several *Coffea arabica* F1 hybrids derived from rejuvenated young somatic embryogenesis-derived plantlets.

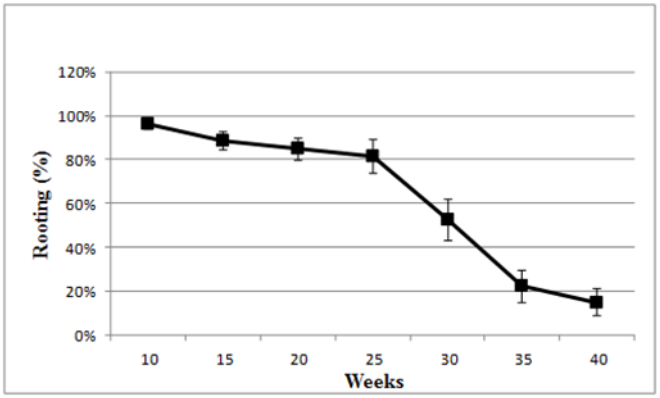

Figure 4. Kinetic of cuttings' rooting ability of *Coffea arabica* plants derived from somatic embryogenesis after 10-40 weeks aclimatization in nursery. Data were obtained for the hybrid H1 *(T5296 x RS).*

The rooting efficiency is up to 90% with 20 weeks rejuvenated SE-derived material (Fig. 3). There's no statistically significant difference between hybrids, therefore no genotypic effect for the rooting ability.

The rooting capacity of the Arabica coffee cuttings harvested in differently aged plants decreases in line with the age of the plants in nursery (Fig. 4). When cuttings are harvested on 10 to 20 weeks young SE plantletss, the rooting rate is up to 90%. In the period comprised between the 25th and the 35th week, this rate drastically decreases from 80 to 20%. When

cuttings are harvested on nursery plants 35 weeks old and beyond rooting efficiency is about 15 to 20%.

In conclusion, the timing for implementing mini-cuttings production programs must be very accurate in Arabica Coffee to have a successful reliable and highly efficient rooting . Indeed, the "action window" is very short on time - approx. between 10 to 20 weeks after the pregerminated embryos planting in nursery - and ephemeral.

The transient ability of cuttings for rooting is probably linked to the rejuvenation process resulting from the successive cell dedifferentiation/re-differentiation mechanisms occurring during SE. The obtained juvenile state consecutive to the *in vitro* culture is then transiently established as it doesn't exceed the nursery phase. Although fleeting, this rejuvenation can be exploited to initiate a new phase of multiplication by rooted cuttings in an Arabica woody species reputed to be recalcitrant to this type of morphogenesis.

Few studies have been done in plants to understand the molecular foundations of aging and rejuvenation. Aging was found to induce cyto-and histomorphological changes as well as the modification of certain metabolic pathways. It has been speculated that DNA methylation could play a key role in the various maturational changes observed in plants during their ontogenetic development. The prevailing hypothesis was that during higher organism development, genomic DNA could become more methylated resulting in the modification or the switching on and off of the gene expression responsible for the variation of the maturational traits noticed. In the heteroblastic tree species *Acacia mangium*, Baurens *et al.* (2004) reported higher DNA methylation rates for the juvenile-like microshoots than for those of the mature type. In tissue-cultured chestnut, Hasbun *et al*. (2005) observed higher levels of methylated DNA in microshoots exhibiting juvenile characteristics than in the more mature-like ones originating from the older parts of the same donor tree. However a study in bristlecone pines found no evidence of the accumulation of somatic mutations in plants aged 23 to 4,713 years.

The integration of such propagation of micropropagated plants method combining somatic embryogenesis and rooted cuttings in the multiplication program of *C. arabica* F1 hybrids will require first the full mastering of the mini-cuttings and rooting process and the assessment of the mini-cuttings-derived plants plant growth characteristics i.e. phenotyping of under field conditions.

High investments in sophisticated greenhouse must be necessary to lead this kind of multiplication program and drastic procedures must be established to avoid sanitary risks especially against fungi attacks and somaclonal variations appearance.

As far as we know, it's the first time that such system is setted-up in nurseries to multiply heterozygous genetic material of *Coffea arabica* F1 hybrid. To our knowledge, such a technology has never been established for the other cultivated species *C. Canephora*. *In vitro* micro cuttings technology has already been described in a lot of species but *ex vitro* mini-cuttings method has never been reported and would bring numerous advantages compared to the former, mainly the simplicity of its implementation.

This study showed that it is possible to micropropagate at the industrial scale and with low cost Arabica F1 hybrids, combining this two complementary SE and rooted cuttings methods. The propagation by mini-cuttings represents the most modern concept for commercial cloning and tends to be very easy to popularize if well managed by professionals. The exponential propagation allowed by cuttings production actually interests the developing countries in

Central and Andin America, which often do not have ressources to develop an expensive and highly sophisticated technology such as SE to propagate heterozygous materials derived from coffee genetic improvement programs.

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Environmental, Economic and Social Impacts of Coffee Varieties from Agronomic Institute in the Main Brazilian Coffee Regions

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SUMMARY

This study aimed to measure the impact of technologies developed at the Agronomic Institute – IAC for coffee in the main producer regions, from 1887 until 2011. For this, it was used a method based on Ambitec and Esac Systems. It were evaluated the following cultivars: Obatã, Tupi, IAC 125, Icatu, Catuaí, Mundo Novo and Apoatã. The results indicate that, among cultivars produced by non-irrigated systems, IAC 125 RN and Apoatã (rootstock) result in greater total impact on Brazilian coffee regions. Economic impacts of cultivars Catuaí and Obatã in irrigated systems are generally positive and higher than those obtained in non-irrigated systems, but they are accompanied by significant adverse environmental impacts. In general, the impacts of coffee cultivars developed by IAC are higher than those observed in the literature for agricultural innovations developed by other institutions.

INTRODUCTION

The foundation of the Agronomic Institute – IAC in 1887, to assist the development of coffee production, marked the beginning of Brazilian coffee research. Chronologically the introduction of coffee cultivation in Brazil and the establishment of the IAC are considered respectively as the second and the third development framework of Brazilian agriculture [1]. Since the nineteenth century, other educational institutions and agricultural research were created in Brazil. Knowledge and technologies generated in these institutions were transferred to the productive chain and progressively used to contribute to its development and environmental preservation. The measurement of their impacts is relevant to the review of the guidelines of the research programs of those institutions. Among the technologies developed at the Agronomic Institute, the coffee varieties are recognized as the most significant for the development of the coffee regions. Therefore, the aim of this study is to quantify the impact of these varieties, as the environmental, social and economic aspects.

MATERIALS AND METHODS

In Brazil, the advances in impact of innovations evaluation result mainly from the Brazilian Agricultural Research / Embrapa and the State University of Campinas / UNICAMP who developed, respectively, Ambitec and ESAC systems. The coffee cultivars developed at IAC were analyzed individually through Ambitec. In this system were incorporated some advantages observed in ESAC, without changing the components of impact indicators and the scoring system. We interviewed the users of cultivars and one of the developers of the coffee

cultivars in IAC. Questionnaires were applied individually or in panels. We applied 194 questionnaires, from 2012 to 2013. We selected cultivars based on their representativeness in relation to its cultivated area (Catuaí and Mundo Novo: 90% of the Brazilian coffee area), regional prominence (Apoatã, Obatã, IAC 125, Catuaí, Icatu, Tupi, Mundo Novo), period of use (Catuaí: since 1972; Mundo Novo since 1952), agronomic characteristics (multiple resistance: Obatã and IAC 125) or problem reported about it (Icatu: loss of resistance). Questionnaires were applied randomly, through contacts in technical or scientific events or in *loco*. Table 1 lists the numbers of interviews per cultivar and region.

Table 1. Number of questionnaires Ambitec / ESAC applied by cultivar, production system and coffee region, 2012.

State	Region	Cultivars: numbers of questionnaires Ambitec/Esac							
		Apoatã	Catuaí	IAC125	Icatu	Obatã	Tupi	Mundo Novo	Total
Minas Gerais	South	-	44	-	-	1	2	-	68
	Cerrado	-	1	12	-	2	2	-	
	Jequitinhonha	-	2	-	-	-	-	-	
	Zona da Mata	-	2	-	-	-	-	-	
Espírito Santo	Serrana	-	22	-	-	-	-	-	28
	Caparaó	-	2	-	-	-	-	-	
	Alto Caparaó	-	4	-	-	-	-	-	
São Paulo	Alta Paulista	6	-	-	-	2	-	-	45
	Garça-Marília	4	-	-	-	5	2	-	
	Mogiana	-	3*	-	3	8	5	1	
	Piraju	-	-	-	-	4	2	-	
Paraná	Norte Novo	3	-	-	-	-	2	-	12
	Norte Velho	-	-	-	-	-	1	2	
Bahia	West	-	8	-	-	3	-	-	41
	Chapada	-	22	-	-	-	-	-	
	Planalto	-	7	-	-	-	1	-	
Brasil	Total	13	117	12	3	29	18	3	194

*- No questionnaires were applied. *Questionnaires correspond to the municipality of low technological level. Source: Data of the study.*

RESULTS AND DISCUSSION

The total impacts of cultivars Apoatã, Catuaí, IAC 125, Icatu, Obatã, Tupi and Mundo Novo, in Brazilian coffee regions, are presented in table 2, and indices of environmental, economic, social and total impact for each of those cultivar are present in Tables 3-8. The indices obtained indicate that IAC 125 and Apoatã have the highest total impacts in without irrigation production systems. IAC 125 stands in the Cerrado of Minas Gerais. The rootstock Apoatã stands out in the regions of Alta Paulista, São Paulo, and in the Northwest of Paraná state.

These results indicate that the environmental and socioeconomic impacts of coffee cultivars of the Agricultural Institute are significant when compared to those obtained in evaluations of technologies developed by other research institutions, when used similar methodology - the

Ambitec System. For instance, two wheat cultivars released after 1986 showed social impact indices respectively equal to 0.93 and 1.39, and indices of environmental impact of 1.91 and 0.01, respectively.

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Table 2. Total impact of cultivar – Apoatã, Catuaí, IAC 125, Icatu, Obatã, Tupi e Mundo Novo – Ambitec/Esac evaluation system, Brazilian regions, 2012.

State	Region	Cultivar: índices of total impact						
		Apoatã	Catuaí	IAC125	Icatu	Obatã	Tupi	Mundo Novo
Minas Gerais	South	-	3,36	-	-	-	0,91	-
	Cerrado	-	-	3,66	-	0,07*	-	-
	Jequitinhonha	-	3,69	-	-	-	-	-
	Zona da Mata	-	3,69	-	-	-	-	-
Espírito Santo	Serrana	-	3,78	-	-	-	-	-
	Caparão	-	-	-	-	-	-	-
	Alto Caparão	-	-	-	-	-	-	-
São Paulo	Alta Paulista**	4,69	-	-	-	3,24	-	-
	Garça-Marília	3,83	-	-	-	2,38*****	-	-
	Mogiana***	-	-	-	0,20	1,3	-	-
	Divinolândia****	-	-0,16	-	-	-0,2	-	-0,14
	Piraju	-	-	-	-	1,3	1,06	-
Paraná	Norte Novo	3,18	-	-	-	-	1,14	-
	Norte Velho	-	-	-	-	-	-	3,35
Bahia	West	-	3,08	-	-	-	-	-
	Chapada	-	2,92	-	-	-	-	-
	Planalto	-	2,92	-	-	-0,19	1,25	-

*- No questionnaires were applied. * Not irrigated ** Pontal of Paranapanema region (not irrigated). *** Not irrigated, without computing the average of questionnaires from Divinolândia. **** Municipality of low technological level. ***** Irrigated. Source: Data of the study.*

Table 3. Total, environmental, social and economic impacts indices of cultivar Apoatã, 2012.

Impact indices	Cultivar: Apoatã / Brazilian regions		
	Alta Paulista (SP)	Garça Marilia (SP)	Northwest (PR)
Environmental	2,84	2,09	1,35
Economic	7,25	6,22	5,76
Social	4,79	4,11	3,57
Total	4,69	3,83	3,18

Source: Data of the study.

Table 4. Total, environmental, social and economic impacts indices of cultivar Catuaí, 2012.

Impact indices	Cultivar: Catuaí / Brazilian regions					
	West of BA	Planalto BA	Espírito Santo	Divinolândia	South MG	Zona da Mata e Jequitinhonha
Environmental	0,38	-2,00	0,00	-1,11	0,00	0,00
Economic	5,26	7,87	5,03	0,37	6,77	5,41
Social	2,55	3,64	3,96	0,14	4,44	4,06
Total	2,92	3,08	3,78	-0,16	3,36	3,69

Source: Data of the study.

Table 5. Total, environmental, social and economic impacts indices of cultivars IAC 125, Icatu and Mundo Novo. Brazilian regions, 2012.

Impact indices	IAC 125	Icatu	Mundo Novo	
	Cerrado MG	Divinolândia (SP)	Norte Velho (PR)	Divinolândia (SP)
Environmental	1,54	0,48	0,00	-0,62
Economic	5,06	0,03	6,91	0,00
Social	4,89	-0,04	4,47	0,00
Total	3,66	0,2	3,35	-0,14

Source: Data of the study.

Table 6. Total, environmental, social and economic impacts indices of cultivar Obatã, 2012.

Impact indices	Cultivar: Obatã / Brazilian regions						
	Oeste BA	Cerrado Minas	Divinolândia	Mogiana	Garça-Marília	Piraju	Pontal Paranapanema
Environmental	-0,04	-0,29	-0,21	-1,59	0,23	0,19	0,42
Economic	-0,3	0,07	-0,39	6,89	2,17	2,8	6,43
Social	-0,05	0,68	-0,03	2,71	2,46	1,59	2,76
Total	-0,19	0,07	-0,2	2,38	1,3	1,3	3,24

Source: Data of the study.

Table 7. Total, environmental, social and economic impacts indices of cultivar Tupi, 2012.

Impact indices	Cultivar: Tupi / Brazilian regions			
	Bahia	Minas Gerais	São Paulo	Paraná
Environmental	0,88	0,17	0,08	0,42
Economic	1,43	1,31	1,92	1,72
Social	2,03	1,54	1,54	1,4
Total	1,25	0,91	1,06	1,14

Source: Data of the study.

Table 8. Total, environmental, social and economic impacts indices of cultivars Catuaí e Obatã in the irrigated production system, Brazilian regions, 2012.

Impact indices	Production systems / Brazilian regions				
	Irrigated			Shaded	
	Cerrado MG Catuaí	Garça-Marília Obatã	West BA Catuaí	Planalto BA Catuaí	Garça-Marília* Obatã
Environmental	-2,74	0,09	-2,00	1,56	-1,02
Economic	9,03	2,13	7,87	5,03	8,23
Social	3,47	1,71	3,64	3,51	2,95
Total	2,94	1,17	3,08	3,43	3,24

**: Shaded and irrigated. Source: Data of the study.*

A Glimpse of Climate Change Impact on *C. arabica* L. and *C. canephora* Pierre ex A. Froehner Physiology – The Combined Effects of Enhanced Growth CO_2 and Temperature

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SUMMARY

The effective impact of climate changes on the coffee plant physiology, promoted by enhanced air [CO_2] and global warming remain to be fully elucidated through biological studies. Therefore, this work aims at linking important coffee physiological responses to environmental changes of enhanced growth [CO_2] and temperature on genotypes from the two major producing species. Potted plants from *C. arabica* cv. IPR 108 and of *C. canephora* cv. Conilon Clone 153 were grown under environmental controlled conditions, either at 380 or 700 μL CO_2 L^{-1} air, for 1 year, without water, nutrient or root development restrictions. After that the temperature was gradually increased from 25/20 ºC (day/night) up to 42/34 ºC. The long-term impacts of enhanced growth [CO_2] and enhanced temperature on the photosynthetic functioning were assessed at 25/20 ºC, 31/25 ºC, 37/30 ºC and 42/34 ºC, through leaf gas exchanges (rates of net photosynthesis, P_n, stomatal conductance, g_s, transpiration, T_r, and photosynthetic capacity, A_{max}), instantaneous water use efficiency (iWUE), fluorescence parameters (photochemical efficiency of the photosystem II under dark, F_v/F_m, and light, F_v'/F_m', conditions, as well as the photochemical, q_P, and non-photochemical, NPQ, quenchings, and quantum yield of the linear electron transport, ϕ_e), photosynthetic pigments (chlorophyll and carotenoids) and some molecules with antioxidant role (ascorbate and α-tocopherol). The results showed that enhanced [CO_2] stimulates photosynthetic functioning, without negative down-regulation. Minor impacts were found in the photochemical performance until 37 ºC, but extensive impacts were shown at 42 ºC, especially in IPR108.

Remarkable was the finding that enhanced [CO_2] preserved a higher functional status (P_n, A_{max}, F_o, F_v/F_m) at high temperatures (37 and 42 ºC), what seems quite relevant under the predicted climate changes and global warming scenarios.

INTRODUCTION

Coffee is one of the world's most traded agricultural products. Modeling studies have predicted that climate change and global warming will have a strong impact on the suitability of current cultivation areas and coffee biodiversity, but these studies did not anticipate potential mitigating effects of the increasing atmospheric [CO_2], as no information exists on the long-term effects of high [CO_2] and temperature on this plant.

The tolerance of the photosynthetic pathway is of crucial importance regarding plant acclimation to environmental variations, including changes in growth [CO_2], to which effects on photosynthesis and stomata were found in *Coffea* spp.. Under enhanced growth [CO_2], C_3 plants often presents 50% increases in the photosynthetic rate (P_n), even if a partial down-regulation (negative acclimation) of the photosynthetic apparatus occurs. The latter is often related to limitations on sink strength that prevents the plant from fully utilizing the higher photosynthate production. That may lead to an increase in non-structural carbohydrates (NSC) that in turn could depress gene expression and the amount/activity of photosynthetic enzymes, including RuBisCO, or reduce the levels of all components of the photosynthetic apparatus. Such impact also depends on the interactions with other environmental limitations. Nonetheless, an increase of P_n is commonly reported, due to the direct effect of a higher substrate (CO_2) availability and to a competitive inhibition of CO_2 over O_2 at the carboxylation sites of RuBisCO, reducing the photorespiration rate. In fact, in C_3 plants such photorespiration reduction under CO_2 enrichment is expected to enhance P_n to a greater degree at high than at low temperature, thereby, at least partially, offsetting the effects of supra-optimal temperatures on yield.

To our knowledge, we report here the first results concerning the physiological responses of the photosynthetic apparatus to elevated atmospheric [CO_2] and temperature in genotypes of the two major coffee producing species.

MATERIALS AND METHODS

Plant material and experimental design

Plants with *ca*. 1.5 years from *C. arabica* L. cv. IPR 108 (IPR108) and *C. canephora* Pierre ex A. Froehner cv. Conilon Clone 153 (CL153), were transferred into walk-in growth chambers (EHHF 10000, ARALAB, Portugal) and grown in 28 L pots under controlled conditions of temperature (25/20 ºC, day/night), irradiance (*ca*. 650-800 μmol m^{-2} s^{-1}), RH (75%), photoperiod (12 h), and either 380 μL CO_2 L^{-1} (380) or 700 μL CO_2 L^{-1} (700) air for 1 year, without water, nutrient or root development restrictions. Thereafter the temperature was increased from 25/20 ºC up to 42/34 ºC, at a rate of 0.5 ºC day^{-1}, with a 7 days temperature stabilization at 31/25, 37/30 and 42/34 ºC to allow analysis. Some parameters were analyzed 4 weeks later at 37/30 ºC to assess plant recovery. Analyses were performed on newly matured leaves.

Leaf gas exchanges

Gas exchanges were evaluated under both [CO_2], at *ca*. 650-800 μmol m^{-2} s^{-1} of irradiance, using an open-system infrared gas analyzer (Li-Cor 6400, LiCor, Lincoln, USA), and

included net photosynthesis (P_n), stomatal conductance (g_s),and transpiration (T_r) rates, as well as the instantaneous water use efficiency (iWUE, calculated as P_n/T_r). The photosynthetic capacity (A_{max}), expressing the photosynthetic apparatus potential under saturating light (900 μmol m^{-2} s^{-1}) and CO_2 (*ca*. 7%) and optimal temperature (25 ºC) conditions, was measured through O_2 evolution in a Clark-type O_2 electrode (LD2/2, Hansatech, UK), according to [3].

Chlorophyll a fluorescence parameters

The fluorescence parameters were determined on the same leaves used for the gas exchange evaluations, using a PAM-2000 system (H. Walz, Effeltrich, Germany), and included the minimal antennae fluorescence (F_o) and the maximal photochemical efficiency of the photosystem (PS) II (F_v/F_m) under dark-adapted conditions, as well as the actual PSII efficiency of energy conversion (F_v'/F_m'), the photochemical (q_P) and non-photochemical (NPQ) quenchings, and quantum yield of the linear electron transport (ϕ_e), all under photosynthetic steady-state conditions [8]. Growth irradiance for photosynthetic stimulation and 7500 μmol m^{-2} s^{-1} saturating flashes were used for these determinations.

Photosynthetic pigments

Total chlorophyll (Chl) and carotenoid contents were evaluated as in [3].

Non-enzyme antioxidants

Molecules with antioxidant role (ascorbate and α-tocopherol) were quantified in frozen samples (collected to liquid N_2 and kept at -80 ºC), as optimized for coffee.

RESULTS AND DISCUSSION

Leaf gas exchanges

Globally, it is known that *C. canephora* usually display better tolerance to higher temperatures than *C. arabica*, due to a higher temperature optimum, whereas the latter species can better cold acclimate, with the photosynthetic apparatus having a crucial role on the plant tolerance. The results showed that, in both genotypes, P_n presented significantly higher values when measured under enhanced [CO_2], irrespective of the temperature, probably linked to the inhibition of photorespiration related to the higher CO2 availability. Such increase was particularly clear in IPR108 that more than doubled the P_n rates found at the control temperature (25ºC). At 37ºC IPR108 showed maximal P_n values, for both [CO_2], whereas CL153 maintained the values observed already at 31ºC. Yet, both genotypes showed significant P_n reductions upon 42 ºC, especially in CL153-380 and IPR108-700. However, at this temperature the 700 plants still presented P_n values 140% (CL153) and 30% (IPR108) higher than their respective 380 μL CO_2 L^{-1} ones (Fig. 1).

The g_s increased at 37 and/or 42 ºC, particularly in the CL153-380 (200 and 50%) and IPR108-380 (210 and 217%) plants, although the maximal values were observed in IPR108-700. Such temperature impact on g_s provoked strong iWUE reductions on both genotypes and growth [CO_2], but the iWUE values were always higher in plants grown in enhanced [CO_2].

The A_{max} values showed somewhat higher absolute values for the plants grown at high [CO_2] at 25/20 ºC and 31/25 ºC in CL153, whereas IPR-700 plants presented similar values to those of IPR-380 for these temperatures. Also, IPR108 showed higher heat sensitivity at 37 ºC than

CL153, when comparing the plants grown under 380 µL CO_2 L^{-1}. For the extreme 42 ºC both genotypes were clearly affected, irrespective of [CO_2], but the 700 plants showed higher absolute values and lower impact than the 380 ones.

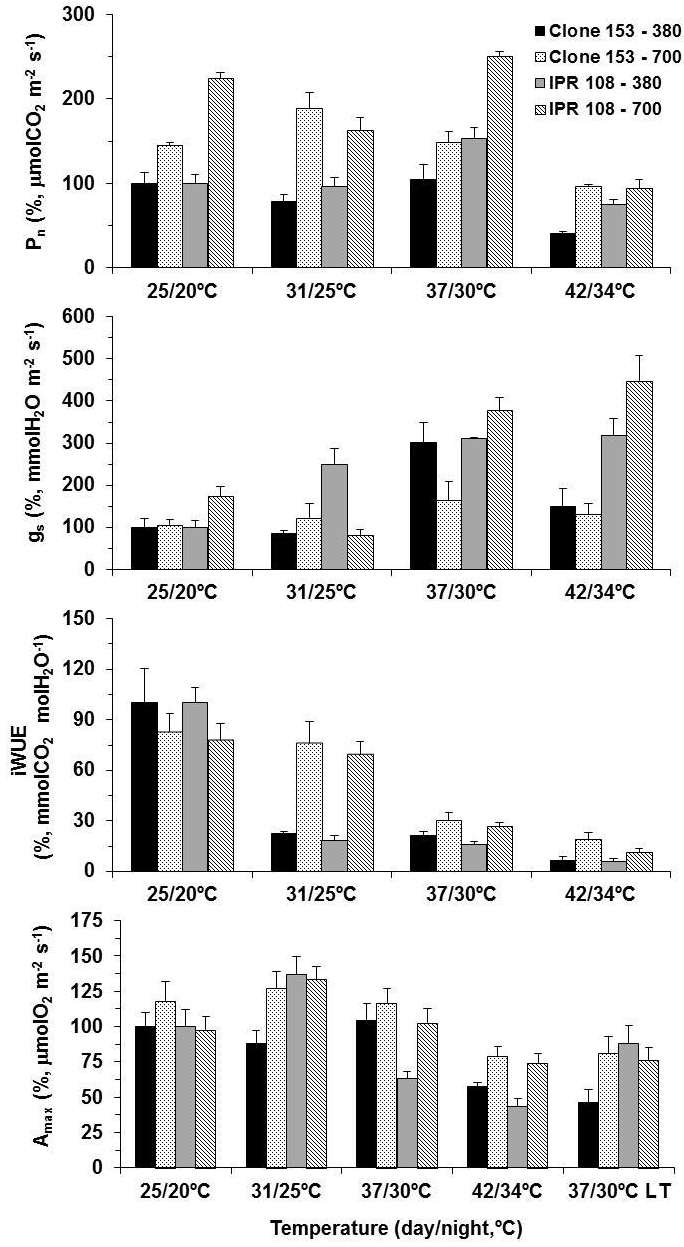

Figure 1. Changes (in %, within each genotype, relative to the 380 µL CO_2 L^{-1} plants at 25/20 ºC) for net photosynthesis (P_n), stomatal conductance (g_s), and photosynthetic capacity (A_{max}), rates, as well as to instantaneous water use efficiency (iWUE). Each value represents the mean ± SE (n=5-8).

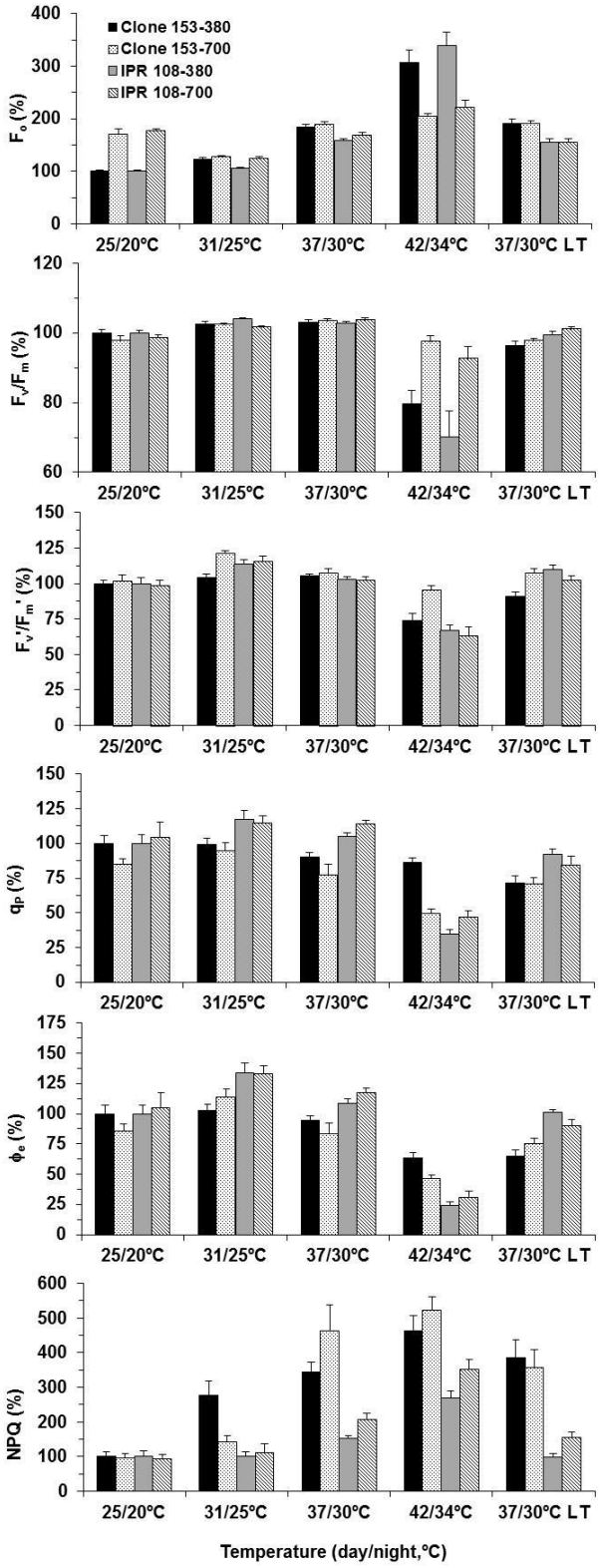

Figure 2. Changes (in %, within each genotype, relative to the 380 µL CO_2 L^{-1} plants at 25/20 ºC) for minimal fluorescence (F_o), maximal photochemical efficiency of the photosystem II (F_v/F_m), actual PSII efficiency of energy conversion (F_v'/F_m'), photochemical (q_P) and non-photochemical, (NPQ) quenchings, and quantum yield of the linear electron transport (ϕ_e). Each value represents the mean ± SE (n=5-8).

The results confirm the absence of photosynthetic down-regulation associated with high [CO_2], either at adequate or supra-optimal temperature. Also, as no stomatal closure occurred, the given P_n reductions would be linked to high temperature mesophyll impairments, as confirmed by the impact on A_{max}.

These were not-easily reverted as they were partially maintained upon the return to 37 ºC. Still, lower impacts were found at 37 ºC and, especially, at 42 ºC, in the plants grown at enhanced [CO_2], suggesting the maintenance of significantly higher functioning capability when compared to plants grown at normal [CO_2]. Moreover, the saturating irradiance to obtain A_{max} was always higher in plants under enhanced CO_2, suggesting that those plants might endure higher irradiance levels (data not shown).

Chlorophyll a fluorescence analysis

The stronger impact on the 380 plants at 42 ºC was further noted by the decline of F_v/F_m associated with an increase of F_o, the latter indicating that the threshold for irreversible damage was reached. This could be attributable to not readily reversible photoinhibitory impairments on PSII centers, possibly related to D1 protein loss, as suggested for coffee under high irradiance levels. Yet, such F_o rise could also be partially linked to the total chlorophyll increase (see below). Furthermore, part of the decrease in F_v/F_m and F_v'/F_m' might be related to photoprotective thermal energy dissipation processes, reflected in the moderate (for a maximum of 1.5) NPQ increase, although the highest NPQ rises occurred in the 700 plants that were less affected than the 380 ones (in F_v/F_m for both genotypes and in F_v'/F_m' for CL153). Negative effects at the extreme temperature of 42 ºC were further observed in q_P and ϕ_e, although without a clear tendency in relation to growth [CO_2].

Photosynthetic pigments and non-enzyme antioxidants

Both total Chl and total carotenoids (expressed on a dry weight basis) were maintained at similar or somewhat lower contents in the 700 plants than in 380 ones in the two genotypes (data not shown). Notably, both pigments tended to increase at higher temperatures, showing maximal values at 37 or 42 ºC, in what seemed to be a reinforcement of photosynthetic structures with heat exposure. Chl increased significantly only in IPR108 (*ca*. 35% at 37 ºC in both [CO_2]), whereas carotenoids showed significant rises in CL153-380 (19% at 42 ºC), IPR108-380 (37% at 37 ºC) and IPR108-700 (35% at 42%). Yet, the Chl (*a*/*b*) ratio followed an opposite trend reflecting a preferential Chl *b* synthesis, suggesting the occurrence of functional readjustments in the photosynthetic structures. These could include a higher proportional reinforcement of light harvesting chlorophyll-protein complex (LHCII) that contains the majority of Chl *b* (and a Chl *a*/*b* ratio around 1.4), instead of PSI that has a much higher Chl *a*/*b* ratio.

The higher functional status of the 700 plants, as compared to the 380 ones, could justify a lower need of antioxidant molecules. Yet, irrespective of genotype and [CO_2], the ascorbate content strongly decreased with the temperature rise, whereas α-tocopherol was increased in IPR108 and maintained in CL153 (data not shown), suggesting different roles in response to heat stress.

In conclusion, enhanced growth [CO_2] stimulates photosynthetic functioning, without down-regulation of photosynthesis. The photochemical functioning of coffee plants remained mostly unaffected until 37 ºC although considerable impacts were depicted at 42 ºC, especially in IPR108 when compared to CL153. Moreover, enhanced [CO_2] preserved a

higher functional status (*e.g.*, P_n, A_{max}, F_o, F_v/F_m) at high temperatures (37 and 42 ºC), what may constitute an important feature under the predicted climate changes and global warming scenarios.

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Sustainable Management of Antestia Bugs, *Antestiopsis* spp.: a Key Approach to Alleviate Potato Taste Defect on Coffee

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SUMMARY

The Eastern Africa region depends heavily on coffee as a major cash and export crop from smallholder, medium and estate coffee farmers. The production of this crop is lately experiencing a rapid decline in quality especially due to the recently reported increasing incidence of potato taste defect in Rwanda and other Eastern Africa countries. The potato taste in coffee is suspected to be facilitated by coffee infestation by Antestia bugs, *Antestiopsis* spp a known major and indigenous insect pest of coffee in Eastern Africa region. Consumer demand for high quality coffee from Eastern Africa remains a major challenge following the reported incidences of potato taste hence the urgent need for sustainable management of Antestia bugs, as a component of possible interventions to alleviate this problem. Management strategies that include use of selective insecticides, cultural and biological control, and Integrated Insect Pest Management (IIPM) that aim to address the problem of potato taste in coffee are discussed.

INTRODUCTION

Coffee is an important commercial crop in the Eastern Africa countries. Its production is challenged by many factors such as coffee pests. Among these pests are the insect pests that are of economic importance. They includes; Antestia bugs, *Antestiopsis* spp and Coffee berry borer (CBB), *Hypothenemus hampei* (Ferrari) among others.

Extent of Antestia bugs spread

The Antestia bugs are key pest of Arabica coffee in East African countries. The adult bug (Fig. 1) is shield-shaped, measures about 6 to 8 mm long and strikingly coloured dark brown with orange and white markings. Females lay eggs in groups of about 12 on the underside of leave (Fig.2) and newly hatched nymphs resembles the adults.

Figure1. (left) Adult Antestia bug.
Figure 2. (right) Masses of Antestia eggs under the leaf.

Damage caused by Antestia bug

Both the adults and nymphs feed no only on green berries, flower buds and also on green twigs. The feeding causes blackening of the flower buds and premature fall of berries. An adult may also feed on mature berries (Fig. 3) where it inserts its proboscis to suck the sap. During this process, the Antestia bug is suspected to introduce some fungi into the berries (Le Pelley, 1968).

Figure 3. Adult Antestia bug feeding on coffee berry.

There are normally no visible surface marks / scars or wounds on the berries that are noticeable until seen on drying parchment coffee on drying beds as "zebra" beans. These "Zebra" beans produce poor quality coffee and are possible avenues for fungal infection. It's also thought that coffee beans when infested by Antestia bugs develop a distinctive 'potato taste', that is indirectly caused by bacteria entering through wounds created by the Antestia (Czerny and Grosch, 2000; Jean, 2009).

In Kenya *Antestiopsis* spp at density of 1-2 bugs per tree are considered as the economic threshold level that requires insecticide spraying so as to avoid economical crop loss (Coffee Research Foundation, 1989). This can be compared with 3 bugs per tree in Tanzania. Antestia bugs are estimated to cause 38% yield loss when bugs' population reaches 15 bugs per tree (Miller, 2012). In Kenya, a crop loss of 15-27% in total bean weight occurs at an infestation of 2-4 Antestia bugs per tree (Wanjala, 1979).

Current and Future Management Strategies

Control of insect pests and especially the Antestia bugs is known to decrease the occurrence of the potato taste in coffee (Cilas et al, 1998). In order to minimize the potato taste associated with Antestia bugs infestation, several sustainable control approaches that include use of selective insecticides (Chemical control), cultural control, biological control , and Integrated Insect Pest Management are emphasized.

Chemical control

The application of insecticide is recommended and justified when the Antestia population has attained economic threshold level (Coffee Research Foundation, 1987). The use of selective insecticides to manage the Antestia bugs is important for conservation of biological control agents that naturally keeps minor coffee insect pests to a sustainable level. A number of

insecticides are available for management of Antestia bugs (Coffee Research Foundation, 1987).

Biological control

The Antestia eggs, nymphs and adults are parasitized by different types of parasitoids (Greathead, 1966). The egg parasitoids; *Telenomous seychellensis* Dodd (*Ascolus seychellensis* Dodd), with parasitism percentage that ranges between 80-91% are common and potentially important in controlling the Antestia bugs (Le Pelley, 1973). Opening up of the coffee bushes through timely and proper pruning promotes the efficiency of the parasitoids. Pruning creates unfavourable conditions for Antestia multiplication, while enhancing the parasitoids ability to search for Antestia egg masses more easily.

Cultural control

Keeping the coffee bushes open by pruning is encouraged as a component of cultural control. It makes the habitat unsuitable for Antestia bugs population increase and favourable one for parasitization. Other cultural practices such as clean weeding are not encouraged (Taylor, 1945) as it interferes with biological control by adversely affecting the population of egg parasitoids as well as being fatal to pupae of Antestia adult parasitoid such as *Bogosia rubens.*

Integrated Insect Pest Management

Integrating the existing cultural, chemical and biological control approaches against the Antestia bugs when properly applied efficiently maintains the Antestia population to below economic threshold levels. Where for instance proper pruning is carried out and parasitoids exist or are introduced, they become more effective. If the population of Antestia is above economic threshold levels, pruning followed by application of selective insecticide is recommended before the parasitoids are released to the coffee farm to biologically control the Antestia bugs

CONCLUSION AND RECOMMENDATIONS

The pest control measures rely heavily on use of synthetic pesticides. Costs of inputs particularly pesticides, have become unaffordable by most farmers in Africa, and the increasing concern about pesticides residue risks have evidently led to the need of developing alternative pest management strategies.

To effectively manage Antestia bugs, combination of various insect pest management strategies which are economically viable, sustainable and environmentally friendly in Eastern Africa region are advocated. Therefore in view of the above, the inclusion of biological, botanicals, cultural and selective insecticides methods are suitable strategies for managing the Antestia bugs.

New approaches to the control of this pest include development of coffee resistant cultivars and the possibility of using compound inorganic and organic fertilizers that can deter or reduce the infestation levels of coffee beans. Work is ongoing at Coffee Research Institute, Kenya to see how manipulation at nutrition levels could reduce the Antestia bugs incidence.

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Phenology and Infestation Pattern of the Coffee Twig Borer, *Xylosandrus compactus*

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SUMMARY

Variations in numbers of different stages of the coffee twig borer, *Xylosandrus compactus* inside infested twigs, with, months, ambient temperature and rainfall; and number of galleries per infested twig was determined during June 2013 to May 2014 at the National Coffee Research Institute (NaCORI), Mukono, Uganda. Mean number of all the stages except eggs varied significantly with months, with peak numbers of the stages occurring during May to July. The number of larvae correlated negatively with temperature suggesting that high temperature either kills the larvae or quickens their development into pupae. Adult numbers correlated negatively with rainfall, indicating that adult *X. compactus* population is at its peak during drought. There were only single galleries in 87% of *X. compactus* infested twigs, suggesting that infestation by pioneers deters others from infesting the same twig. Implications of these findings in the management of the pest and future research direction are discussed.

INTRODUCTION

The coffee twig borer, *Xylosandrus compactus* (Eichhoff) (Coleoptera: Scolytidae) is a serious polyphagus pest of over 255 tree species worldwide (CABI, 2013). In Uganda, the pest causes an estimated loss of US$ 40 million from coffee export revenue (Egonyu et al 2009; Kagezi et al, 2013). The losses due to the pest result from death of infested twigs which would otherwise bear the coffee berries. Management of this pest in the country currently relies on phytosanitation through cutting and burning of infested twigs, and insecticide application. However, phytosanitation is too laborious, while insecticides are too costly and may be hazardous to human and environmental health. To effectively manage this pest, a good understanding of its population dynamics is essential.

Here we report variation of numbers of different stages of *X. compactus* with months, ambient temperature and rainfall; and the possible influence of infestation by pioneers on subsequent infestation of the same twig by others. These results may be useful for targeted interventions of managing *X. compactus* at the population peaks, and offer prospects of identifying deterrence cues for repelling the pest.

MATERIALS AND METHODS

The experiment was conducted from June 2013 to May 2014, replicated in three coffee blocks, at NaCORI, Mukono, Uganda. Twenty coffee twigs infested with *X. compactus* were randomly sampled monthly from each block. The twigs were dissected under a microscope to count eggs, larvae, pupae and adults inside galleries. Data on temperature and rainfall were

obtained from NaCORI meteorological station. The number of *X. compactus* galleries per twig was recorded.

Counts of different stages of *X. compactus* per month were subjected to binomial generalized linear modeling with binomial distribution error and logit link to determine variations of numbers of the different stages with months. Means of the different stages of *X. compactus* were subjected to Pearson's product-moment correlation with monthly total rainfall and mean temperature. Number of twigs with one gallery was compared to that with two galleries using χ^2 test. All analyses were conducted in R statistical software version 3.1.1.

RESULTS AND DISCUSSION

There was no significant variation in number of *X. compactus* eggs with months (Figure 1). This may be partly due to very low numbers per gallery. The lower number of eggs of *X. compactus* in the galleries than other stages may be a result of their lifespan being the the shortest life stage lasting 3-5 days compared to other stages which take 7-9 days (CABI 2013). The number of larvae, pupae and adult females varied significantly with months (P = 0.002, P =0.007 and $P < 0.001$, respectively). These stages were at their peaks during May to July. It may therefore be helpful to target management interventions at this period. There is however a need to establish if adult female peak flight activity also coincides with the period of adult abundance in the galleries. Determining peak adult female flight periods would be helpful in applying management tactics such as baited traps against the females (Miller and Rabaglia 2009).

The monthly seasonality of *X. compactus* may be influenced by a number of biotic and abiotic factors which may vary with the months. Temperature and rainfall are among the key factors that fluctuate markedly with months (Faccoli 2009). We therefore correlated monthly total rainfall and mean temperature during the study period with means of different stages of *X. compactus* inside galleries. There was no significant correlation between mean number of eggs per twig with either temperature or rainfall. The mean number of larvae per twig was significantly negatively correlated with temperature (P = 0.02), but the correlation between the mean number of larvae per twig with rainfall was not significant. Mean number of pupae per twig was not significantly correlated with both monthly mean temperature and total rainfall. Mean number of adult females and males were significantly negatively correlated with rainfall (P = 0.03 and P = 0.01, respectively), but there were no significant correlations of the adult numbers with temperature. These results demonstrate that weather patterns play a critical role in the population dynamics of *X. compactus,* although the prevailing mean temperatures and total rainfall during this study may not have reached developmental threshold limits for eggs and pupae. It would therefore be useful to investigate the role of these weather variables in the development of *X. compactus* under a broader range of these variables either under regulated laboratory conditions or in diverse agro-ecologies.

Temperature is one of the key parameters that influences development and population dynamics of insects. In tandem, our data–albeit being from a narrow ambient mean temperature range –showed a reduction in number of *X. compactus* larvae with increasing temperature. This suggests that high temperature either kills the larvae or hastens their development into pupae thus facilitating a shorter lifecycle of *X. compactus*.

Some insect pest outbreaks and damage, especially wood borers, are reportedly rampant during drought (Faccoli, 2009). This hypothesis concurs with our findings above that X. *compactus* adults are most abundant inside galleries during dry weather. This phenomenon is explained by a number of hypotheses depending on the pest species (Mattson and Haack,

1987; Faccoli, 2009). With our knowledge of the biology and ecology of *X. compactus* (CABI 2013), we have selected the following three hypotheses as possible explanations for the relationship of *X. compactus* adult numbers with rainfall. Firstly water stressed coffee may become less resistant to *X. compactus* boring and subsequent development, and also to the ambrosia fungi, thus providing more abundant food for *X. compactus*. Secondly, drought is normally associated with high temperature which may indirectly shorten the lifecycle of *X. compactus* and/or favour the growth of the ambrosia fungi. Thirdly, water stress may induce aggregation of *X. compactus* inside galleries since most scolytid pheromones are derived from host monoterpenes such as α-pinene which is abundant in water stressed plants (Mattson and Haack, 1987). The third proposition is substantiated by the report by Miller and Rabaglia (2009) that α-pinene attracts *X. compactus*. However, validation of these propositions on *X. compactus* outbreaks requires further research.

This study revealed that the maximum number of galleries per *X. compactus* infested twig was six, with only 1.28 ± 0.93% of the twigs having more than two galleries, of which the percentages of the infested twigs with 4, 5 and 6 galleries were 0.1 ± 0.1for each category (Figure 2). The percentage of *X. compactus* infested with 1 gallery (87.07 ± 1.26%) was significantly higher than 10.65 ± 1.61% of the twigs which had two galleries (P <0.001). This suggests that infestation by *X. compactus* pioneers elicits a signal to deter conspecifics from infesting the same twig. This may be either as a result of modification of plant odors due to boring by the pioneer (Visser 1986; Du et al 1998; Meiners and Hilker 2000; Wegener et al 2001) or its release of deterrence pheromones to prevent competition for the same twig (Byers, 1989). Increased concentration of attractive host monoterpenes such as α-pinene for example leads to repellence of beetles by the host volatiles (Seybold et al 2006). This semiochemical communication, if elucidated in *X. compactus*-host interaction can be applied in development of repellence techniques against the pest.

In summary, we have shown that *X. compactus* larval, pupal and adult female numbers are variable during the year in Mukono, Uganda with peaks occurring during May to July. The seasonality in larval numbers is influenced by fluctuations in temperatures during the months, where high temperatures may be either lethal or favourable for a speedy eclosion of the larvae into pupae. We have also shown that water stress favours high *X. compactus* adult populations, leading to outbreaks during drought. Lastly, our results suggest that infestation by pioneer *X. compactus* females may deter others from infesting the same twig, although the mechanism for this observation remains unknown. These findings may be useful for targeted interventions in managing *X. compactus* at the population peaks, and offer prospects of identifying cues to repel the pest.

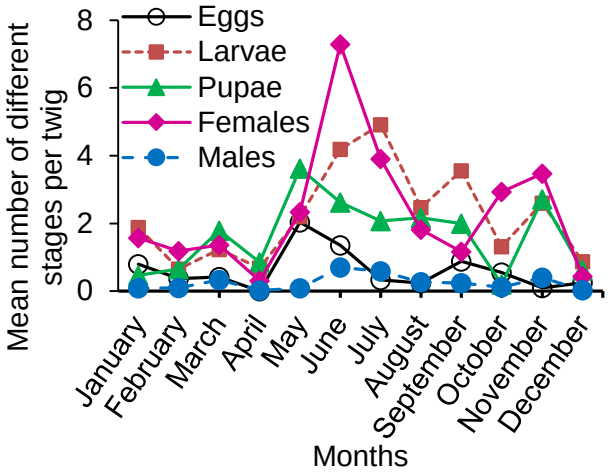

Figure 1. Variation of numbers of different stages of *Xylosandrus compactus* per infested twig with months from June 2013 to May 2014 at the NaCORI, Mukono, Uganda

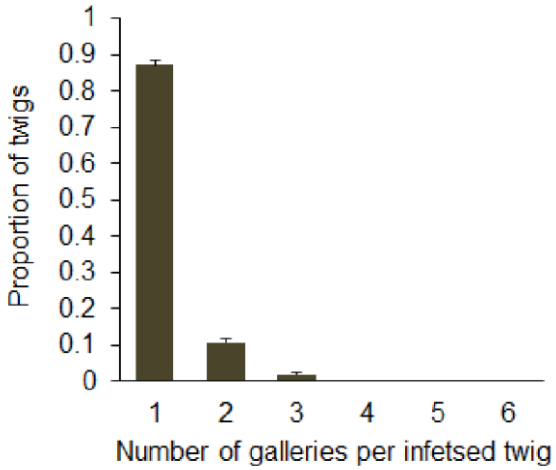

Figure 2. Proportions of *Xylosandrus compactus* infested twigs with 1-6 galleries during June 2013 to May 2014 2014 at the NaCORI, Mukono, Uganda

ACKNOWLEDGEMENTS

We thank Uganda Coffee Development Authority and the Inter African Coffee Organisation for facilitating the presentation of this work at the 25th International Scientific Conference on Coffee; and the conference organisers for the waiver of the registration fees. This study was conducted with funding from the International Development Agency through the Agricultural Technology and Agribusiness Advisory Services project.

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Climate Change Impacts on Arabica Coffee in Brazil

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SUMMARY

Brazil is the world's largest producer of Arabica coffee. Adverse climatic events in its major production regions have global repercussions through market effects. Climate change impacts on the Brazilian coffee production are thus of high interest to understand long term trends on global coffee markets. Nevertheless, this aspect has not been looked at using a rigid modelling framework.

To investigate the climate change impacts on the Brazilian Arabica production, we employed three different modeling approaches to generate an ensemble climate envelope model of current and future Arabica suitability distributions. First, based on high-resolution census data an unbiased database of Arabica production locations was assembled. Three different classification methods from ecological niche theory, Hypervolume, Maxent and Bioclim, were trained and evaluated against this true distribution of current production using various feasible parameter settings. We thus extrapolated these models on downscaled climate data from three global climate models of the 4th IPCC Assessment report for 2030, 2050 and 2080 in the SRES A2 emission scenario and constructed consensus maps for each time slice.

The results of the consensus across all models show a marginal migration of areas suitable for coffee production towards the Southern states of Santa Catarina and Rio Grande do Sul, while more Northern locations in Bahia, Rondonia and Goias are projected to experience drastic losses of area. By 2050 50% (70% by 2080) of models in the ensemble project a loss of suitable area in Minas Gerais as compared to current climate if cultivation practices are adapted to progressive climate change. When we applied a more restrictive analysis, demanding a high agreement of models comparable to the agreement for current conditions, we found that area with such ideal conditions could be reduced entirely by 2080 for all of Brazil. Our results confirm previously hypothesized trends caused by climate change, but dispute the extent of opportunities from novel areas in the extreme South of Brazil. Once full climate change effects are experienced, Brazil may face challenges to remain a major coffee producing country.

INTRODUCTION

About one third of global green coffee production is produced in Brazil (FAO 2012). An estimated 80% of this is Arabica coffee (IBGE 2013), making Brazil the largest producer of this commodity worldwide. Climate change impacts on the Brazilian coffee production are thus of high interest to understand long term trends on global coffee markets. To investigate the climate change impacts on the Brazilian Arabica production we employed three different modeling approaches to generate an ensemble climate envelope model of current and future Arabica suitability distributions.

METHODS

First, based on high-resolution census data an unbiased database of Arabica production locations was assembled. For Brazil detailed production statistics are available on municipal level from IBGE (2013). Using census data we identified municipalities that are characterized by at least 75% of production being *C. arabica*. For the municipalities from the highest three percentiles that are Arabica dominated we generate 3 to 1 random sample points at a minimum distance between points of 0.05 decimal degrees. Thus, municipalities with high production are sampled more than municipalities with little production. This way a total of 975 putative Arabica point locations was generated.

To characterize the general environment at potential coffee growing locations we pick an equally sized random sample from all locations that are within the range of annual mean temperature as can be found in the presence sample.

For the current climate (1950–2000) we use the WorldClim global climate data set on 5" ArcMin resolution (Hijmans et al. 2005). Correlation analysis is conducted in order to remove highly collinear climate information from the dataset. For correlation analysis the Pearson correlation coefficients are calculated. Coefficients $|R| > .7$ are regarded critical and at least one of the variables had to be removed from the dataset.

Three different classification methods from ecological niche theory, Hypervolume (Blonder et al. 2014), Maxent (Phillips et al. 2006) and Bioclim (Nix 1986), were trained and evaluated against the distribution of current production. Based on omission of current presence locations a threshold was determined beneath which a score is converted to 0, or to 1 above the threshold to make results comparable. 5 different omission levels were considered for training: 1%, 2.5%, 5%, 7.5%, and 10% omission.

To evaluate the ability of the resulting ensemble model for current climate to be able to reflect the actual point distribution with high confidence we use a calibrated AUC measure (cAUC) on each individual model as proposed by Hijmans (2012). Algorithm training is conducted on 80% of the total sample and the resulting models are applied to the remainder 20% for evaluation.

We thus extrapolated the models derived from the training step on downscaled climate data for current and future conditions. For current conditions we used the same climate data from WorldClim as before. For future conditions we used data from three global climate models (cnrm_cm3, mri_cgcm2_3_2a, ukmo_hadgem1) of the 4th IPCC Assessment report for 2030, 2050 and 2080 in the SRES A2 emission scenario. For each time slice consensus maps for were constructed by averaging the individual results.

To differentiate suitability scores that represent areas currently used for *C. arabica* coffee production from suitability scores in areas not used for production we define a threshold. This threshold was determined by the maximum sum of sensitivity and specificity criterion (MaxSSS) as suggested by Liu et al. (2013).

In order to quantify impacts for each Brazilian state we used two measures. First, we sum all suitability scores in grid cells by each state, and express impacts as percentages of the current sum of suitability scores. Second, for each state we count the number of 5ArcMin grid cells that show scores higher than the previously defined threshold.

RESULTS

The final variables chosen to be included in the model are listed in Table 1. The final variables are not significantly correlated.

Table 1. Variables used in model.

Bioclimatic variable	Description	Unit
BIO 2	Mean Diurnal Range (Mean of monthly (max temp - min temp))	**°C**
BIO 5	Max Temperature of Warmest Month	**°C**
BIO 6	Min Temperature of Coldest Month	**°C**
BIO 12	Annual Precipitation	**mm**
BIO 15	Precipitation Seasonality (Coefficient of Variation)	-
BIO 18	Precipitation of Warmest Quarter	**mm**

On average across all 25 repeats cAUC values are high. The total average cAUC is .426. This indicates a high predictive performance.

Based on the MaxSSS criterion we determine the threshold that under current conditions distinguishes areas with current presence of coffee production from areas that putatively are not used for C. arabica coffee production. The threshold was determined to be .78. This means that a location can be seen as suitable if 78% of the independent model runs classify the location as likely to be suitable.

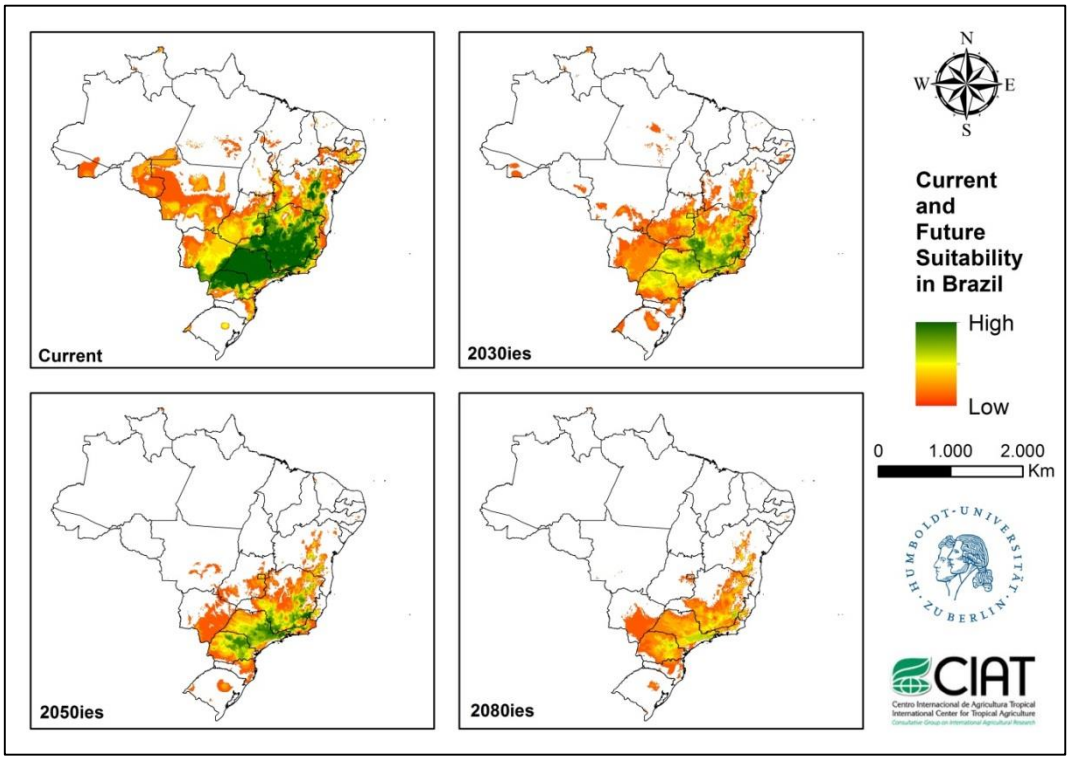

Figure 1. Model consensus map of current and future suitability in Brazil. Green colors indicate high suitability, red colors low suitability.

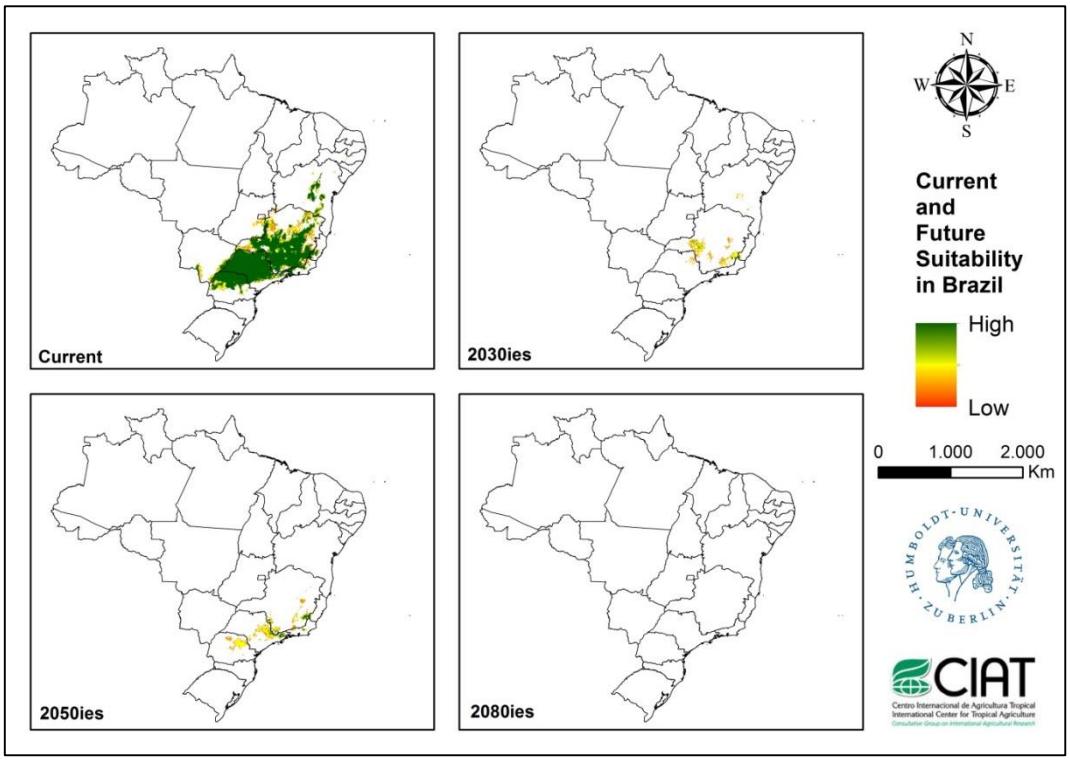

Figure 2. Model consensus map of current and future suitability in Brazil showing only grid cells with suitability scores found at the majority of current presence locations. Green colors indicate high suitability, red colors low suitability.

The distribution of suitability scores as given by the average score across all independent model runs is shown in
Figure 1 for current and future climate conditions. Under current conditions the models agree that the most highly suitable area can be found in the traditional Brazilian coffee states of Minas Gerais, Sao Paulo, Parana and Bahia. Under progressive climate change suitable area is about halved every 20-30 year time step. Northern and Eastern locations are more impacted than South-Western locations. By the 2080ies comparatively small areas retain high suitability scores.

Restricting the analysis to grid cells with suitability scores above the threshold of current production areas shows that by the 2080ies no area has scores higher than the threshold. The state of Parana is projected to lose area suitable according to threshold analysis by the 2030ies, but regains some of this area until the 2050ies (
Figure 2).

By the 2050ies most states are projected to experience drastic impacts. Nearly all states lose more than 50% of their current suitability score total. Exceptions are Rio Grande do Sul and Santa Catarina which up to this point experience positive total changes.

Some states could lose all of their current suitability by the 2080ies. Only Rio Grande do Sul retains current levels of suitability. Also the state of Santa Catarina could well be a relative winner of climate change as impacts at 20% losses are comparatively low. The largest *C. arabica* producer in Brazil, Minas Gerais, loses up to 80% of its total suitability by the same time.

Summing suitability scores for the entire country Brazil losses in total are 49% by 2030, 64% in the 2050ies and 79% until the 2080ies. Until the 2080ies all area above the 78% threshold would be lost. Regarding the entire country losses of area according to this analysis would be 92% for the 2030ies, 89% by the 205ies and 100% until the 2080ies.

DISCUSSION

Despite its importance as the world leader in coffee production until now climate change impacts on Brazilian *C. arabica* cultivation have not been studied with sufficient rigor. Our aim for this study was to use ecological niche theory derived approaches that use statistical or machine learning methods to gain better insight into possible impacts.

Our results confirm previously hypothesized trends caused by climate change, but dispute the extent of opportunities from novel areas in the extreme South of Brazil. A map indicating the distribution of suitability scores in Brazil reflects the current distribution of coffee production. Its equivalents for future time steps demonstrate early (2030) losses in North-Eastern states and less harsh impacts in South-Eastern states even by the 2080ies. Applying a threshold to the suitability scores aggravates this picture. While using this threshold the distribution of most of the Arabica production in Brazil is reflected well under current conditions under 2080 conditions no such area remains.

Once full climate change effects are experienced Brazil may thus face challenges to remain a major coffee producing country. The differing results with or without threshold applications demonstrates the need to invest in adaptation measures.

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Impact Assessment of AAA Sustainable Quality™ Program on Small Farmers in Colombia

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SUMMARY

As part of CRECE´s research program to assess the impact and outcomes of adopting sustainable practices in coffee, this study is aimed to analyse the impact that the implementation of the Nespresso AAA Sustainable Quality™ Program has had on social, environmental and economic conditions of the coffee growers involved. Three aggregate indexes were calculated by implementing the Polychoric Principal Component Analysis technique to compare target producers against conventional producers operating as a control group for a period of three years - 2009, 2011 and 2012. The results account for a positive and significant impact of the program on Economic and Social issues.

AAA PROGRAM EXPECTED OUTCOMES

Launched in 2003 in collaboration with the NGO the Rainforest Alliance, the *Nespresso* AAA Sustainable Quality™ Program implements a vision of sustainability based on quality, and integrating the spheres of environmental conservation, social equity and economic viability, with the aim of creating long-term shared value. The AAA Program links quality and sustainability through a program of integrated coffee farm management practices for long term social and economic value.

The implementation of the Program is supported by a set of activities including special projects and programs with the partners to support farmers' investments; training for farmers and agronomists; and technical assistance to the farmers in order to improve sustainability and productivity. It is expected that the sequence of activities will be reflected in changes in knowledge, skills and attitudes leading to better farm productivity, quality consistency, and positive environmental and socioeconomic impact.

METHODS

The study is based on a probabilistic sample taken from the population of coffee growers beginning their participation in 2009 in one of the three components of the AAA Program in Colombia (Quality, Productivity and Sustainability) at the Departments of Antioquia, Caldas, Cauca, Huila and Nariño. The sample is composed by 1.222 target and 563 conventional farmers randomly selected from AAA Program clusters, with 95% of confidence and errors of 7% or less. This sample was followed-up for three years with annual measurements for the same group of producers, allowing a balanced panel.

The focus of the study is based on the development and application of an internationally-recognized methodology and data gathering process to monitor and assess sustainability initiatives promoted by COSA organization and adapted to Colombia by CRECE [1] The

treatment is represented by the set of activities carried out by the Nespresso AAA Program in concordance with the expected impacts and outcomes of the Program [2]. The control group operated as the counterfactual, to estimate what the target group would have achieved if not been exposed to the Program activities.

As a quantitative quasi-experimental panel analysis, the method is focused on isolating as much as possible the effect of factors other than the intervention. Propensity Score Matching and Difference in Difference techniques were implemented to control by factors that could affect indicators performance. However, it has to be recognized that despite the thorough procedures implemented to data cleaning and comparability, some factors could keep influencing the results. Therefore, some differences could persist between target and control producers, even after controlling for Propensity Score Matching, probably due to pre-treatment influencing factors.

In order to measure the aggregated sustainability and its social, environmental and economic dimensions, the Polychoric Principal Component Analysis technique was implemented [3]. This method allows to synthetize multiple variables in one simple index that ranges from 0 to 100[1]. It uses discrete variables and calculates what would be their correlation as if they were on a continuous scale [4]. In the case in which there are two variables (X_1 and X_2) that represent the binary discretized form (this discretization is defined by thresholds) of two continuous variables (Y_1 and Y_2), the polychoric correlation of both variables would suppose that there exists a common continuous latent trait (T) defined by the interaction of both variables. The variables used for the indexes are dichotomous, categorical and discrete. Some of them are transformations of continuous numerical variables into categorical variables.

RESULTS

After three rounds (2009, 2011 and 2012) of measurement of the two groups of farmers, the results reveal improvement in the performance of economic, environmental and social conditions. Although the overall Sustainability Index of both AAA farms and the control group increased over the three year period, the difference remained constant by 25%.

The calculated indexes have an upward trend that indicates an improvement for the producers in the overall sustainability over time. This improvement is occurring in both groups -except for the social dimension in conventional producers- (Figure 1). In regards to the performance of the indicators, the variables that explain the increase of the economic index were mainly yield[2], 61% of AAA farmers sell tolerable levels of low quality beans -compared to 35% of control-, record keeping of fertilizer's application, training in market topics that doubled to 56% and knowledge of the domestic price. However, variables such as the search for new customers, the use of organic fertilizers and the proportion of farmers falling in high and very

[1] The Economic index includes variables such as yield and net income that comes from the coffee production (the variables were categorized in an ascending order). It also includes the level of knowledge that the producer has on the market and the access to it, technical use of the inputs, perception of the economic situation of the farm, diseases and pests and percentage of coffee sold as low quality. The Social index includes indicators of farm's resilience, working conditions at the farm, producers' perception of their household quality of life and their relationships with their employees. The environmental index includes information about recycling programs at farm level, conservation practices, training in environmental topics and subjective perception of the environment conditions of the farm and the village.

[2] The percentage of farmers in high productivity categories of the index increased from 39% in 2009 to 43% in 2012. At the same time, the proportions of farmers that fall in low productivity categories are slightly decreasing.

high income (revenue) level[3] are not growing by the last period of observation, despite that AAA farmers' net income doubled the control group in 2012.

The social index is being driven by the progress in variables such as worker's living conditions (conditions to cook and safe water availability for workers), possession of household assets increased, proportion of AAA Farmers that use increasing items of protective gear to work, Occupational safety and health such as access to medical attention, first aid kits and restrictions to work for vulnerable people and perception of the household's quality of life as high increased from 30 to 59%. However, variables such as the proportion of farms with crop production for family consumption and revenues from sales of crops other than coffee did not grow. Meanwhile, all the variables inside the environmental index improved for AAA farmers. Main drivers that contributed to the improvement in Environmental conditions were increased training in environmental practices, soil conservation implementation on AAA farms, better water treatment practices, lower water usage for wet milling (1 liter less water to mill 1 kg of parchment), farm and village environment care perception.

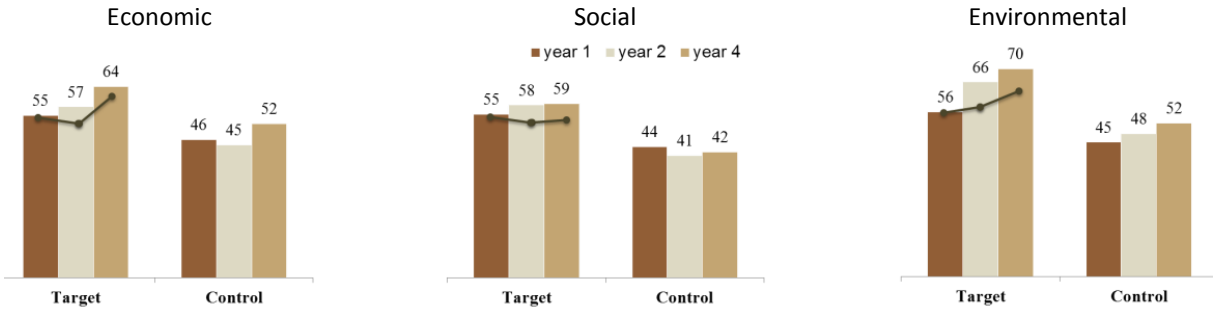

Figure 1. Sustainability index by year. Target and Control

The estimated impact of the adoption of the Good Agricultural and Quality Practices recommended by the AAA Program through the TASQ™[4] [6] resulted positive for the aggregate indexes of sustainability (Table 1). In order to control bias as much as possible to quantify the impact of the AAA Program, PSM + DID methods were implemented. In line with these methods, the projection line in every graph depicts the trend that the target group should have had if the baseline difference to control were imposed for the period of evaluation. This shows what would be the average scores of the producers if they had not been part of the program. Therefore, the impact of the Program over the corresponding index is the difference between the score of the target group and the projection line.

[3] The proportions of farmers in high and very high income levels of the index passed from 28% in 2009 to 24% in 2012.

[4] Tool For the Assessment of Sustainability Quality™

Table 1. AAA Program sustainability impacts

Indicator	DD 1st follow-up		DD 2nd follow-up	
Social Index	3,5		2,6	**
Environmental Index	7,3	*	-2,2	
Economic Index	8,3	*	4,1	*
Productivity (kilos GBE per ha)	83,8	**	48,9	
Net income (USD per kilo GBE)	$ 0,03		$ 0,22	**

significant at the 90%; ** at the 95%; * at the 99%*

After controlling by factors influencing the participation, statistically significant impacts were found over the social (+2.6 points) and economic (+4.1) conditions (Table 1). In contrast, despite the huge progress achieved in the accomplishment of environment care requirements by AAA farmers, there was no significant net effect in this regard. The cancellation of the effect of the outcome is due to the intervention received by the control group, from institutions other than the program[5], especially in soil and water conservation practices.

Additionally to the aggregated impacts of the program, it was estimated the effect over two important expected effects of the program: the change in productivity and the change in farmer's Net income. Productivity approached by yield (kilos of Green Bean Equivalent per hectare) showed a positive significant effect of 8.4 kilos GBE by the second year although it turned out not significant by the fourth one. To this result contributes the fact that the conventional farms started to recover their levels of yield by the last year of the survey. The program has a positive and significant effect on the farmers' net income for the last observation, in line with the program expected impacts.

CONCLUSION

The producers who participate in the Nespresso AAA Sustainable Quality™ Program are progressing over time in the social, economic and environmental dimensions of sustainability. The increasing adoption of better sustainable practices is associated to technical assistance provided by the program. After controlling by influencing factors, it is concluded that the situation for the farmers is better than that in absence of the program, for the social and the economic dimension. The effects on environmental conditions were overlapped by the also good performance by the control group as a consequence of programs other than AAA.

The program achieved positive impact on productivity by the second year which was diluted by the recovering of the control group at the end of the period of observation. By contrast, a significant effect on farmer's net income was observed compared to the situation in absence of the program. Despite the increasing accomplishment of the program requirements, there is still enough space to improve the adoption of practices by the farmers.

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[5] In Colombia, other organizations, such as the FNC, also work with farmers to enhance environment management, hence the positive impact observed for the control group

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Diversity of Ugandan *Coffea canephora* Pierre Biochemical Compounds as Measured by Simple Sequence Repeats and Near Infra Red Spectroscopy

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SUMMARY

About 1.25 million households grow Robusta in 270,000 hectares, earning at least US $3.2 million annually. This study aimed at establishing Ugandan leaf DNA and biochemical fingerprint variability that influence quality in *C. canephora* green bean. Leaf DNA of 135 *C. canephora* germplasm collections was evaluated with SSR markers. More 454 bean samples from 21 districts and research stations were assessed using Near Infra Red Spectroscopy (NIRS) at the National Crops Resources Research Institute at Namulonge, Uganda. Caffeine content of 16 Robusta accessions was estimated using HPLC and NIRS to verify the precision of the two methods. Transformation of the spectral data into numerical data using predictive models and DNA screening was done at the Centre for International Agricultural Research Development, France. Correlation coefficients quantified the strength of paired biochemical compound relationship at 5% significance level. PCA of biochemical compounds with tree age and altitude was compared with multivariate groups from the factorial step discriminant analysis. The results revealed three major genetic diversity groups of heritable traits and four multivariate groups that reflected a combined influence of inherent traits and environment. Caffeine content from HPLC and NIRS technique which is fast and cheaper was insignificantly different. Ugandan collections had more fat (10.44 to 15.94% of dry matter), wide sucrose range (2.48-7.34% dm) and chlorogenic acid (10.88 to 15.64 % of dry matter) than those reported in previous studies, reflecting high diverse flavours and aroma. The more CGA content than caffeine alkaloid (1.41-3.29% dm) meant not all bean CGA was converted to caffeine chlorogenate as previously reported. The significantly negative correlation between caffeine, chlorogenic acid with trigonelline, sucrose and fat contents implied coffee brew with less caffeine was less bitter and rich in flavour and aroma. Enormous Ugandan genetic and biochemical compound diversity is useful for conservation and crop improvement.

INTRODUCTION

Coffee contributes 20% of Uganda's foreign earnings estimated at US $400 M per year with 8 M peasants involved in its growing and enterprises for income. Robusta constitutes 80% of Ugandan coffee. Markets grade Robusta as bitter with no favour although Ugandan is reported to be mild. Genes and environment influence biochemical precursors that determine coffee bean quality (Leroy et al., 2006). Aluka et al., (2006) found some Ugandan Robusta genotypes had as good cup as Arabica, while Durand et al.,(2006) and Leroy et al., (2006) revealed polymorphism in genes related to sucrose metabolism. This study aimed at

establishing leaf DNA and green bean biochemical compound variability influencing bean quality of Ugandan *C. canephora* for strategic conservation and use.

MATERIALS AND METHODS

Leaf DNA of 135 *C. canephora* germplasm collections were assessed for genetic diversity using SSRs. More 454 accessions were evaluated with near infrared spectroscopy (NIRS) calibrations developed for green coffee (Davrieux et al., 2003). Factorial Dissimilarity matrix of paired genetic distances was graphically represented using Un-Weighted Neighbour Joining Arithmetic Mean analysis (Fig 1). Caffeine content for 16 accessions derived from HPLC and NIRS was compared using one-way ANOVA and a bar plot (Fig 2) to verify precision. The 6 biochemical compound contents quantified by predictive models were summarized (Table 1). Correlation coefficients (Table 2) quantified the strength of paired biochemical compound relationship on an ordinal scale at 5% significance level. PCA of biochemical compounds with tree age and altitude was compared with multivariate groups from the factorial step discriminant analyses (Fig 3).

RESULTS AND DISCUSSION

The 3 major genetic diversity groups from Ugandan 135 *C. canephora* leaf DNA unveiled variable heritable traits that can be conserved, used to contain epidemics as was the case of *Fusarium xylariodes* in Uganda and crop improvement.

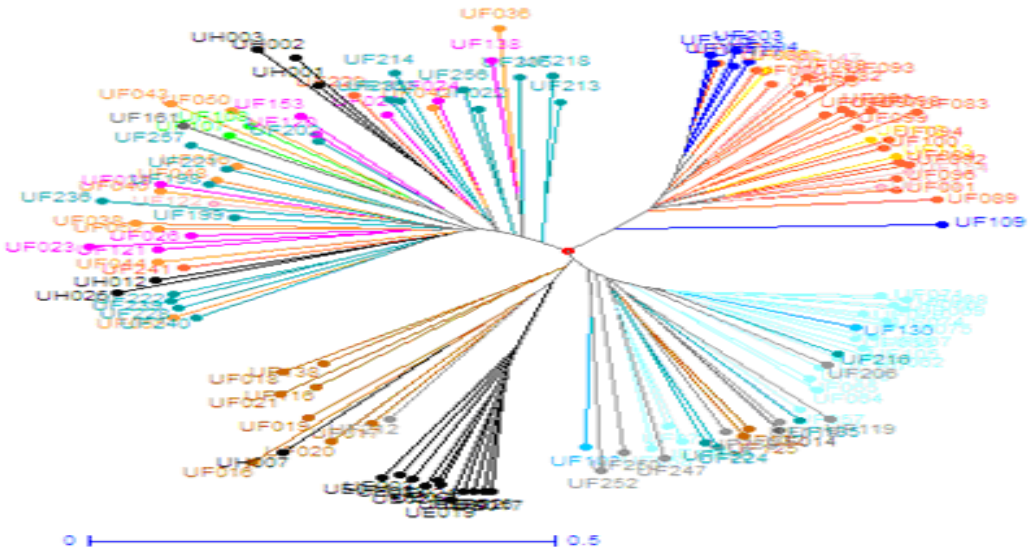

Figure 1. Darwin Neighbour Joining tree for 135 Ugandan Robusta coffee DNA analyzed with 18 SSR markers; Key: letter codes represent different districts.

Insignificant difference between caffeine content derived from NIRS and HPLC implied the NIRS technique may preferably be used to estimate caffeine. However, need to validate efficiency of the two methods for other bean biochemical compounds that predict coffee quality.

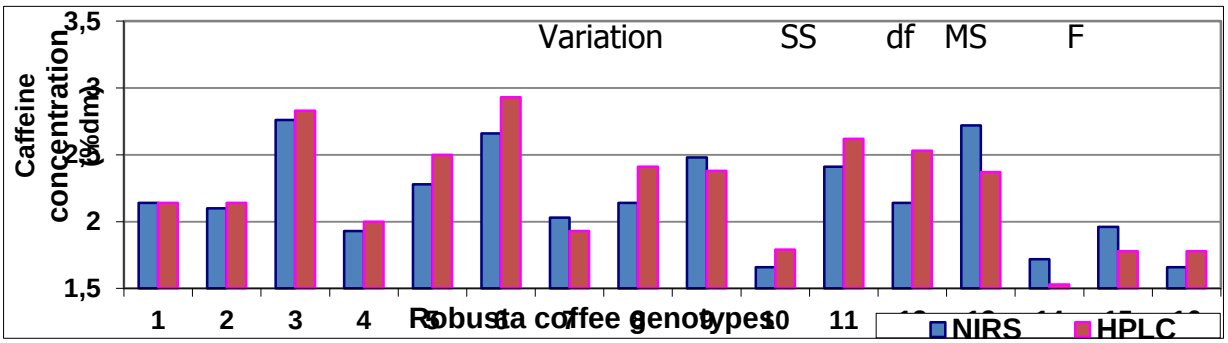

Figure 2. One-way ANOVA and a bar plot comparison for caffeine concentration derived from High Performance Liquid Chromatography and Near Infra Red spectroscopy.

The high bean fat content in Ugandan Robusta (10.44-15.94% dm) as compared to 7.0-11.0%dm reported by Wintgens, (2004) with a wide sucrose range (2.48-7.34%dm) increased flavor and aroma. More CGA content than caffeine alkaloid (1.41-3.29% dm) implied not all bean CGA converted to caffeine chlorogenate as reported by Campa et al., 2005b, reducing brew bitterness. Beans with high heritability values for weight (0.73), fat (0.74) and caffeine (0.80) make good parents crosses for improving flavour and reducing bitterness (Leroy et al., 2006).

Table 1. Summary of the 6 major biochemical compounds quantified in the 454 Robusta coffee genotypes using NIRs (% dry matter)

Stat	Dry matter	Caffeine	CGA	Trigonelline	Sucrose	Fat
Min	85.82	1.41	10.88	0.66	2.48	10.44
Max	91.52	3.29	15.64	1.03	7.34	15.94
Mean	88.26	2.33	12.92	0.79	5.20	13.79
V.R	5.7	1.88	4.76	0.37	4.86	5.50
SD	0.83	0.32	0.71	0.07	0.78	0.78

Key: CGA=chlorogenic acid; SD= standard deviation; Caf=caffeine; Stat= statistics; trigo=trigonelline; V.R=value range; Min=minimum; Max=maximum

Positive and significantly correlated bean caffeine and chlorogenic acid contents meant the two compounds occured in proportional amounts. The significantly negative relation between caffeine, chlorogenic acid with trigonelline, sucrose and fat contents implied with less caffeine, the brew is less bitter with high flavour and aroma.

Table 2. Correlation coefficients of 454 Ugandan *C. canephora* green bean biochemical compounds (% dry matter).

Variables	Caffeine	CGA	Trigonelline	Sucrose	Fat
Caffeine	1				
CGA	0.51***	1			
Trigonelline	-0.43***	-0.06	1		
Sucrose	-0.12**	0.21***	0.21***	1	
Fat	-0.17***	0.38***	0.37***	0.22***	1

, **, * significant at 0.05, 0.008, 0.0005 levels of probability*

Bean biochemical compounds varied with tree age and altitude. Group 1 had dense beans from young trees with low caffeine and CGA. Group 4 beans were bitter beans from older trees. Unlike group 4, group 3 had more fat, sucrose and trigonelline with more flavour and aroma.

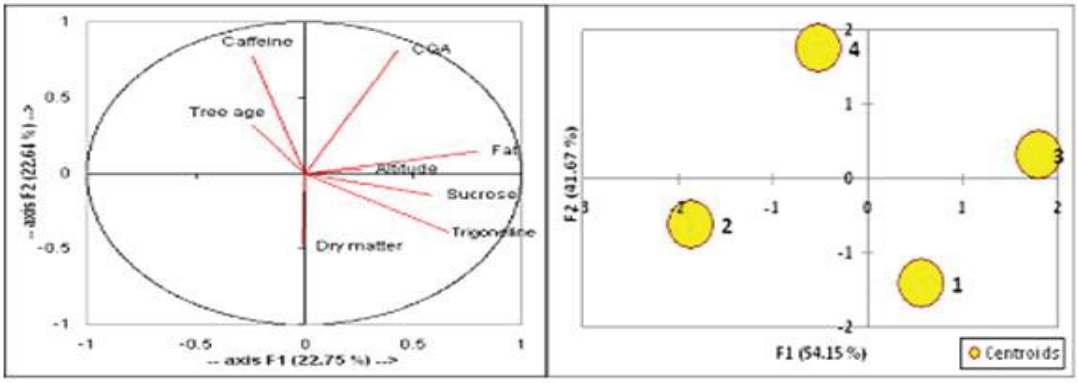

Figure 2. (a) Comparison of PCA plot of green bean biochemical compounds, altitude, tree age with (b) multivariate groups derived from factorial step discriminant analysis. Note: Mahalanobis distance range between groups; 5.11 - 14.22. Confusion matrix mean correct genotype group placement range; 93.20% - 94.17% with a mean of 93.80%.

CONCLUSION

Uganda cultivated *Coffea canephora* has a wide genetic and green bean biochemical compound variability that can be utilized in crop improvement and explore market opportunities. The high fat and sucrose values provide high fragrance, aroma and flavor in the brew.

RECOMMENDATIONS

Investigate and identify diverse traits in Ugandan *C. canephora* for *in situ* and *ex situ* custody and crop improvement. Profile Uganda coffee growing areas according to bean biochemical compounds for targeting specific markets.

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Endogenous Factors Associated with Somatic Embryogenesis of Coffee (*Coffea arabica* L.): a Review

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SUMMARY

Many *Coffea* species have difficulty regenerating somatic embryos in tissue culture, in spite of the great progress accomplished in development of embryogenic cell induction protocols. This difficulty results in regeneration of few embryos during the induction process and subsequently fewer coffee seedlings that do not meet farmers' demands of new disease resistant coffee varieties. Although some studies report the origin of embryogenic cells, much of the early developmental processes and factors in coffee somatic embryos remain unclear, especially those related to the regulation of the induction and development of somatic embryos. Understanding the mechanisms and factors that trigger induction and expression of somatic embryogenesis (SE) in coffee could lead to more rational regeneration protocols that would increase the number of plantlets available for distribution to coffee growers. This review explores some selected endogenous inhibitors and stimulators present during somatic embryogenesis of coffee in order to understand their role in somatic embryogenesis.

INTRODUCTION

Plant tissue culture plays an important role in agricultural biotechnology. It allows *in vitro* regeneration and multiplication of plants under aseptic conditions through a process known as micropropagation. To date, micropropagation is the most common application of tissue culture. High cost of micropropagation, however, limits its application mostly to high-value ornamental, plantation and forestry plant species. Plant regeneration in tissue culture occurs through two developmentally and morphologically divergent processes: (a) Somatic embryogenesis (SE) and (b) organogenesis. Somatic embryogenesis is a type of vegetative propagation based on plant cell totipotency, which offers a powerful alternative to other vegetative propagation methods, i.e. cuttings or grafting. In the case of coffee, its main use is for F1 hybrid propagation, thereby avoiding manual hybrid seed production and cuttings which are costly and difficult to root. Whereas some species exhibit both regenerative processes, in others, morphogenesis occurs preferentially by either SE or organogenesis. *In vitro* regeneration of coffee is mainly through somatic embryogenesis because it presents the highest rate of multiplication.

Coffee is a perfect plant model for applied research on the development strategies for large-scale production of somatic seedlings. It is also a relevant model species for fundamental research in molecular biology, biochemical and biotechnological research particularly for the investigations on the release of inhibitors as well as stimulating compounds *in vitro*.

SUBSTANCES INFLUENCING SOMATIC EMBRYOGENESIS

Phenols

Tissue culture often involves extensive cutting and stress injury of tissues. Such stresses cause programmed physiological and chemical changes in plants. The chemical changes involve production of phenols. Being an important group of secondary metabolites, phenolics may act as modulators of plant development by regulating indole acetic acid (IAA) catabolism. They may combine with proteins either reversibly by hydrogen bonding or irreversibly by oxidation. There are two opinions on interactions between phenolics and plant growth and development. One indicates that phenolics are negatively related with plant *in vitro* proliferation while the other mentions the opposite. In tissue culture studies, phenolic substances, especially oxidized phenolics generally effect *in vitro* proliferation negatively. Phenolics are normally viewed as deleterious compounds during *in vitro* culture, since their exudation and oxidation negatively affect the explants, causing browning and necrosis, especially when mature explants of woody plants are used. Previous studies have shown that phenolic compounds are often associated with somatic embryo formation. For instance, for coffee explants, it was observed that embryogenic calli developed only after browning of the initial explant through a necrosis-like process which does not impair somatic embryo formation. These results demonstrate that somatic embryogenesis induction is not incompatible with phenolic compound production during *in vitro* culture.

Microscopic analysis showed a strong relationship between somatic embryo development and phenolic-rich cells. Reis *et al*., found that the levels of total phenolics changed during the embryogenic process and that the inclusion of exogenous phenols in the induction medium modified the type of phenolic compounds present in the embryogenic explants and their relative proportions. An increasing number of data seem to indicate that the role of phenolic compounds in vitro cultures should be analysed more carefully since, in some systems, phenols promote in vitro morphogenic processes. It is not yet clear how phenolic compounds affect somatic embryogenesis induction and somatic embryo development. A possible interference of phenolic compounds with auxin metabolism and, as a consequence, with the levels of this type of plant growth regulator in the explants has been suggested by some authors.

Carbohydrates and Reducing Sugars

Carbohydrate metabolism plays an important role in organogenesis as well as in somatic embryogenesis. Carbohydrates partly exert their effect on growth and morphogenesis through their nutritional value, and partly through their varying osmotic potential, which influences the rate of cell division or the degree of morphogenesis of the cells. In addition, carbon sources perform function in synthetic pathway of many compounds as building blocks of macromolecules and may control several developmental processes in the cell. Reducing sugars are important in callus formation and differentiation because they are necessary for the formation of reserved cell wall polysaccharides.

Proteins and Amino Acids

Besides having a function in the plant defense system, plant-related (PR) proteins have a function in development of the plant. Plant-related protein accumulation occurred under different stress conditions, whether caused by wounding, plant hormones, salicylic acid, heavy metals, heat, cold or UV light. Mobilisation of nutrients such as amino acids, proteins and lipids in the culture medium is used by different biosynthetic pathways for embryo

growth and development. Nienenak *et al*. reported that differences in the concentrations of total amino acids were especially evident for *Theobroma cacao* L. globular somatic embryos grown on solid medium and in temporary immersion system (TIS). The contribution of gamma amino butyric acid (GABA) to the total free amino acids in embryogenic callus and further developmental stages of cacao somatic embryos is substantial compared to non-embryogenic callus. These authors concluded that their finding fits the evidence that acquisition of embryogenic competence is a stress-response process.

Plant Hormones

Plant hormones play a vital role in the control of growth, not only within the plant as whole but apparently also within individual organs. Although auxins, which are known to mediate the transition from somatic to embryogenic cells, are the agents generally used to induce embryogenesis, the effect of other plant growth regulators on this phenomenon must not be overlooked. It has been frequently observed that embryogenic competent and incompetent callus sections are produced in the same explant, indicating that even genetically identical cells respond differently to a particular stimulus, with a minority the lesser part of the cells being responsive. Anatomical and physiological differences between embryogenic and non-embryogenic cultures are expressed when such changes occur. Among these differences, the endogenous hormone levels should be of great importance, since they regulate the processes of explant differentiation in culture. For this reason, the endogenous hormone levels and their relation to the embryogenic competence of the explants, may be the key to achieve the induction and expression of somatic embryogenesis in recalcitrant genotypes through amendments to the culture medium, with substances that may mimic the inductive condition (supplying a deficiency or counteracting an excess), to develop or optimize in vitro protocols for somatic embryo production, maturation, and conversion to plantlets.

CONCLUSION

Somatic embryogenesis is an important means to regenerate and propagate plants for commercial propagation of valuable genotypes. However, several events in somatic embryo development remain a mystery and need to be unveiled. Comprehensive work in future will clarify somatic embryogenesis in coffee by studying the interactions of various endogenous factors thereby the entire picture of regulatory mechanism of embryogenesis would be transparent.

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A Dramatic Change in Humidity Induces Rapid and Significant Changes in the Leaves of Immature *Coffea canephora* Plants

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SUMMARY

Plants react to changes in the levels of atmospheric water vapour (RH, relative humidity) in various ways. As there is relatively little information on the reactions of coffee to changes in humidity, we set out to explore this by studying the effects induced by a strong "humidity shock" on one variety of *Coffea canephora* (Robusta). To do this, small Robusta plants were acclimatized to high humidity conditions (RH >85%, 25-30°C), and then transferred to much lower humidity conditions (RH 30%, 27°C) for 2 hours, followed by a recovery under the original growth conditions. Physiological and gene expression measurements were carried on the leaves at different times during the treatment, and after a 24 hour recovery period. The results presented show that the applied "humidity shock" rapidly induces leaf wilting/ drooping, as well as resulting in significant reductions in leaf water content. As the leaves lost water, noticeable physiological changes often associated with drought stress in whole plants were also observed, and related gene expression data indicated that several genes whose expression are induced in the leaves by lack of water in the soil, are also induced in leaves of plants subjected to the "humidity shock".

INTRODUCTION

Future climate change scenarios indicate some coffee growing regions will experience a combination of drier atmospheric conditions and higher temperatures in the coming years (Davis et al, 2012). In addition, it is possible that parts of these "at risk" areas may also be subjected to more frequent and dramatic fluctuations in weather conditions, including modifications in atmospheric water vapour (RH) concentrations. Atmospheric humidity often influences plant growth and performance through its effect on water movement between the intercellular air spaces of the leaf and the atmosphere (Sellin et al. 2014). Despite its global significance as an important constraint on plant growth, the impact of variations in humidity has received little research attention in coffee, or in other plants. The aim of this work was to examine the the physiological and molecular implications associated with a sudden decrease of relative humidity on immature *Coffea canephora* (Robusta) plants that have been acclimatized to very high relative humidity conditions.

MATERIALS AND METHODS

Eight month old coffee plants acclimatized to high humidity conditions (RH > 85-90% -25-30°C- VPD below 0.32 - 0.64 kPa;) were used in this study. A humidity shock was applied by transferring test plants to a growth chamber at 27^0C with a relative humidity of 30% (VPD 2.49 kPa) and approximately 50 µmol.m^{-2}.s^{-1} irradiance while control plants remained under the high humidity conditions. After 2 hours of this "humidity shock" the test plants were returned to the initial conditions. At different time points, the visual appearance of the plants

was recorded, and a series of physiological measurements were performed. Photosynthetic CO_2 assimilation and stomatal conductance were measured on two leaves (an upper near fully expanded leaf and a lower leaf of each plant) using a Li-COR 64000Xt instrument with a CO_2 concentration set at 400µmol.m^{-2}.s^{-1}. Photosynthetically Active Radiation (PAR) was maintained at 1000 µmol.m^{-2}.s^{-1} and the data were recorded when the key parameters (Photosynthesis, Conductance and Ci) were stable. At each time point, the upper leaf was measured before the lower leaf. Less than 5 minutes was required to record the measurements for each leaf. The ratio of the variable to maximum fluorescence (Fv/Fm) was also measured for the two selected leaves at each time point (upper and lower leaves) using a Handy-PEA (Hansatech®). Each plant was first dark-adapted for 10 min. using leaf-clips supplied with the Handy-Pea® instrument and the Fv/Fm recorded after applying a pulse of saturating light.
At each time point, a leaf was also harvested and divided in half along its midrib axis with a scalpel. One half of the leaf was used to determine the relative water content (Gonzales & al 2001) by firstly obtaining its fresh weight, and then measuring the fully hydrated (turgid) weight by rehydrating the leaf section in distilled water over-night and re-weighing. Finally, the leaf fragment was then transferred to an incubator at 65^{0}C for approx. 24 hours to obtain its dry weight. The relative water content was determined using the formula below

$$\mathbf{RWC} = \frac{(\boldsymbol{fresh\ weight - dry\ weight})}{(\boldsymbol{turgid\ weight - dry\ weight}} \boldsymbol{x\ 100})$$

The other half of the leaf sample was stored at -80°C for RNA extraction. The RNA obtained was then used for quantitative gene expression analysis by RT-PCR (Simkin et al 2008).

RESULTS

Humidity shocked plants exhibited rapid leaf wilting/drooping after 15 minutes under low RH, with the upper leaves generally being more affected. One day after returning to high RH, the leaves recovered turgor, but many developed lesions (Fig 1). A large reduction in RWC was also seen after 15 minutes at low RH, followed by further slight decreases up to 45 minutes afterwards. Between 45-120 minutes, RWC then increased, and this continued during the 24 hour "recovery" period at the original higher RH. (Fig 2- A).

Figure 1. Visual appearance of coffee plants submitted to a short humidity shock. (A) under normal growing conditions RH >85-90 %. (B): 15 minutes after transfer to RH 30%. (C) 45 minutes after transfer to RH 30% (D): 24 hrs after return to RH 85-90 %. Note: missing leaves at later times used for RWC and gene expression analysis.

For upper leaves, a progressive decrease in Fv/Fm was observed over the 2 hours at the lower RH treatment, and continued during the 24 hour recovery period (no recovery in fluorescence was observed). It was noted that the standard deviation of the Fv/Fm for the upper leaves increased with time, suggesting a variation in the response. For the lower leaves, the Fv/Fm values only showed a small decrease during the first 45 minutes of the treatment, and then appeared to revert back to the levels seen in the control plants (Fig. 2, B1 and B2). For both

upper and lower leaves, a significant decrease of CO_2 assimilation was seen after only 15 minutes at low RH and these recovered only slightly after the 24 hour "recovery" period (Fig. 2, C1 and C2). Similarly stomatal conductance (Fig. 2, D1 and D2) was significantly reduced after 15 minutes at low RH for both leaves. For the upper leaf, only a small increase in stomatal conductance was seen between the end of the low RH treatment and the end of the 24 hour "recovery" period, but the lower leaf showed a better recovery in stomatal conductance over this period.

We also examined the expression of a number of genes previously shown to be influenced by drought stress. The results obtained (Figure 3) indicate that the levels of dehydrin DH1a transcripts increased significantly in the humidity shocked leaves between 30-60 minutes after the start of the low RH treatment, and increased further over the 120 minute treatment (with leaves with a lower water content having higher DH1a levels after 120 minutes, data not shown). These transcripts then decreased during the 24 hour "recovery" period (Fig. 3, A)**.** For the drought-inducible transcription factor DREB1 (Marraccini et al. 2012), low levels of transcripts were detected at T=0 (before RH stress), followed by a strong increase in transcripts 30 minutes after the start of the low RH treatment. DREB1 transcript levels then fell after 60 min, with the levels of both 120 min samples being similar to T=0 levels. After the 24 hour recovery period, DREB1 levels decreased further (Fig 3, Panel B)**.** For the putative drought-induced MYB transcription factor**,** transcript levels were low at T=0 and at T=30 minutes, and then started to rise at 60 minutes and 120 minutes. The transcript levels of this Myb gene then returned to T=0 levels after the 24 hour "recovery" period. (Fig 3, C).

DISCUSSION

The transfer of Robusta coffee plants grown under high humidity conditions to low humidity produced very rapid leaf wilting. This response appears visually similar to the leaf wilting seen during a classical drought stress (soil water deficit) experiment. RWC measurements confirmed the visual observation, that is, after only 15 minutes of humidity stress, the leaves had lost significant amounts of water (38%), presumably due to excessive transpiration via the stomata. Although physiological measurements of CO_2 assimilation and stomatal conductance suggests stomatal closure occurs over the first 15-45 minutes of the stress, this closure appears too slow to avoid a large loss of water after the dramatic change in humidity imposed in our experiments.

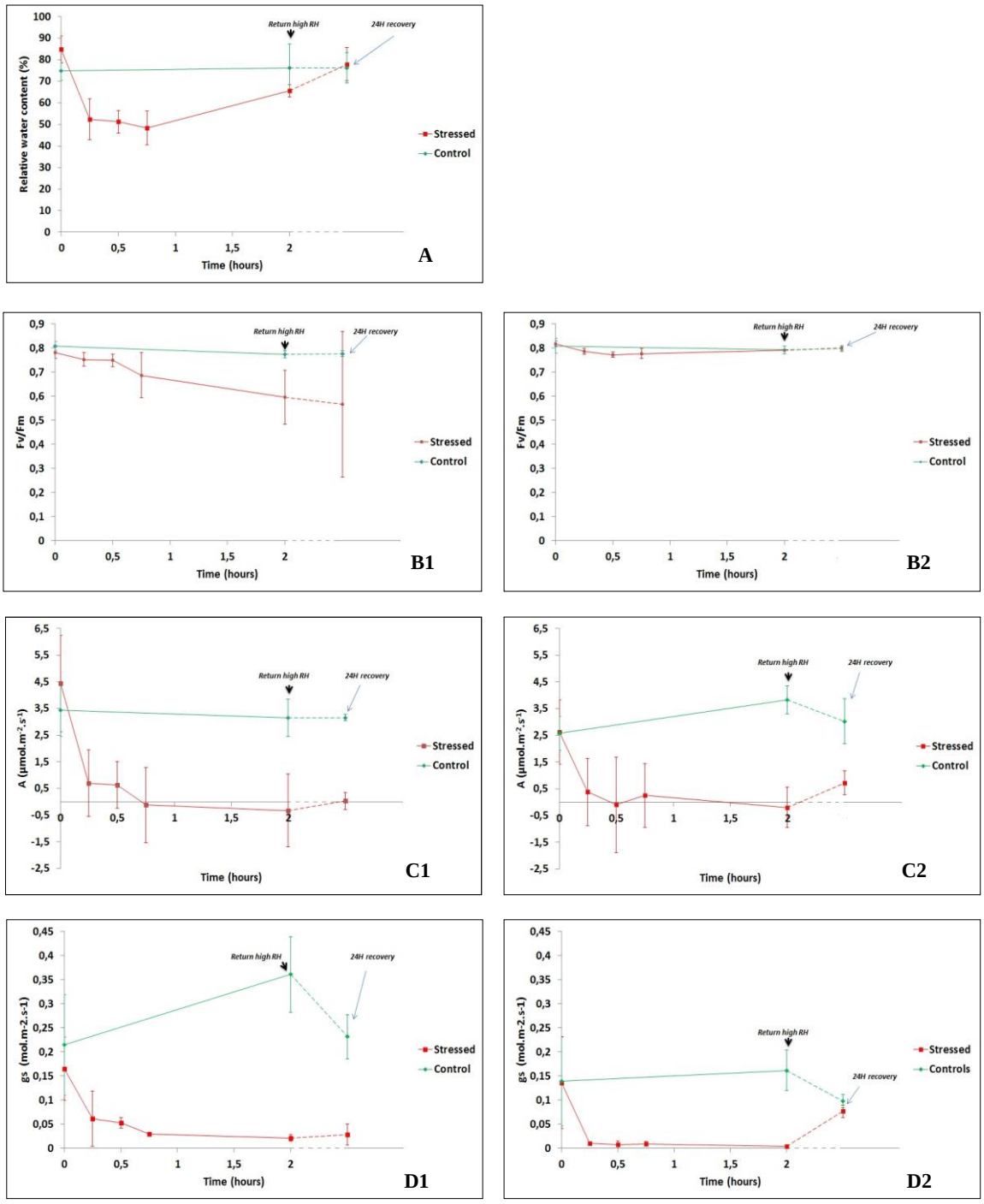

Figure 2. Physiological measurements on Coffea canaphora plants subjected to a humidity shock. Physiological data at different times for leaves of Coffea canaphora (variety FRT 07) plants subjected to a "humidity shock" (RH >85-90% at 25-30°C to RH 30%) and recovery. Measurement times: T=0, T= 15 min, 30 min, 45 min, 120 min, and 24 hours after recovery. The data presented here represents measurements and samples from three independent humidity shock experiments. The red squares/line represent the average values ±SD from 3 -7 treated (stressed) plants, and the green circles/line represent the average values ±SD for 3 plants (controls) that remained under the high the humidity conditions. <u>Panel (A)</u> Leaf Relative Water Content (RWC). PANEL (B1) and PANEL (B1): Leaf Chlorophyll Fluorescence (Fv/Fm) upper and lower leaves respectively. PANEL (C1) and PANEL (C2) CO_2 assimilation (A; µmol CO_2 $.m^{-2}.s^{-1}$) of upper and lower leaves. PANEL (D1) and PANEL (D2) stomatal conductance (g_s; mol H_2O $.m^{-2}.s^{-1}$) of upper and lower leaves.

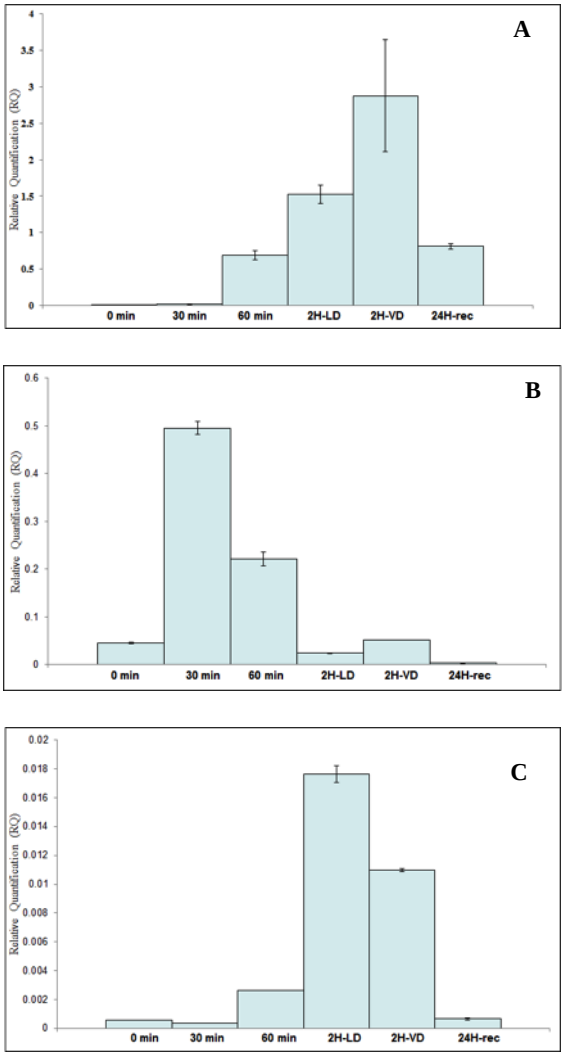

Figure 3. Gene expression alterations during the "humidity shock" Relative expression analysis using QRT-PCR of genes up-regulated in coffee leaves of "humidity shock" treated plants. (PANEL A) Dehydrin 1a (DH1a). (PANEL B): Dehydration-Responsive Element-Binding 1 (DREB1). (Panel C) Myb transcription factor. Gene expression was measured relative to the expression of the ribosomal gene rpl-39. Data presented are average expression values for 2 plants at the times indicated. (2HLD = dry leaves and 2HVD = very dry leaves sampled at 2 hours)

Interestingly, the upper leaves seemed slightly more physiologically active at T=0 than the lower leaves (higher A and g_s), but were more sensitive and, therefore, probably more damaged by the "humidity shock", as seen by the tendency of Fv/Fm values to fall in the upper leaves. We observed much lower changes in Fv/Fm in the lower leaves. It has been noted previously that in the absence watering (soil water deficit), little change in Fv/Fm is observed for coffee leaves until water is witheld for long periods and a high level of stress is reached (Marraccini et al 2012). This supports the notion that the upper leaves are more sensitive to the "humidity shock" and suggests future experiments directed at following individual leaves for Fv/Fm performance and gene expression analysis may uncover some subtle gene expression differences associated with different Fv/Fm values in the same plant. Expression analysis of several genes known to be induced by a classical drought stress in coffee indicated that the DREB1 transcription factor gene (Viera et al 2013) was induced early (after 30 min), and that the DH1a dehydrin gene, encoding a structural protein proposed

to protect cells from stress related to water loss (Hinniger et al. 2006, Brombini Santos and Mazzafera 2012, Radwan et al. 2014) is induced after DREB1. Finally, a gene proposed to encode a drought inducible MYB transcription factor (M Lepelley, C. Perrois, JMcCarthy unpublished data) was seen to be induced after the DH1a gene**.**

The data presented here demonstrates that a large, rapid change in relative humidity (RH) can induce a dramatic stress response in Robusta coffee leaves. Our physiological and gene expression data suggests that a "humidity shock" may mimic the effects of a "classical" and severe drought response in leaves. Future work will determine the relationship between responses to a "humidity shock" stress versus a classical drought stress by doing comparative measurements on FRT07 Robusta plants subjected to both atmospheric and soil water deficits. If the two stress responses have strong similarities, it will be of interest to explore whether coffee varieties showing different drought tolerance properties in the field also show different responses to a large humidity change. Such research could, in the future, enable the utilisation of small immature plants, in combination with a "humidity shock" regime, as a rapid drought tolerance screening tool for identifying coffee plants with enhanced tolerance to water deficits.

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Gene Expression in *C. arabica* with High Tolerance to *Mycena citricolor*

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SUMMARY

In recent years ICAFE of Costa Rica identified coffee plants with high tolerance to Ojo de Gallo, including selections of Villa Sarchí variety. The expression of six genomic sequences related with defense-related proteins were evaluated in tolerant and susceptible Villa Sarchí plants during initial stages of *M. citricolor* infection. The RNA was extracted from leaves collected 0, 4, 8, 12 and 24 hours after inoculation of *Mycena citricolor* and gene expression analysis was made by qPCR (RT-qPCR). Increase in the expression of all the studied sequences was detected during *M. citricolor* infection in the tolerant plant, contrary than the susceptible plant without expression. Higher expression levels were obtained eight and twelve hours after *M. citricolor* inoculation for the PR-1 protein.

INTRODUCTION

Mycena citricolor is the causal agent for Ojo de Gallo disease that causes defoliation of coffee trees and decreases in crop yield. Ojo de Gallo traditionally is controlled by the application of agrochemicals, which has environmental and economic implications for the producer (ICAFE 2009-2011). Genetic improvement by including materials with some tolerance to *M. citricolor* is an important alternative to be incorporated in the integrated control of this disease. ICAFE of Costa Rica in the recent years has been working on the identification of tolerant plants to Ojo de Gallo disease with promising results that include some Villa Sarchí selections with 60% more tolerance than Caturra (ICAFE, 2011).

The identification of genes involved in the defense response against *Mycena citricolor* fungus is important for increase the knowledge about defense mechanism of coffee plants specifically about Coffea - Ojo de Gallo disease interaction, a regional disease poorly studied. Further on genes involved in the process of defense against Ojo de Gallo could be used to identify tolerant materials.

Plants have defense mechanisms against phytopathogens, which involve expression of group of genes and the massive synthesis of molecules that act directly against the infection or indirectly inducing other compounds synthesis. During plant-pathogen interaction, gene expression is modified to respond for the pathogen attack, the susceptibility or resistance are determinate besides the expression of a group of genes by other factors as timing, speed and magnitude of that expression as well as their effectiveness to counteract a specific pathogen infection (Van Loon et al., 2006).

Genes related with plant defense include which codify for proteins induced during pathogenic infection. Defense-related proteins accumulate during infections on infected tissues and around infection points (Van Loon y Van Strien, 1999) and have been used as molecular markers for systemic acquired resistance induction (Ryals y Jones, 1996). PR protein and defense-related proteins were firstly identified in Tobacco (Antoniw et al, 1980) but then in

other crops including coffee. Authors as Guzzo and Martins (1996), Guzzo (2004), (Jesus, 2009) studied PR-proteins induction by different stress types on coffee plants.

The objective for this research was to evaluate the relative expression of genomic sequences related to defense-related proteins during initial stages of *Mycena citricolor* infection on coffee plants.

MATERIALS AND METHODS

Plants

C. arabica plants Villa Sarchí variety of 2 years old were tested against *M.citricolor* in Laboratory conditions and were selected those with higher tolerance (60% higher than Caturra) and greater susceptibility (similar to Caturra).

Pathogen

Mycena citricolor isolation used is the most aggressive against coffee of pathogens collection of Costa Rican Coffee Research Center.

EST sequences of defense-related proteins

Peroxidase, β-1,3 glucanase, chitinase class III, thaumatin, PR-10 and PR-1 induced by different stress types were selected from other publication and Genbank data base program and were standardized the conditions for Real-Time PCR quantification (Applied Biosystems SBRY ® Green PCR Master)

Plants were inoculated with *Mycena citricolor* on Laboratory conditions, in a humidity chamber with 21°C temperature, 100% relative humidity and micro-sprinkler irrigation every hour. Two plants were inoculated, one with high tolerance to Ojo de Gallo and other with high susceptibility. Ten leaves of each plant were inoculated with four "gems" of *Mycena citricolor* each. Leaves were collected before inoculation and 4, 8, 12 and 24 hours after *M.citricolor* inoculation. The leaves were frozen by submersion on liquid nitrogen and the ARN was extracted. Relative expression of six genomic sequences in tolerant and susceptible plant was carried out by Real Time PCR System (*Step One Plus, Applied Biosystems*) and the Software Step One v2.2.2 (*Applied Biosystems*).

RESULTS

Expression of the sequences studied increased during *M. citricolor* infection in the tolerant plant contrary in the susceptible plant no expression was observed. Higher levels of expression were obtained eight and twelve hours after inoculation of *M. citricolor* and were higher for PR-1 protein (Figure 1-2).

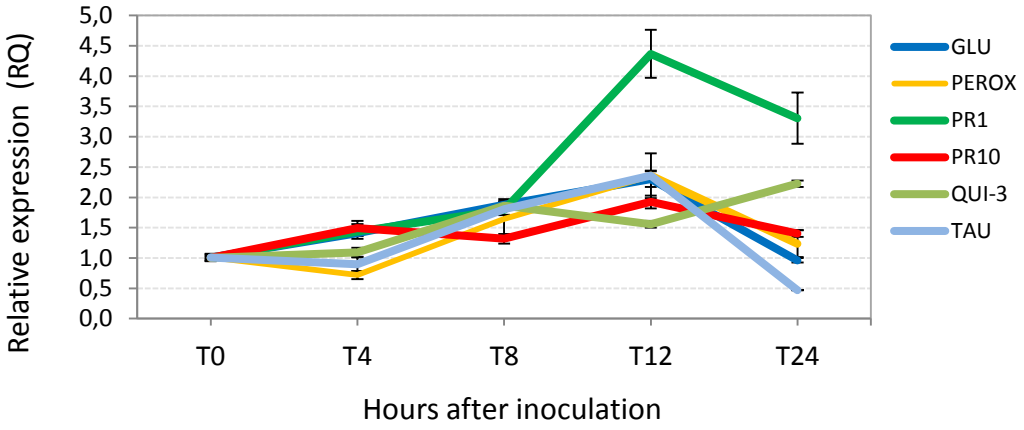

Figure 1. Relative expression of β-1,3-glucanase (GLU), peroxidase (PEROX), PR-1, PR-10, chitinase class III (QUI-3) and thaumatin (TAU) in tolerant plant, 24 hours *after M. citricolor* inoculation.

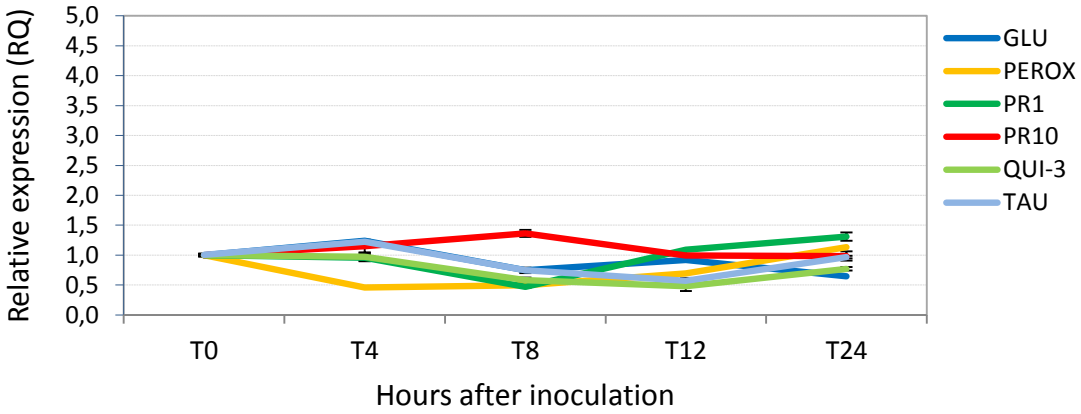

Figure 2. Relative expression of β-1,3-glucanase (GLU), peroxidase (PEROX), PR-1, PR-10, chitinase class III (QUI-3) and thaumatin (TAU) in susceptible plant, 24 hours after *M. citricolor* inoculation.

The activation of genomic sequences of defense-related protein is consistent with plant tolerance against Ojo de Gallo. Stereoscopic observations allow to visualize that in tolerant plants only between 10-20% of inoculated points develop lesions and the signs of the disease were visible just 30 hours after inoculation. While in plants with high susceptibility to this disease (Caturra and Catimor) these signs are observed 12 hours after inoculation, and furthermore between 80-100% of inoculated points formed a lesion (Robles, 2014). The delay in the disease symptom manifestation in tolerant plants may be related to the rapid activation of the defense result of expression of genes involved in defense against this pathogen, as those analyzed on this study. Defense-related proteins have diverse functions, peroxidase activity is related with death of cell surrounding pathogen penetration points during hipersensibility response (Silva et al., 2006), chitinases and glucanases hydrolyzed polymers of N-acetyl-glucosamine, chitin and glucan components of the cell walls of fungi (Farkas, 1979), and also thaumatin is able to hydrolyse polymers of β-1,3-glucan (Grenier et al. 1999). Moreover PR-10 protein with nuclease activity related with death of cells near to pathogen sites of infection (He et al., 2012, Xu et al., 2014). And PR-1 protein has unknown function but normally this protein is used as indicator of SAR induction (Van Loon y Van Strien, 1999). In this study was observed increase on expression of those enzymes during initial stages of Ojo de Gallo infection and mainly PR-1 protein with an increase of 4-fold initial level.

CONCLUSION

The increased level of expression of the genomic sequences of defense-related proteins during early stages of *Mycena citricolor* infection, are evidence of plant defense against this pathogen and appear to relate to the tolerance showed by C. arabica plants. This tolerance was observed as a lower amount of lesions on inoculated leaves as well as a delay of first signs of the disease. Variable expression of PR-1 protein and other defense-related proteins could be used as a molecular marker of tolerant coffee plants to Ojo de Gallo disease, reducing the time required for selection of tolerant plants; however this information should be confirmed by other studies.

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Aquaporins in *Coffea arabica* L.: Physiological Activity and Gene Expression in Roots and Leaves, Under Different Light Intensities and Water Status

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SUMMARY

Plant aquaporins (AQPs) belong to a large superfamily of conserved membrane proteins called major intrinsic protein (MIPs), involved in the membrane transport of water and other small solutes. Aquaporins are known to play major roles in the regulation of plant water balance and transport, as well as in growth regulation and response to abiotic stress factors. To the best of our knowledge, no information is available in the literature about the presence and role of AQPs in *C. arabica*. Our research identified candidate AQP genes by screening a proprietary *C. arabica* transcriptome database, resulting in the selection of eight putative aquaporins. A phylogenetic analysis was performed using previously characterized MIPs from *Arabidopsis thaliana* and *Solanum tuberosum*, specifically assigning the coffee sequences to the Tonoplast (TIP) and Plasma membrane (PIP) MIP's subfamilies.

After bioinformatic analysis, the possible functional role of putative Arabica AQPs was explored by means of physiological and biomolecular experiments. Hydraulic conductance and aquaporin gene expression were analyzed on leaf and root tissues of two-year-old plants (*C. arabica* cv. Pacamara), under two different experimental conditions.

The first experiment tested the plants before dawn and at mid-day, to verify the influence of light and photosynthesis on AQP activity. In a second experiment, we compared plant hydraulic response to different water stress level as eventually affected by changes in aquaporin expression levels.

The results shed the first light on the possible roles of aquaporins in the regulation of *C. arabica* water balance, opening a new field of research that could become increasingly important for the sustainability of coffee cultivation in a scenario of global climate change.

INTRODUCTION

Plant aquaporins (AQPs) belong to a large superfamily of conserved membrane proteins called major intrinsic protein (MIPs), involved in the membrane transport of water and other small solutes. Aquaporins are known to play major roles in the regulation of plant water balance and transport, as well as in growth regulation and response to abiotic stress factors (Kaldenhoff *et al* 2008). To the best of our knowledge, no information is available in the literature about the presence and role of AQPs in *C. arabica*. Our research identified candidate AQP genes by screening a proprietary *C. arabica* transcriptome database, resulting in the selection of eight putative aquaporins. Considering that this is the first study on coffee

AQPs, we also investigated *in vivo* the two major environmental factors influencing plant water status (light and water availability).

MATERIALS AND METHODS

The analysis of physiological parameters and of aquaporin gene expression were made on leaves and roots of 2-year old *C. arabica* cv Pacamara, grown in the greenhouse of the University of Trieste (Italy).

The plants were subjected to two distinct experimental conditions. One experiment investigated the response of plants to light intensity: on consecutive days, plants were sampled either before dawn or at mid-day. Physiological measurements (see below) were taken, and total RNA was immediately extracted from samples of root and leaf tissue, for subsequent gene expression analysis.

The second experiment tested the effect of drought stress on plant hydraulic parameters and AQP expression levels. Three groups of 15 plants each were subjected for 20 days to different irrigation conditions: 100 ml water/day (fully irrigated control), 25 ml water/day (mild water stress) and 10 ml water/day (severe water stress). After the test period, physiological parameters were measured and RNA samples taken, in the same way as the previous experiment.

For all samples, the following physiological parameters were recorded: plant hydraulic conductance (with a High Pressure Flow Meter); leaf conductance to water vapor (gL; mmol $m^{-2}s^{-1}$) with a porometer (Leaf Porometer, Decagon Device Inc.) and leaf water potential (ψL, MPa) with a pressure bomb (Soil moisture mod. 3005).

AQP gene expression analysis was conducted as follows: total RNA was isolated from leaves and roots (adaptation of the CTAB protocol by Chang *et al*, 1993) and cDNA was synthesized. Real time quantitative expression analysis (RT-PCR) was performed using SsoAdvanced Universal SYBR Green Supermix (BioRad, Hercules, CA, USA). The Sigma Plot 11.0 (Systat Software Inc.) and SigmaStat 2.03 (SPSS Inc.) softwares were used for statistical analysis. Statistically significant differences were highlighted by the use of one-way ANOVA or t-test.

RESULTS AND DISCUSSION

No significant hydraulic response to variations in light intensity was detected, in both leaves and roots of Pacamara plants (data not shown). This could be a result of the shade adaptation typical of *C. arabica*.

Instead, the values of all physiological parameters decreased with increasing levels of drought stress. (Fig. 1, 2)

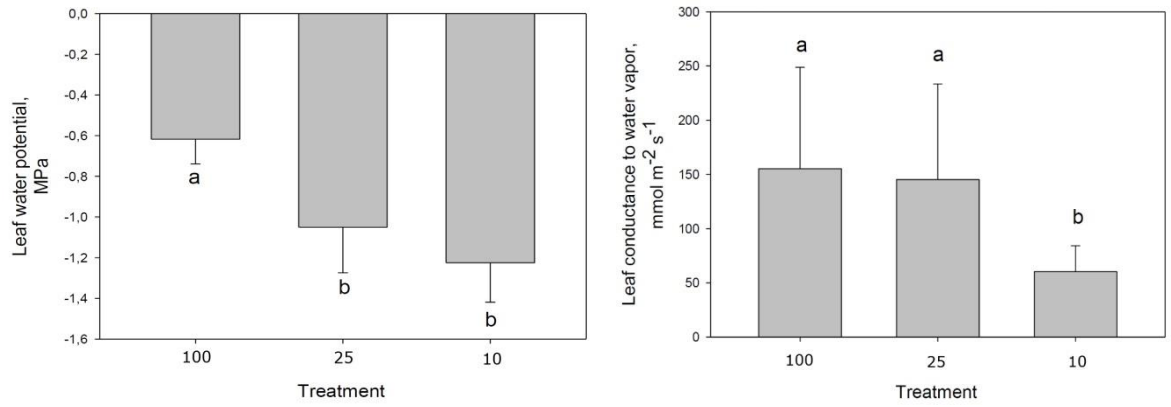

Figure 1. Leaf water potential and leaf conductance to water vapor in 3 different water stress regimes (100, 25 and 10 ml water/day).

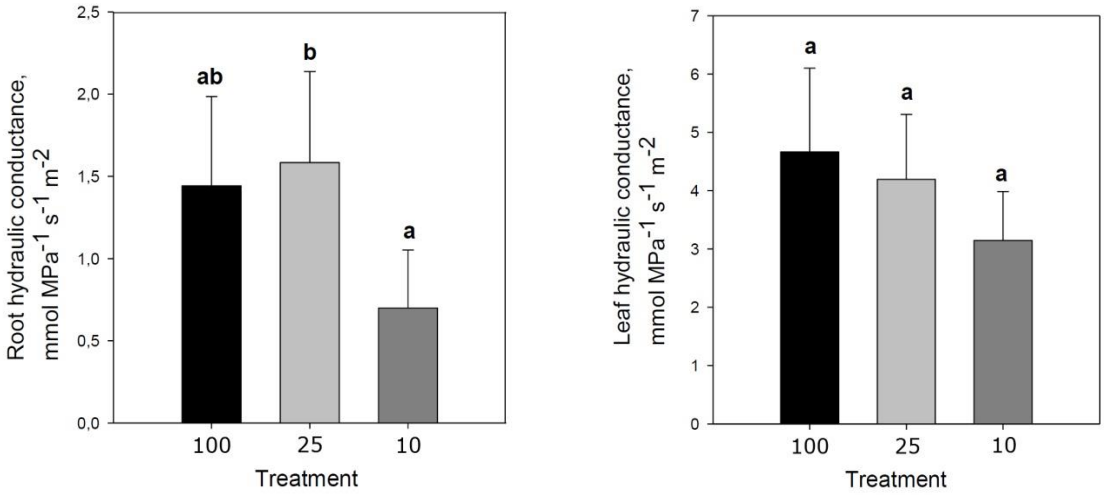

Figure 2. hydraulic conductance of root and leaf in 3 different water stress regimes (100, 25 and 10 ml water/day).

In the roots, all AQP genes (except AQP8) showed decreased expression levels with the decrease of hydration status (Fig.3).

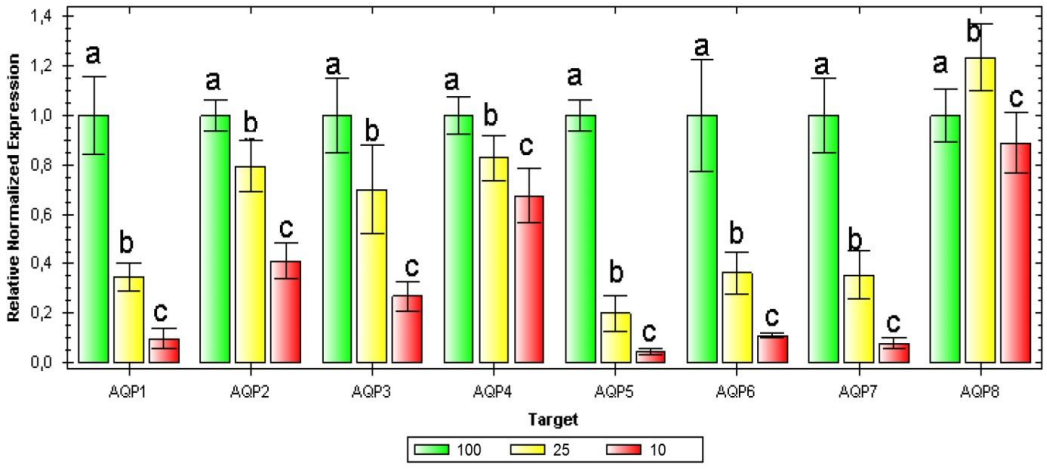

Figure 3. AQPs gene expression in root.

In leaf tissue, instead, different behaviours were observed among different aquaporin transcripts (Fig.4). In particular, AQP1, AQP2 and AQP4 showed strong correlation between their gene expression levels in leaves and the measures of relative hydraulic conductance and relative water potential (data not shown). This finding is supported by bioinformatic analysis that putatively classified these three transcripts as water channels.

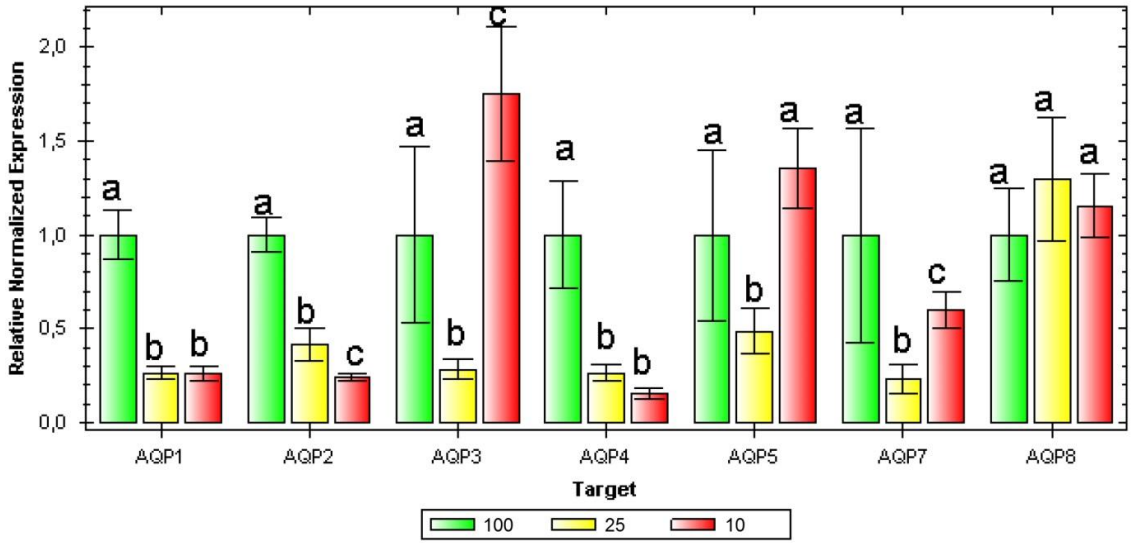

Figure 4. AQPs gene expression in leaf.

CONCLUSION

The global and regional modifications of temperatures and precipitation patterns are challenging several crops in different areas of the globe. Coffee is one of the most threatened crops, and a recent study has shown that even under the most conservative climate change scenarios, the extension of localities favourable for the persistence of coffee populations might decrease by 65% within 2080 (Davis *et al*. 2012). This finding is particularly alarming, as coffee represents an important basis for the economy of several developing countries.

This is the first study investigating coffee aquaporins, and their involvement in the response of the plant to environmental stresses. This information might help in better understanding the physiology of the coffee crop and in identifying cultivars best suited to the changing climatic conditions.

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Development of a Male Sterility Based Reproductive System to Ensure a Cost Effective and Massive Propagation of New Outstanding F1 *Arabica* Hybrids by Seed

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SUMMARY

Arabica as self-pollinated species is mainly disseminated as homozygous varieties (pure lines) and can be reproduced by seed. Hybrids often present genetic and agronomic advantages: higher and more stable yields, homogeneity, speed of combination of favorable dominant genes in the same genotype. In Arabica, breeding strategies in view of developing F1 hybrid cultivars have been adopted in Ethiopia, East Africa and Central America.

In this study, we developed a reproductive and innovative method to produce by seed Arabica F1 hybrids using male sterility.

INTRODUCTION

Once a new 'improved' variety is created, it has to be maintained and propagated, and disseminated to potential users. The modalities for such maintenance and propagation depend, on the one hand, on the reproductive pattern of the species (sexual self-pollinated, sexual cross-pollinated, asexual) and, on the other, on the variety's genetic structure (homozygous and stable over generations of sexual propagation or heterozygous and unstable). Dissemination modalities also have to include an economic angle, as it is usually through the sale of seeds that the investment made in the plant breeding activity can be recovered.

Arabica as self-pollinated species is mainly disseminated as homozygous varieties (pure lines). Because of their stability over generations, these varieties can be reproduced from year to year on the farm.

Heterozygous genetic structures often present genetic and agronomic advantages: higher and more stable yields, homogeneity, speed of combination of favourable dominant genes in the same genotype, etc. They encourage breeders to increasingly use hybrid varietal formulas, even for self-pollinated species. In Arabica, the advantages of hybrid cultivars were demonstrated in the complex hybrid cultivar "Ruiru 11". Then breeding strategies of developing hybrid cultivars of *Arabica* coffee have been adopted in Ethiopia, Central America and Tanzania. Here we present the development of a reproductive technique of F1 hybrids based on the use of malesterility. We show how this mutant can be used for the seed production of new F1 hybrid varieties.

MATERIALS AND METHODS

We discovered a natural mutant (named CIR-MS01) without pollen in a population of 20 000 wild trees from Sudanese Ethiopian origin The plants were reproduced through tissue culture via somatic embryogenesis.

The male sterile genetic determinism was studied on four F2 population respectively derived from four F1 hybrids obtained by manual pollination between Caturra, Catuai, T5296 *(Sarchimor line)* and IAPAR *(Sarchimor line)* as elite lines pollen donor and CIR-MS01.For this experiment more than 1300 F2 plants were studied in the field for sterile male character segregation.

Then, other four combinations of hybrids with recurrent CIR-MS01 male sterile were tested this time with natural pollination conditions in Nicaragua and Mexico in two field plots (respectively CIR-MS01 x Caturra, x Naryelis *(Colombian line)*, x IAPAR59, x Marsellesa (*Sarchimor line*)).

The proportion of sterile male and elite varieties plants tested to establish the seed gardens was one donor pollinating for eight sterile male plants. The purpose of the experiment was to show that natural pollination was possible to produce F1 hybrid seed.

At least, the best F1 hybrid (CIR-MS01 x Marsellesa) combination was planted in a plot of 25 000 plants to produce coffee in 2011.

RESULTS AND DISCUSSION

Male sterile genetic determinism

Table 1.

F1 Hybrid	Total of F2 descendants	Number of sterile male observed in F2 descendant	% of F2 sterile male mutant
CIR-MS01 x Caturra	321	7	2.18% [a]
CIR-MS01 x Catuai	365	17	4.66% [a]
CIR-MS01 x T5296	386	66	17.10% [b]
CIR-MS01 x IAPAR59	314	39	12.42% [b]

χ^2test. P<0.01

The F2 populations are a mixture of sterile and fertile plants.Segregation of the character ranges from 3.5% for Caturra and Catuai to 15% for T5296 and IAPAR59. The genetic determinism of such male sterility seems to be more epigenetic than mendelian and could be dependent of the genetic background.

Hybrid combinations tested

From the four combination tested in two conditions, the best cross was the F1 population « CIR-MS01 x Marsellesa » .This population produced 20-30% more than Marsellesa and presented a standard to good beverage quality with rust and CBD resistance. All plants were fertile.

Proportion of pollen donor vs elite variety to set up seed garden (Figure 1a)

An experimental seed garden (2000 trees), isolated from alien pollinic contamination was established with 'Marsellesa' cv. as a pollen donor. Experiments showed that the number of pollen donors (1 donor pollinating for 8 sterile male plants) was not sufficient to well pollinate the sterile male trees. Indeed, the hybrid seed production was increased by more than 40% when additional pollen was manually applied. We thought that a proportion of 1 donor for 4 sterile male trees would be convenient for a fully efficient natural pollination

Set up of F1 productive parcel with "CIR-MS01 x Marsellesa" combination's seed (Fig. 1b)

The hybrids produced in « seed garden » were then planted in a plot of 25000 plants. The farm trial was planted in late 2011 and came into first commercial production in 2013.As mentioned above, plants are more vigorous than Marsellesa and have a good production (up to 30% more than the progenitor line) but we observed a segregation of characters up to 25% which was confirmed by SSR markers. This segregation appears mainly related to remanent heterozygous level in Marsellesa cv. and in the CIR-MS01 clone. Indeed, based on SSR markers and SNP markers we estimated that heterozygosity of Marsellesa was about 15% and that of clone MS was over 25%.

So, to provide a stable and consistent F1 hybrid population, completely homozygous maternal lines should be used. This work is underway for IAPAR59, Marsellesa and Caturra.

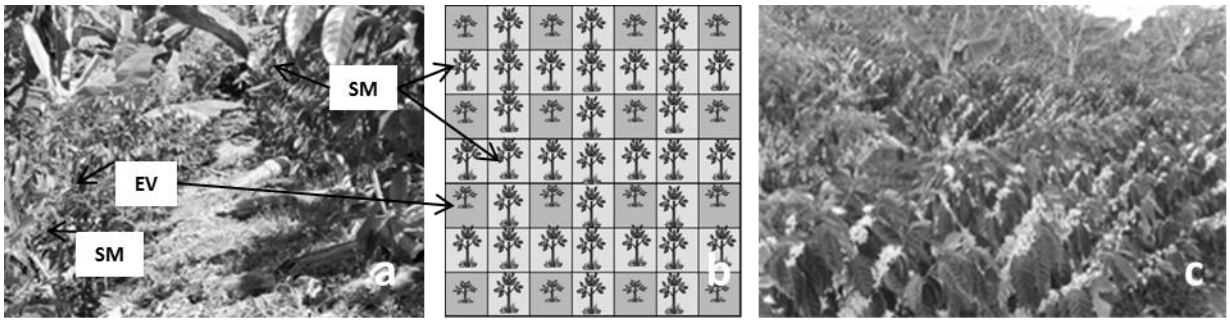

Figure 1. a) First experimental seed garden using male sterility strategy established in Nicaragua since 2009; b) Scheme of planting "Elite variety (EV)" x "Sterile male (SM)" to produce Arabica hybrid seeds: 1 pollen donor variety pollinates 8 sterile male plants; c) First flowering for CIR-MS01 x Marsellesa cv. plots in 2014.

CONCLUSION

We discovered a stable sterile male genitor (CIR-MS01) which confers strong hybrid vigor when it is crossed with traditional Arabica American lines. The cross of CIR-MS01 with Marsellesa (*a Sarchimor line*) provides a hybrid population whose performances are highly superior to those of Marsellesa control. We demonstrated that the establishment of seed garden under natural pollination is possible. A proportion of 1 donor for 4 sterile male trees is convenient for a fully efficient natural pollination. Due to heterozygosity of CIR-MS01, the crosses made with this parent are hybrid populations. The donor should be close to full homozygosity to prevent segregation for important agronomical traits such as tree phenotype and rust and CBD resistance. In Arabica, as it does not exist any true commercial pure lines, it

appears essential to auto-pollinated at least two or three time before using commercial lines as pollen donors.

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Embryogenic Potential of Hybrids Originated from *Coffea arabica* (Caturra X Timor Hybrid) X Wild Ethiopian Accessions Crosses (Pb212)

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SUMMARY

Embryogenic potential is a key trait for the multiplication of promising hybrid genotypes of coffee for experimental and commercial purposes. Therefore, the embryogenic potential was determined in 29 two year-old hybrids resulting from crosses between 10 wild *C. arabica* accessions from Ethiopia with 3 maternal lines (CU1842, CX2385 and CX2848) obtained from crosses between *C. arabica* var. Caturra and the Timor Hybrid. First, 100 to 200 leaf explants from each hybrid were planted on NPC media (NPC salts, 2.4D and 2IP); one month later, the explants were transferred to Embryogenic Tissue (ET) inducer medium (MS salts, 2,4D and BAP), with ET regeneration starting after six months, followed by subculture of regenerated tissues in either Liquid Proliferation Medium (MS salts, 2,4 D and Kinetin) or Embryo Liquid differentiation Medium (MS salts, ANA and Kinetin).

ET regeneration as well as embryo and plantlet production were observed in 65% of the hybrids planted (19 out of 29). Hybrids derived from the maternal line CX2848 showed the highest regeneration potential, with 8 out of 8 derived hybrids regenerated. Next was line CU1842, with 6 out of 10 hybrids showing regeneration, and last was line CX2385, where only 5 out of 11 hybrids have regenerated. These results are probably associated to the maternal line ancestry and to the introgression level from the Timor Hybrid, where line CX2385 had the largest introgression levels as determined by microsatellite markers, followed by line CU1842, both lines sharing a common ancestor at the F1 generation. The smallest introgression levels were present in line CX2848, that comes from an unrelated cross.

INTRODUCTION

Embryogenic potential is a key trait for the multiplication of promising hybrid genotypes of coffee for experimental and commercial purposes. The objective of this work was to determine the embryogenic potential in 29 two year-old hybrids resulting from crosses between 10 wild C. arabica accessions from Ethiopia with 3 maternal lines (CU1842, CX2385 and CX2848) obtained from crosses between C. arabica var. Caturra and the Timor Hybrid (Table 1).

Table 1. Material identification.

TYPE	MATERNAL	PATERNAL	MATERNAL	PATERNAL	MATERNAL	PATERNAL
	LYNE	LYNE	LYNE	LYNE	LYNE	LYNE
MOTHER	CU1842		CX2385		CX2848	
HYBRID	CU1842	E554	CX2385	E554	CX2848	E554
HYBRID	CU1842	E286	CX2385	E286		
HYBRID	CU1842	E057	CX2385	E057	CX2848	E057
HYBRID	CU1842	E054	CX2385	E054		
HYBRID	CU1842	E114	CX2385	E114	CX2848	E114
HYBRID	CU1842	E047	CX2385	E114 (2)		
HYBRID	CU1842	E069	CX2385	E047	CX2848	E047
HYBRID	CU1842	E291	CX2385	E069	CX2848	E069
HYBRID	CU1842	E464	CX2385	E291	CX2848	E291
HYBRID	CU1842	E501	CX2385	E464	CX2848	E464
HYBRID			CX2385	E501	CX2848	E501

MATERIALS AND METHODS

Explants

During the months of January to March of 2013 leaves from all the materials were collected. For each plant 5 to 10 young leaves from the second pair of the principal or secondary branches of the trees were taken. Once in the laboratory the leaves were disinfected by washing them with liquid soap and tap water. Then 5 leaves per plant were immersed in a 500 ml of commercial Sodium hypochlorite solution diluted at 50% for 15min stirred at 40 rpm. Then the solution was replaced for sterile distilled water. The leaves were left in water for 5 min and two more water changes were done. Then, the water was eliminated and under the laminar flow hood the leaves were dried out with sterile napkins and cut in order to obtain the explants. From each leaf between 10 to 20 explants pieces of 1 x 1 cm were obtained. Each explant contained part of the secondary nervure and both the principal nervure and the leaf border were eliminated.

Culture Media

For each plant, 100 explants were placed in the solid NPC induction media, with ten explants for petri dish, and they were incubated under dark conditions at 24°C. After one moth, explants were subcultured to solid Embryogenic Tissue (ET) induction medium (Figure 1). The materials were observed each 15 days and, if contamination was higher that 30%, the material was collected again. ET regeneration starting after six months in some of the samples (Figure 2). Once the ET was formed it was subcultured (Figure 3) in either Liquid Proliferation ET Medium (Figure 4) or Liquid Embryos regeneration Medium (Figure 5). Tissues were subcultured to the same medium every 15 days. Once the embryos were formed they were transferred to solid plant germination medium (Figure 6). Table 2 shows the media composition (Murashige & Skoog 1962. Murashige 1974. Samson et al., 2006. van Boxtel et al., 1996. Acuña personal communication).

Table 1. Media composition

Media Components	NPCM Induction Medium/L (PGR1 modified. Samson et al 2006)	ET Induction Medium /L (modified van Boxtel et al 1996)	Liquid Proliferation ET Medium/L (CP modified. van Bostel et al 1996)	Liquid Embryos Regeneration Medium/L (Acuña personal communication)	Plant Germination Medium /L
Macro/ Micro nutrients NPC	(1X)				
Macronutrients M&S Murashigue and Skoog		(1/2 X)	(1/2 X)	(1/2 X)	(1/2 X)
Na2 EDTA_ Ferrous Sulfate (37.3mg /ml EDTA, 27.8 mg/ml Ferrous Sulfate)	1.25 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml
Thiamine 10 mg/ml	1.5 ml	2.0 ml	0.5 ml	1.0 ml	1.0 ml
Nicotinic Acid 1 mg/ml	1.0 ml		0.5 ml		1.0 ml
Potassium Iodine 0.42 mg/ml	-	1 ml	1.0 ml	1.0 ml	1.0 ml
Pyridoxine HCL 1 mg/ml	1ml		0.5 ml		
Potassium Nitrate				2.85 g	
2,4D 0.5 mg/ml	1 ml	1 ml	4.0 ml		
2IP 1mg/ml	1 ml				
BAP 1 mg/ml		8 ml			
Kinetin 1 mg/ml			2.0 ml	0.5 ml	
ANA 1 mg/ml				50 ml	
Inositol	0.13gr	0.2 g	0.05 g	0.1 g	0.1 g
Cysteine		0.04 g	0.1 g	0.037 g	
Adenine Sulfate		0.06 g			
Glycine 20 mg/ml		1ml			
Casein hydrolase		0.2 g	0.1 g		
Malt Extract		0.8 g	0.2 g		
Sucrose	30 g	30 g	15.0 g	15.0 g	20 g
Gel Rite	3gr	3gr			7.0 g
pH	5.6	5.6			

RESULTS AND DISCUSSION

The regeneration of the ET started after 6 months in some of the materials. The first regenerated hybrid corresponded to CU1842xE286, followed by CU1842xE501, CX2848xE069, CX2848xE464, CX2848xE554 and CX2848xE291. Non-differentiated tissue was transferred to Liquid Proliferation Medium after the formation of the ET and embryos. On the other side, tissue already forming pre-embryos was transferred to Embryo Liquid differentiation Medium. Once the embryos started to develop roots in the liquid medium, they were transferred to solid Plant Germination medium were plantlets grew. Table 3 shows the regeneration capacity of the materials. The results indicated that population 3, Hybrids derived from the maternal line CX2848, had the higher regeneration potential (100%). From eight different CX2848 lines evaluated, all of them regenerated, showing the largest number of embryos and plantlets regenerated so far. In addition, the mother CX2848 is the only one that has regenerated ET until now. Next was line CX2385, where 9 out of 11 hybrids have regenerated (82%), and last was line CU1842, with 6 out of 10 hybrids (60%) showing regeneration.

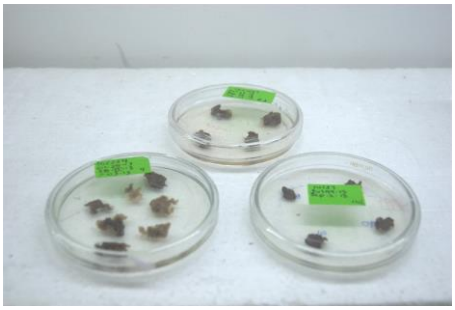
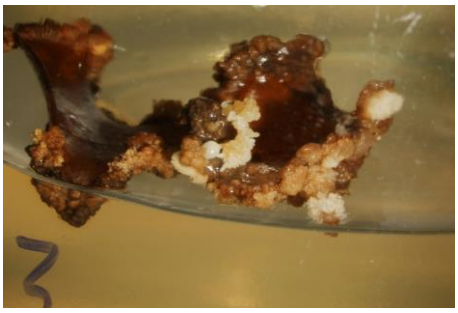
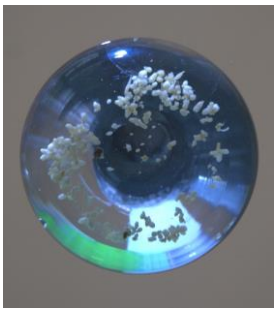


Figure 1. (left) Explants in Embryogenic Tissue (ET) induction medium.
Figure 3. (right) Embryogenic tissue in Embryo Liquid Medium





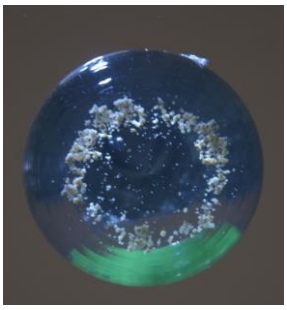


Figure 2. (left) Embryogenic tissue regenerated from CX2385xE114 after 6 months in induction medium.
Figure 4. (center) Embryogenic tissue in Liquid Proliferation Medium
Figure 5. (right) Embryos in Liquid Embryo Differentiation Medium

Figure 6. Embryos and plants in solid plant Germination Medium

These results are probably associated to the maternal line ancestry and to the introgression level from the Timor Hybrid, where the smallest introgression levels were present in line CX2848, that comes from an unrelated cross, compared with line CX2385 had the largest introgression levels as determined by microsatellite markers (Fig. 7), followed by line CU1842, both lines sharing a common ancestor at the F1 generation.

Table 3. Regeneration capacity of the crosses between 10 wild C. arabica accessions from Ethiopia with 3 maternal lines (CU1842, CX2385 and CX2848) obtained from crosses between *C. arabica* var. Caturra and the Timor Hybrid.

FAMILY	REG. TYPE	PROL.	REG.	N° EMBRYOS	N° PLANTLETS
CU1842	NO				
CU1842xE554	**NO**				
CU1842xE286	**YES**	21	15	**14**	**13**
CU1842xE057	**NO**				
CU1842xE054	**NO**				
CU1842xE114	**YES**	2			
CU1842xE047	**YES**	2	2	**9**	**9**
CU1842xE069	**YES**	2	3	**26**	
CU1842xE291	**NO**				
CU1842xE464	**YES**	6	7		
CU1842xE501	**YES**	2			
CX2385	**NO**				
CX2385xE554	**YES**	1			
CX2385xE286	**YES**		6		
CX2385xE057	**YES**	5	6		
CX2385xE054	**NO**				
CX2385xE114	**YES**	8	16	**65**	**85**
CX2385xE114	**YES**	1			
CX2385xE047	**YES**	4	9		
CX2385xE069	**YES**	5	10	**4**	**2**
CX2385xE291	**YES**	2	2		
CX2385xE464	**NO**				
CX2385xE501	**YES**	11	9		
CX2848	**YES**	5	2		
CX2848xE554	**YES**	5	16	**38**	**105**
CX2848xE057	**YES**	11	9		
CX2848xE114	**YES**	1	2	49	52
CX2848xE047	**YES**	1	4	**30**	**71**
CX2848xE069	**YES**	1	1	**8**	**17**
CX2848xE291	**YES**	12	18	**65**	**124**
CX2848xE464	**YES**	24	16	**18**	**66**
CX2848xE501	**YES**		8	108	74

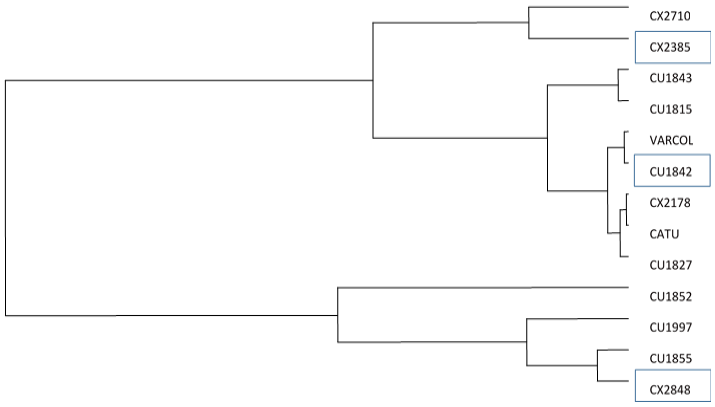


Figure 7. Dendrogram showing the relationship among the parental lines using microsatellites markers

ACKNOWLEDGEMENTS

AIRD for funding in France and the Administrative Department of Science, Technology and Innovation - Colciencias for project financing BASES GENETICAS Y MOLECULARES Y PREDICCIÓN DE LA HETEROSIS EN UNA ESPECIE ALOPOLIPLOIDE AUTOGAMA (coffea arabica L) No. RC-0148-2013.

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Molecular Marker for Fingerprinting of Brazilian Coffee Cultivars

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SUMMARY

Every year new improved *Coffea arabica* cultivars are released to meet market demands. However, with the narrow genetic base of this species, current cultivars are phenotypically close. This fact hinders a precise differentiation of these materials by morphological descriptors in distinctness, uniformity and stability tests (DUS) required for the registration of a new cultivar. In this sense, molecular markers can discriminate more accurately and safely cultivars which are intended to be registered and/or protected. Thus, this study aimed to establish a set of microsatellite markers for molecular characterization (fingerprinting) of coffee cultivars. Thirty-four Brazilian cultivars/progenies of *C. arabica*, which belongs to the National Coffee Cultivars Trial, were accessed. From 31 primers microsatellites analyzed, 27 amplified DNA, and 20 polymorphic markers were used to establish molecular profiles of cultivars. Results showed that microsatellite markers are able to differentiate related genotypes, which are phenotypically close, and it may help in the DUS test of cultivars. The molecular profile of the cultivars/progenies in study was established.

INTRODUCTION

There are currently 127 *C. arabica* registered cultivars in Brazil, which combine high yield and vigor, excellent cup quality and adaptation to several soil and climatic conditions. Among these, 71 are resistant or tolerant to coffee rust, which is considered to be the most important disease in this crop.

New cultivars can only be registered if they are distinct, uniform and stable (DUS). Morphological descriptors have been used for DUS test for cultivar registration in Brazil. However, commercial cultivars are increasingly coming phenotypically closer. This hinders a precise discrimination of these materials by such descriptors.

Finding out new and alternative descriptors that allow, in a rational and practical way, to discriminate the different coffee cultivars will be fundamental in the breeding programs of this species. This strategy can help to avoid problems with their registration and with the Plant Variety Protection Law.

Molecular markers may complement morphological descriptors for this species, since they have allowed identifying, with precision, the genetic variations directly into the DNA of an organism, and therefore has been used since 2009 by the National Service for Plant Variety Protection in Brazil. Therefore, this study aimed to establish a set of microsatellite markers for molecular characterization (fingerprinting) of coffee cultivars.

MATERIALS AND METHODS

Twenty-six cultivars and five elite progenies resistant to rust and three susceptible cultivars were studied. Out of the three susceptible cultivars, two are Catuaí, belonging to the group of the most planted cultivars in Brazil, and the other is Bourbon, considered to be standard for good cup quality. The 34 coffee plants were selected for the study due to their importance, and for being difficult to phenotypically discriminate. These 34 cultivars/progenies comprise the National Coffee Cultivar Trial, which justifies the importance of these materials in Brazil. These trials are set in the main coffee producing regions in Brazil and studies are ongoing.

For laboratory analysis, fully developed and young leaves of each cultivar/progeny were collected. Genomic DNA was extracted according to the protocol described by Diniz et al., and amplified with 31 microsatellite markers, following the methodology described by Missio et al.. The products of PCR reaction were separated by electrophoresis in 6% denaturing polyacrylamide gel, and visualized by silver nitrate staining, according to the protocol described by Brito et al..

Data were coded as codominant. Thus, if locus presented four alleles, there was the representation 1, 2, 3 and 4 for the homozygous forms, and 12, 13, 14, 23 and 24 for heterozygous forms. The analysis of the cultivars profiles was carried out using GENES software. Only polymorphic primers were considered in this study.

RESULTS AND DISCUSSION

Six plants were analyzed for each cultivar/progeny. A total of 31 primers were analyzed in 34 genotypes, of which four showed no sharp bands and therefore were discarded. Out of the remaining ones, 20 markers were polymorphic.

With 20 polymorphic microsatellite markers, 47 bands were amplified. It was observed an average of 2.35 bands per primer, ranging from two to four bands. The percentage of polymorphic primers was 74.04%. This high percentage does not indicate high variability among cultivars/progenies, since the primers used in this study were selected for being polymorphic in other previous studies. In a study with F_2 population resulting from a cross between Catuaí Amarelo IAC 64 (UFV 2148-57) and Híbrido de Timor UFV 443-03, it was obtained 7.34% of polymorphism in 286 SSR primers. Reduced number of polymorphic SSR loci for *C. arabica* was also observed by Vieira et al..

The average number of bands obtained per primer (2.35) indicated that there was narrow genetic base among cultivars/progenies. This can be explained by the low number of plants introduced in Brazil, which are the genetic basis of current cultivars. Recent studies have shown that the genetic basis of 121 cultivars released in Brazil between 1939 and 2009 is due to only 13 different parents. These authors also found that seven parents contributed with 97.55% of the genetic basis of *C. arabica* cultivars in Brazil.

Based on polymorphic markers, molecular profiles of cultivars/progenies were obtained from the individual data of the genotypes that constitute them (Table 1). The analysis of the pattern of bands enabled to observe polymorphism between all cultivars/progenies and segregation between and within cultivars. Thus, some cultivars/progenies had more than one genotype per locus. In this case, in the construction of the molecular profile, all genotypes presented by cultivar/progeny were considered.

Eight cultivars/progenies (Catuaí Vermelho IAC 144, Catuaí Vermelho IAC 15, Bourbon Amarelo UFV535, Catucaí Vermelho 20/15, Sabiá tardio, Obatã IAC 1669-20, IPR 103 and H 419-3-3-7-16-4-1) had no segregation between individuals within cultivar, in any of the 20 loci analyzed. In four cultivars (IAC 125 RN, IPR 99, IPR 100 e MGS Catiguá 3), polymorphism was observed within a single locus (5%). The others varied from two (10 genotypes) to 11 markers (1 genotype).

The results showed that microsatellite markers are able to differentiate genotypes which are related and phenotipically close, and they can be used to help in tests of distinctiveness, homogeneity and stability of cultivars which are intended to be protected and registered. Moreover, based on these markers, the molecular profile of the cultivars/progenies in study was established.

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Evaluation the Wild Ethiopian *Coffea arabica* Accessions in Three Environments by Spectral Signature and Chemical Compounds

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SUMMARY

In order to characterize a sample of wild materials present in the Colombian Coffee Collection (CCC) for chemicals associated with quality,16 wild Ethiopian and 4 controls were evaluated in in a complete randomized block (BCA) design with three replicates in three contrasting localities of the Colombian coffee zone (Venecia - Antioquia, Chinchiná - Caldas and Floridablanca - Santander), quantification of 15 chemical compounds was performed using prediction equations developed in Cenicafé and NIRS spectral signature. Information was analyzed using the Mahalanobis Distance (MD) and discriminant analysis (DA). Are highly Significant differences were found between locations in the spectral fingerprint based on the DM and chemical compounds, where the shortest distances were 2.40 and 6.11 for the spectral fingerprints of chemical compounds between Rosario and Floridablanca. The overall percentage of correct classification was 83 % using chemical compounds and 85% with the spectrum. Within each locality the materials showed a similar trend in the chemical composition and spectral signature. These results suggest that local behavior has the greatest effect on the variability among genotypes regarding the expression of chemical composition and spectral fingerprints.

INTRODUCTION

The search for chemical compounds correlated with the quality of the cup, is a topic of wide interest in the coffee commercialization. The market demands require product diversity (by origin, varieties, production systems, decaffeinated natural, exotic sensorial attributes, etc.) to fulfill the customer requirements.

Contributing to the previously exposed reasons, Cenicafé has created a germplasm bank of *Coffea arabica,* known as the Colombian Coffee Collection (CCC) which has 471 accessions from the collecting expeditions made in Ethiopia (Fig. 1) by the FAO (Bellachew, 1997) and OSRTOM (1978).

Germplasm evaluation and characterization of genetic variability provide a strategic line of research to solve current and future problems in regards to productivity, adaptation and development of new alternatives to improved varieties. The possibility of identifying genotypes of wild coffee with traits of interest for genetic improvement, and diversity in chemical compounds, allow the identification of each of these features to extent the possible development of new varieties of coffee with good agronomic characteristics.
The aim of the present research is to evaluate the Ethiopian accessions present in the CCC, as an important genetic resource for the development of new varieties with high quality attributes.

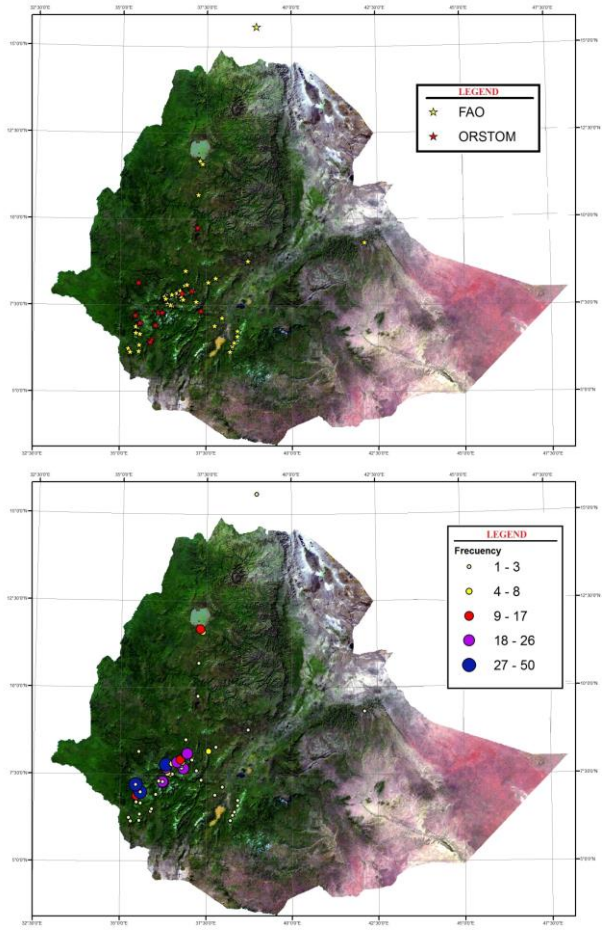

Figure 1. Maps of the collecting expeditions made in Ethiopia and its frequency

MATERIALS AND METHODS

In order to characterize a sample of wild materials present in the CCC for chemicals Associated with Quality, 16 wild Ethiopian coffee samples and four controls were evaluated in a Randomized Complete Block Design with three replicates in three contrasting Localities of the Colombian coffee region: Venicia - Antioquia, Chinchiná - Caldas and Floridablanca - Santander.

Biochemical and spectral analysis of Coffee samples were carried out by near infrared spectroscopy (NIRS). The reflectance spectra (R), (Downey & Boussion, 1996), was collected by a monochromator spectrophotometer (Fig. 2) (FOSS, model 6500, Perstrop Analytical Inc., 1201 Tech Road, Silver Spring, MD 20904, USA), driven by software ISIscan v.2.71 responsible for obtaining the sample spectra and mathematical processing software WINISI III (v.1.50e Intrasoft Intl, LLC, RD109, Sellers Lane, Port Matilda, PA 16870, USA) used to convert the spectra in varying scale and perform statistical treatment of the data. The quantification of 15 chemical compounds was performed using prediction equations developed in Cenicafé. Information was Analyzed using the Mahalanobis Distance (MD), discriminant analysis (DA) and Principal component analysis (PCA).

RESULTS AND DISCUSSION

Highly Significant Differences Between locations were found in the chemical compounds (Table 1) and in the spectral fingerprint based on the MD (Table 2) , where the shortest distances Were 2.40 and 6.11 for the spectral fingerprints of chemical compounds Between Rosario and Floridablanca. The overall percentage of correct classification was 83% using chemical compounds and 85% With the spectrum. PCA showed that 1 principal Component explain more than 97% of the data variation (Table 3). Within each locality the materials Showed to a similar trend in the chemical composition (Fig. 2) and spectral signature (Fig. 3).

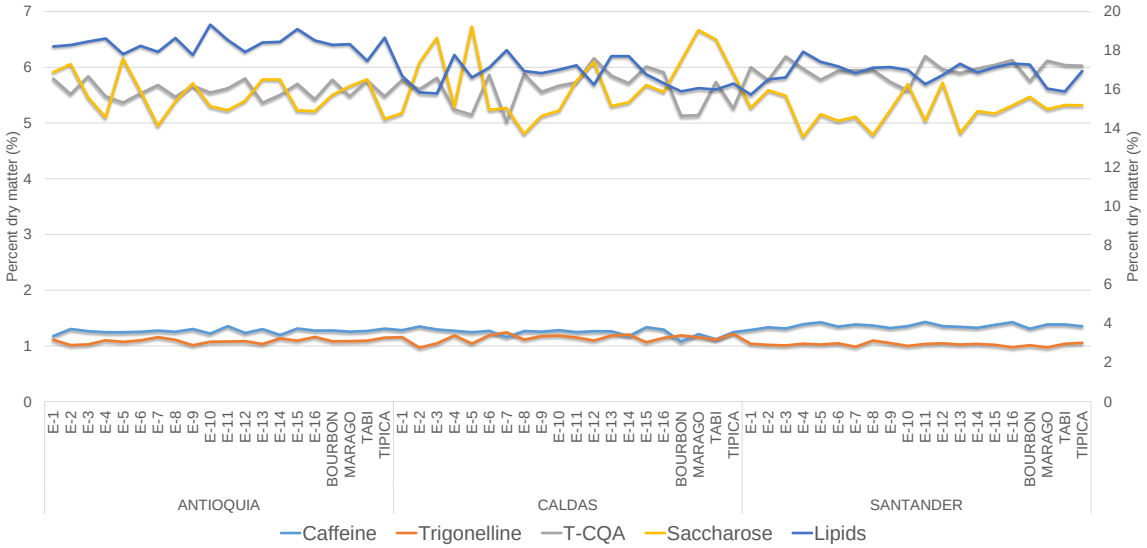

Figure 2. Chemical composition by Genotype and Locality.

Table 1. Chemical composition by locality.

Compound	ANTIOQUIA			CALDAS			SANTANDER		
	Minimum (%)	Mean (%)	Maximum (%)	Minimum (%)	Mean (%)	Maximum (%)	Minimum (%)	Mean (%)	Maximum (%)
Caffeine	1.18	1.27 ± 0.04	1.36	1.08	1.25 ± 0.07	1.35	1.29	1.36 ± 0.04	1.43
Trigonelline	1.01	1.09 ± 0.04	1.16	0.97	1.14 ± 0.07	1.24	0.97	1.03 ± 0.03	1.10
3-CQA	0.38	0.43 ± 0.03	0.47	0.38	0.42 ± 0.02	0.46	0.40	0.45 ± 0.03	0.50
4-CQA	0.56	0.6 ± 0.02	0.62	0.53	0.59 ± 0.03	0.62	0.56	0.62 ± 0.03	0.68
5-CQA	3.42	3.67 ± 0.21	4.48	2.66	3.29 ± 0.19	3.56	3.27	3.6 ± 0.17	3.92
T-CQA	5.35	5.58 ± 0.16	5.84	5.02	5.61 ± 0.33	6.16	5.58	5.95 ± 0.16	6.20
Saccharose	4.95	5.52 ± 0.33	6.16	4.81	5.72 ± 0.58	6.73	4.75	5.24 ± 0.27	5.72
Lipids	17.48	18.49 ± 0.76	21.25	15.80	16.75 ± 0.69	18.02	15.74	16.87 ± 0.55	17.94
Aracidhidic Acid	2.49	3.04 ± 0.21	3.52	3.10	3.36 ± 0.16	3.61	3.10	3.35 ± 0.16	3.65
Behenic Acid	0.44	0.76 ± 0.1	0.92	0.92	1.01 ± 0.05	1.13	0.74	0.83 ± 0.05	0.92
Oleic Acid	10.17	11.63 ± 0.98	15.13	9.62	10.73 ± 0.65	12.11	9.22	10.18 ± 0.5	11.10
Linoleic Acid	35.49	36.85 ± 0.85	38.27	37.02	38.34 ± 0.95	40.48	35.67	37.21 ± 0.8	38.34
Linolenic Acid	1.29	1.43 ± 0.06	1.50	1.16	1.33 ± 0.07	1.46	1.36	1.44 ± 0.03	1.50
Palmitic Acid	35.34	37.16 ± 0.88	38.74	31.02	34.34 ± 1.24	35.95	36.61	38.06 ± 0.84	40.02
Stearic Acid	8.45	9.02 ± 0.32	9.75	8.31	8.89 ± 0.35	9.65	8.27	8.61 ± 0.27	9.29

Table 2. Mahalanobis distances by locality.

Locality	Caldas	Antioquia	Santander
Caldas	0	13.736	12.984
Antioquia	13.736	0	4.942
Santander	12.984	4.942	0

Table 3. PCA results

PCA	PC1	PC2	PC3
Standard deviation	11.657	1.720	0.866
Proportion of Variance	0.970	0.0211	0.005
% Cumulative	0.970	0.991	0.997

CONCLUSION

These results suggest that place or locality has the greatest effect in behavior on the variability among genotypes, regarding the expression of chemical composition and spectral fingerprints.

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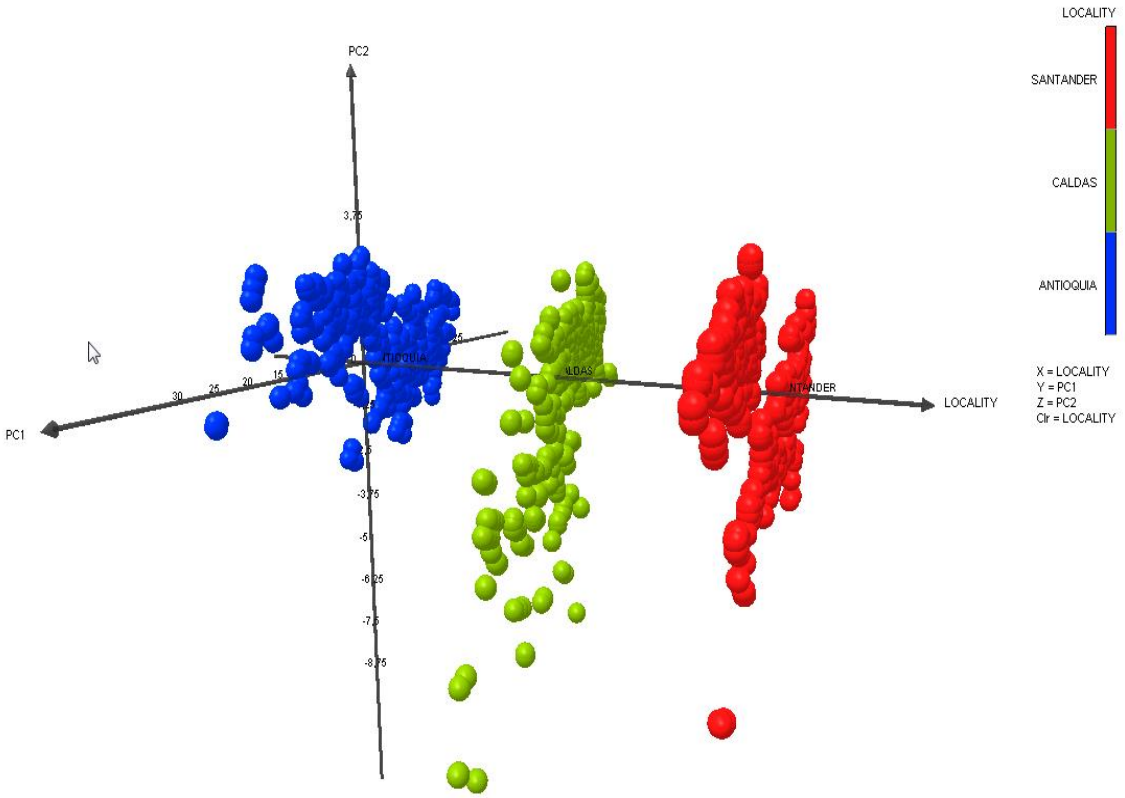

Figure 3. Spectral signature by locality (Biplot of the first 2 Principal Components)

Identification and Characterization Of Molecular Markers Linked to Introgressions from *Coffea canephora* Pierre Ex Froehner in F5 Coffee Lines with Resistance to Coffee Leaf Rust

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SUMMARY

In the way to accelerate coffee breeding is necessary to identify genomic regions introgressed and associated with the genotype of interest. Using 36 pairs of amplified fragment length polymorphism (AFLPs) primer combinations in a sample that includes a total of 75 introgressed and non-introgressed individuals distributed in bulks, 47 fragments corresponding to possible introgression markers were identified, It was possible to isolate and clone successfully 28 of these markers from which eleven showed 30-66% homology with proteins that may be involved in plant resistance against pathogens. In addition, in order to identify molecular marker linked or associated with introgression and also possible resistance genes, 329 primer combinations were evaluated in the same group of genotypes, integrating the techniques of AFLP-RGAs (amplified fragment length polymorphism-resistance gene analogs), SSR-RGAs (simple sequence repeat-resistance gene analog) and RGAs (resistance gene analogs). It was possible to identify more than 200 polymorphisms and at least 12 introgression markers caused by more than 60 new primers combinations. The present study contributes to the identification of markers that are linked to the foreign introgression of C. canephora (Coffee-Rust resistant genotype) in breeding F5 lines of C. arabica, particularly in the resistant components of the Castillo Variety of Colombia. These markers have highly potential use in a system of marker-assisted selection for coffee breeding programs.

INTRODUCTION

Coffee is one of the world's most important agricultural products, provides the most popular drink and consolidates itself as the mainstay of the economies of many developing countries. Breeding of agronomic traits of interest mainly disease resistance has been achieved through gene introgression from diploid species such as *Coffea canephora* using the Timor Hybrid (TH) as a bridge. Gene introgression in coffee, particularly for resistance coming from diploid species, is not simple. The difficulty for coffee mainly arises from problems inherent to interspecific hybridization, which commonly results in hybrid instability, infertility, non-Mendelian segregation, and low levels of intergenomic recombination, among others (Stebbins 1958).

The best known interspecific hybrid is the Timor Hybrid (TH) (2n = 4x = 44). TH was produced spontaneously between *Coffea arabica* and *Coffea canephora* on the island of Timor in 1927 from 1960 several clones and offspring's of TH accessions CIFC832-1, CIFC832-2 and CIFC1343, among others, were distributed to several coffee producing countries, including Colombia, by de *Centro de Investigaçao das Ferrugens do cafeeiro* (CIFC). As result, a number of commercial varieties were produced at the end of 80's and

planted in extensive areas of Central and South America (Herrera et al. 2014). To accelerate coffee breeding it is necessary to identify introgressed genomic regions and associate them with the genotype of interest.

The present study contributes to the identification of markers that are linked to the foreign introgression of *C. canephora* (Coffea-Rust resistant genotype) in breeding F5 lines of *C. arabica*, particularly in the resistant components of the Castillo Variety of Colombia. These markers have high potential to be employed in marker-assisted selection for coffee breeding.

MATERIALS AND METHODS

Plant material was obtained from the germplasm bank from the Naranjal experimental station at Cenicafé. The population was composed of 75 introgressed and non-introgressed individuals that were distributed either in bulk or individually (Fig.1 and Table. 1). In this study, a population formed by bulks of TH, *C. canephora*, four non-introgressed susceptible arabica genotypes (Caturra, Bourbon, Typica, and Ethiopian), two bulks with improved lines introgressed with TH in other producer countries (Costa Rica 95 and Iapar 59), and 11 elite lines of the Castillo variety were individually evaluated using the different molecular techniques such as AFLPs, RGAs and SSRs as well as a new systems including combinations of different types of primers AFLP-RGA as well as SSR-RGA.

Genomic DNA was isolated from young leaves following the CTAB method with some modifications for coffee. To generate the bulks, equimolar quantities of DNA from each individual coffee plant were mixed together. Then were included in order to identify molecular markers linked with the *Coffea canephora* genotype. The candidate introgression markers were selected according with the following criteria: *"markers that were present in TH 1343, C. canephora and at least in one of the elite Castillo F5 lines and absent in the susceptible non-introgressed C. arabica genotypes (Caturra, Ethiopian, Bourbon, and Typica).* Then some candidates markers were further analyzed by sequencing in order to identify the potential associations with gene functions based on the categories of Gene Ontology.

Table 1. Genotypes used and bulks conformations for the introgression marker identification.

Sample	Genotype	Number of individuals per bulk	Species	Type
Bulk 1	Caturra	7		No introgressed
Bulk 2	Ethiopian	4		No introgressed
Bulk 3	Bourbon	10		No introgressed
Bulk 4	Typica	10		No introgressed
Bulk 5	Timor hybrid 1343	4		-
Bulk 6	*C. canephora* 1	**3**		No introgressed
Bulk 7	*C. canephora* 2	**6**		No introgressed
Bulk 8	Costa Rica 95	10		Introgressed
Bulk 9	Iapar 59	10		Introgressed[a]
10	Elite Castillo	CX2848		Introgressed[b]
11	Elite Castillo	CU1855		Introgressed[c]
12	Elite Castillo	CU1852		Introgressed
13	Elite Castillo	CU1815		Introgressed
14	Elite Castillo	CX2197		Introgressed
15	Elite Castillo	CX2827		Introgressed
16	Elite Castillo	CX2710		Introgressed
17	Elite Castillo	CU1997		Introgressed
18	Elite Castillo	CU1827		Introgressed
19	Elite Castillo	CX2178		Introgressed
20	Elite Castillo	CU1843		Introgressed

[a] Introgression from TH CIFC832-1 (Coffea arabica x Coffea Canephora). [b] Introgression from TH CIFC 832-2 (Coffea arabica x Coffea Canephora). [c] Introgression from TH CIFC 1343 (Coffea arabica x Coffea Canephora.

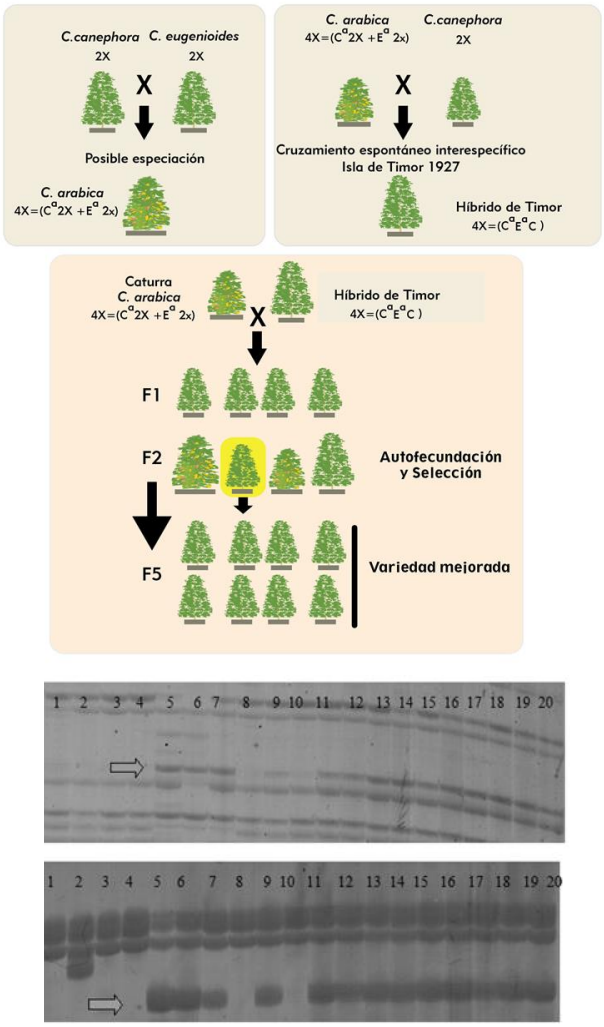


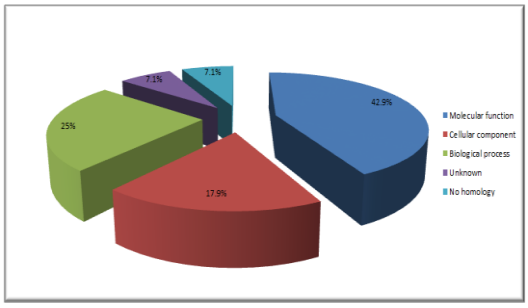
Figure 1. (top) General outline of the origin of the species evaluated. *Coffea arabica* (Caturra, Bourbon, Typica and Ethiopian), Timor Hybrid, breeding variety (Elite Castillo Lines) Adapted from (Herrera et al. 2002). (bottom) Acrylamide gels showing the presence of polymorphic introgression bands. Columns 1-4: bulks *Coffea arabica*. Columns 5 and 6*: bulks Coffea canephora*. Column 7: bulk Timor hybrid. Column 8: bulk Costa Rica 95. Column 9: bulk Iapar 59. Column 10-20: F5 elite Castillo® lines.

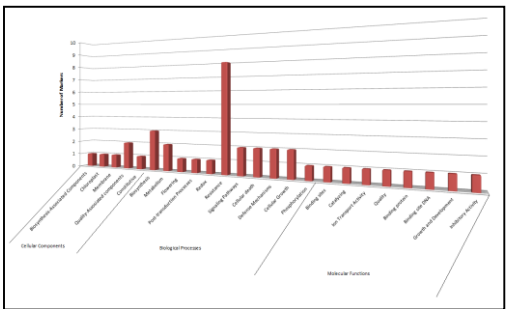
RESULTS AND DISCUSSION

Through PCR-based molecular approaches (AFLP, AFLP-RGA, SSR. SSR-RGA and RGAs) were identified more than 90 markers associated with the phenomenon of introgression. 28 of these markers obtained with the AFLP technique were sequenced. And then analysed using BlastX search against the public data base in GenBank as well as the private data base Quimbaya-Cenicafé, 26 out of 28 proved to be similar to know protein sequences (Fig. 2). Annotation of the sequences were grouped in to three major functional categories of the Gen Ontology (Fig. 2) To gain a deeper insight into the molecular biology of the defense system in coffee, defense-related genes were analyzed. According to this analysis, ten of the markers may correspond to introgressed genomic regions associated with genes involved in plant immunity. This was the case for AFLP markers 13, 26, 17, 15, 21, and 4; these markers showed similarity with gene sequences coding for proteins with NBS, LRR, or TIR domains, which are typical of resistance proteins. AFLP markers 9 and 22 showed similarity with a gene involved in biological processes related to signaling pathways and defense in plants.

AFLP marker 14 showed similarity with the family of transcription factors WRKY, which modulates the expression of genes involved in plant responses to biotic stress, including wounds produced by insects and infections caused by pathogens. Finally, AFLP marker 2 showed similarity with Miraculin, which could be related to biological processes that are linked to quality and has been observed to be involved in defense mechanisms in plants (Theerasilp et al. 1989; Gahloth et al. 2011). these markers will be evaluate for possible implementation in the breeding program on a strategy of marker assisted selection (MAS).

Primer combination	MrI. ID	Size bp	Posible function[1]	Homologue sequence[2]	Similarity	e-value
E-AGC/ M-CAA	AFLP-24	418	MF: binding site DNA	RNA polymerase beta *[Coffea arabica]* YP_817473.1	83%	4,00E-14
E-ACA/ M-CAA	AFLP-6	270	CC: Biosynthesis	fibrillin 8 *[Coffea canephora]* ABD39695.1	80%	2,10E+00
E-ACT/ M-AAC	AFLP-13	196	MF: Resistance	Putative NBS domain resistance protein *[Coffea spp.]* ABU51680.1	66%	2,70E+00
E-AGG/ M-CTG	AFLP-26	172	MF: Resistance	Putative NBS domain resistance protein *[Coffea spp.]* ABU51823.1	66%	1,00E+00
E-CAC/ M-CAT	AFLP-27	179	CC: Constitutive	Photosystem I P700 apoprotein A2 *[Coffea arabica]* YP_817481.1	66%	6,60E-01
E-AAC/ M-CTA	AFLP-1	111	-	Hypothetical protein *[Coffea canephora]* 46C02.16 ABZ89190.1	63%	2,50E-01
E-ACT/ M-CAA	AFLP-17	170	MF: Resistance	Putative NBS domain resistance protein *[Coffea spp.]* ABU51866.1	63%	7,50E-02
E-AAC/ M-CTC	AFLP-2	185	MF: Resistance, biosynthesis	Miraculin-like protein *[Coffea arabica]* ABK01288.1	62%	2,40E+00
E-ACG/ M-CTA	AFLP-10	100	MF: Growth and development	Putative oxygenase *[Coffea arabica]* AAK27512.1	61%	3,50E-03
E-ACA/ M-CAT	AFLP-7	384	MF: Binding protein	Ethylene receptor *[Coffea canephora]* ADI44158.1	57%	7,30E-01
E-ACT/ M-AAC	AFLP-14	121	BP: Resistance	Putative WRKY1b transcription factor *[Coffea arabica]* ABC86708.1	57%	4,00E-01
E-ACG/ M-CTA	AFLP-9	290	BP: Signaling, cellular death, defense mechanism, cellular growth, signaling phatways.	Somatic embryogenesis receptor-like kinase *[Coffea canephora]* CAI10726.1	56%	6,80E-01
E-AAC/ M-CTC	AFLP-5	135	MF :metabolic functions, phosphorylation, biosynthesis, qualty.	P-coumaroyl quinate/shikimate 3'-*hydroxylase [Coffea canephora]* ABO77958.1	55%	1,30E+00
E-ACT/ M-AAC	AFLP-15	108	MF: Resistance	Putative NBS-LRR protein *[Coffea canephora]* ABS82612.1	54%	7,60E-01
E-ACA/ M-CAT	AFLP-8	144	BP MF: biosynthesis	Caffeic acid O-methyltransferase *[Coffea canephora]* AAN03726.1	53%	7,90E-01
E-ACT/ M-CAT	AFLP-18	207	Unknow	Putative protein *[Coffea canephora*] ABZ89184.1	53%	3,30E-02
E-AGG/ M-CTC	AFLP-25	163	BP: Biosynthesis, metabolism	Cell-wall invertase *[Coffea canephora*] ABI17893.1	53%	1,50E-02
E-ACG/ M-CTA	AFLP-11	61	MF: inhibitory activity, quality components	Invertase inhibitor *[Coffea canephora]* ABI17896.1	50%	4,50E-01
E-AAC/ M-CTC	AFLP-3	129	BP: redox processes	Plastid terminal oxidase *[Coffea canephora*] ABB70513.1	42%	3,10E-01
E-ACT/ M-CTT	AFLP-21	70	MF: Resistance	Putative TIR-NBS-LRR disease resistance protein *[Coffea arabica]* ABW76508.1	41%	2,10E-02
E-AGC/ M-ACA	AFLP-22	70	BP: Signaling, cellular death, defense mechanism, cellular growth, signaling pathways	Somatic embryogenesis receptor-like *kinase [Coffea canephora]* ABN42681.1	40%	8,10E-03
E-AAC/ M-CTC	AFLP-4	108	MF: Resistance	Putative NBS-LRR protein *[Coffea canephora]* ABS82610.1	38%	3,00E-01
E-ACT/ M-CTG	AFLP-19	201	CC: constitutive MF: Ion transport activity	ATP synthase CF0 subunit IV *[Coffea arabica]* YP_817470.1	37%	2,60E-02
E-AGC/ M-ACA	AFLP-23	88	CC: Components associated with quality, MF: Catalyzing	Mannan synthase *[Coffea canephora*] ACF33171.1	33%	1,60E-02
E-CAC/ M-CAT	AFLP-28	114	BP: Flowering, post-transduction processes, MF: Binding sites	Putative protein >gb|ADZ55302.1| cyclophilin *[Coffea canephora]* ABZ89184.1	33%	1,80E-01
E-ACT/ M-CAA	AFLP-16	181	CC: Chloroplast, membrane	Hypothetical chloroplast RF1 *[Coffea arabica]* YP_817540.1	30%	3,70E-02
E-ACT/ M-CTT	AFLP-20	100	No homology	-	-	-
E-ACG/ M-CTA	AFLP-12	74	No homology	-	-	-





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Durable Resistance to Coffee Leaf Rust Provided by the SH_3 Gene Evaluated at the Instituto Agronômico de Campinas, SP, Brazil

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SUMMARY

Before the arrival of coffee leaf rust (*Hemileia vastatrix* Berk. et Br.) in Brazil, the Instituto Agronômico de Campinas (IAC/APTA) introduced in 1953 and 1965 from India and Africa several accessions carrying resistance genes SH_1, SH_2, SH_3 and SH_4. With the arrival of rust race II (v_5) in 1970, all the commercial cultivars planted at that time in Brazil such as Bourbon, Mundo Novo and Catuaí showed to be susceptible to the disease. From that year, many crosses were made between Indian, African varieties with Brazilian cultivars in order to develop rust resistant cultivars in successive selfed progenies. Hybrids of tall and short stature were planted in Campinas in 1972. Few years later, new races matching the SH_1, SH_2 and SH_4 genes appeared in the selection fields. After 44 years of disease epidemics in Brazil, the SH_3 gene is the only resistance factor not yet overcome by *H. vastatrix*. Among the introductions from India, two accessions B. A. 21 (IAC1107) and B. A. 10 (IAC 1110) containing SH_3, are still resistant to prevalent races of rust in Brazil. However, the SH_3 gene appeared to be strongly associated with an important bean defect (elephant beans). From 1992 onward, large scale assessment of resistance was initiated in two trials containing progenies segregating for SH_3. The current study focused on the determination of the homozygous/heterozygous condition of the SH_3 gene in F_1 progenies and on selection of successive generations for presence of the SH_3 gene and low elephant bean percentage. It was shown that IAC progenies 1110-8-5, 1110-10 and 1110-10-1 behaved as homozygous (SH_3SH_3) and 1107-4, 1107-4-1 and 1107-5-6 progenies as heterozygous (SH_3sh_3). Advanced productive progenies (F_5, F_6), derived from crosses between IAC 1110-8-5, 1110-10 and 1110-10-1with the cultivars Mundo Novo, Acaiá and Catuai, have been obtained that carry the SH_3 gene in homozygous condition and present low elephant bean percentage.

INTRODUCTION

The coffee rust (*Hemileia vastatrix*) is the most important disease of coffee, causing damage ranging of 30-50% in productivity when chemical control is not performed. The best alternative for rust control is the use of resistant cultivars which represent an economical and not harmful to the environment method. With the evolution of rust, currently more than 45 physiological races were identified by CIFC (Centro de Investigação das Ferrugens do Cafeeiro), nullifying resistance comes from different genetic materials. So far, the SH_3 gene is the only known factor that conditions resistance (immunity) in coffee. However, the SH_3 gene appears to be strongly linked to the large amount of elephant beans in the plant coffee that is an important defect of the commercial product. The aim of this study was to present the behavior of genetic resistance hybrids carrying the SH_3 allele, 44 years after the entry of rust

in Brazil and also show selections of Catuaí, and Mundo Novo descendants of India BA 10 access with SH_3 allele in homozygous and with normal grains obtained by successive selfing.

MATERIALS AND METHODS

In 1972, two experiments (short and tall stature) of hybrids obtained from crosses made in 1970 between accessions from India, Africa and Catuaí and Mundo Novo cultivars were established with the purpose of transferring genetic resistance present in these coffee plants. The experimental design in both experiments was completely randomized. In the short stature experiment, 14 treatments and a susceptible control cultivar Catuaí Vermelho IAC 81, nine replicates and one plant per plot plant were used. In the experiment with the tall stature 24 treatments and a susceptible control cv. Acaiá IAC 474-7, six replicates and one plant per plot were employed. The rust evaluations of the experiments were carried out before harvesting, beginning in 1992, using a range of types of rust reaction (RT) with 5 points. In 2012, was performed the last evaluation of rust in the experiments before coffee pruning. Furthermore, characteristics of beans such as types of flat, peaberry and elephant beans percentage, 100 beans weight in grams and average size sieve of F6 generation of Catuaí and Mundo Novo progenies carrying the SH_3 allele in homozygous.

RESULTS AND DISCUSSION

In the tables 1 and 2, some treatments and corresponding hybrids, their parents, their origins and also average of types of rust reaction (RT) observed in experiments are shown.

Table 1. Parents, their origins and rust resistance of 10 short stature hybrids, and control cv. Catuaí Vermelho IAC 81 evaluated in the experiment installed in 1972, in Campinas/SP.

Treatment	Hybrids	Parents	Origin	RT*
1	H 8088 (H2077-2-5-24 x 1120-16)	Catuaí x X 321	Brazil x Africa	3.8
2	H 8089 ((H2077-2-5-24 x 1137-5)	Catuaí x Geisha	Brazil x Africa	3.2
3	H 8105 (H2077-2-5-81 x 1110-10)	Catuaí x BA 10	Brazil x India	0.2
4	H 8107 (H2077-2-4 x 1125-3)	Catuaí x Cioiccie	Brazil x Africa	3.0
5	H 8113 (H2077-2-5-81 x 1517-1)	Catuaí x Kaffa	Brazil x Africa	3.1
6	H 8114 (H2077-2-5-81 x 1521-2)	Catuaí x Wush-Wush	Brazil x Africa	3.2
7	H 8129 (1107-4-1 x H2077-2-5-24)	BA 21 x Catuaí	India x Brazil	2.0
8	H 8130 (1107-4 x H2077-2-5-81)	BA 21 x Catuaí	India x Brazil	2.1
9	H 8142 (1107-5-6 x 1518-2)	BA 21 x S 333	India x India	1.9
13	H 8188 (1110-8-5 x H2077-2-5-81)	BA 10 x Catuaí	India x Brazil	0.4
15	H2077-2-5-81 (control)	Catuaí A. x M. Novo	Brazil	3.5

**Mean of treatmentin the range of 0 to 4 points.*

Table 2. Parents, their origins and rust resistance of 10 tall stature hybrids, and control cv. Acaiá IAC 474-7 evaluated in the experiment installed in 1972, in Campinas/SP.

Treatment	Hybrids	Parents	Origin	RT*
1	H 8126 (1107-4-1 x CP 474-4)	BA 21 x Acaiá	India x Brazil	1.5
3	H 8162 [1109-7-6 x (1109-7 x CP387-17)]	BA 8 x (BA 8 x M. Novo)	India x Brazil	3.3
4	H 8359 (1475-5 x CP 474-4)	Cioiccie x Acaiá	Africa x Brazil	3.0
5	H 8141 (1107-5-6 x 1120-35)	BA 21 x X 321	Brazil x Africa	2.2
6	H 8355 (1475-5 x 1110-10)	Cioiccie x BA 10	Africa x Brazil	0
7	H 8187 (1110-8-5 x CP 474-7)	BA 10 x Acaiá	India x Brazil	0.2
13	H 8414 (CP 467-1 x 1133-2)	M. Novo x Harar	Brazil x Africa	3.2
14	Acaiá IAC 474-7 (control)	Bourbon V. x Sumatra	Brazil	3.4
16	H 8420 (IAC 471-5 x 1110-1-1)	M. Novo x BA 10	Brazil x India	0
17	H 8421 (IAC 471-5 x 1110-10)	M. Novo x BA 10	Brazil x India	0.2
24	H 8518 (IAC 386-2 x 1110-1-1)	M. Novo x BA 10	Brazil x India	0.8

*Mean of treatment in the range of 0 to 4 points.

The treatments 3, 7, 8, 9, 10 and 13 of short stature and the treatments 1, 5, 6, 7, 16, 17 and 24 of tall stature experiments derived from accessions of India BA 10 (IAC 1110) and BA 21 (IAC 1107) were resistant, with an average of RT ranging from 0 to 2.2 points indicating the presence of SH_3 gene. While hybrids of African origin, whose parents are X321, Geisha, Cioiccie, Kaffa, Wush-Wush and Harar carriers of SH_1, SH_2 and SH_4 alleles (associated or not), all showed RT above 3 points (susceptibility), in accordance with previous studies by CIFC. The frequency of the types of rust reactions observed in coffee plants from resistant treatments to identify the genetic constitution of the resistant parents was studied too (Table 3).

Table 3. Rust segregation in progenies of 12 F1 generation hybrids derived from SH_3 gene carriers BA 10 and BA 21 access and susceptible controls Catuaí Vermelho IAC 81 and Acaiá IAC 474-7, evaluated in experiments in Campinas/SP.

				Number of Plants				
	Short stature			---------RT*---------				
Treatment	Hybrids	Parents	Total	0	1	2	3	4
3	H 8105 (Catuaí V. IAC 81 x 1110-10)	Catuaí x BA 10	5	4	1	0	0	0
7	H 8129 (1107-4-1 x Catuaí V. IAC 24)	BA 21 x Catuaí	7	3	0	0	1	3
8	H 8130 (1107-4 x Catuaí V. IAC 81)	BA 21 x Catuaí	7	2	1	0	2	2
9	H 8142(1107-5-6 x 1518-2)	BA 21 x S 333	9	3	1	0	4	1
10	H 8142 (1107-5-6 x 1518-2)	BA 21 x S 333	7	4	0	0	2	1
13	H 8188 [1110-8-5 x Catuaí V. IAC 81]	BA 10 x Catuaí	9	6	3	0	0	0
15	Catuaí Vermelho IAC 81	Catuaí A. x M. Novo	9	0	0	0	4	5
	Tall stature							
1	H 8126 (1107-4-1 x IAC 474-4)	BA 21 x Acaiá	6	3	0	0	3	0
5	H 8141 (1107-5-6 x 1120-35)	BA 21 x X 321	5	2	0	0	1	2
6	H 8355 (1475-5 x 1110-10)	Cioiccie x BA 10	4	4	0	0	0	0
7	H 8187 (1110-8-5 x IAC 474-7)	BA 10 x Acaiá	6	5	1	0	0	0
14	Acaiá IAC 474-7	Bourbon x Sumatra	6	0	0	0	6	0
16	H 8420 (IAC 471-5 x 1110-1-1)	M. Novo x BA 10	6	6	0	0	0	0
17	H 8421 (IAC 471-5 x 1110-10)	M. Novo x BA 10	6	5	1	0	0	0
24	H 8518 (IAC 386-2 x 1110-1-1)	M. Novo x BA 10	4	3	1	0	0	0

**RT = Types of rust reaction: 0 e 1= Resistant; 2= Moderately resistant; 3 e 4 =Susceptible.*

The coffee plants of the short stature treatments 3 (H8105), 13 (H8188) and the tall stature treatments 6 (H8355), 7 (H8187), 16 (H8420), 17 (H8421) and 24 (H8518), were all resistant (RT= 0 and 1) indicating that the IAC 1110-10, 1110-1-1 and 1110-8-5 parents behaved as homozygous (SH_3SH_3). The tall stature treatments 7 (H8129), 8 (H8130), 9 and 10 (H8142), and the short stature treatments 1 (H8126) and 5 (H8141) segregated in a ratio of 1:1, indicating that probably progenies IAC 1107-4, 1107-4-1 and 1107-5-6 are heterozygous (SH_3sh_3). The coffee beans characteristics of the eight F6 generation of Catuaí SH_3 and Mundo Novo SH_3 and the cultivars Catuaí Amarelo IAC 62 and Mundo Novo IAC 376-4 used as controls are shown in Table 4.

Table 4. Data of beans characteristics of eight F6 progenies of Catuaí SH_3 and Mundo Novo SH_3 and, comercial cultivars controls Catuaí Amarelo IAC 62 and Mundo Novo IAC 376-4 obtained from an experiment in Campinas/SP.

		Coffee beans characteristics				
Samples	**Progeny**	**Flat**	**Peaberry**	**Elephant**	**Weight of 100 beans (g)**	**ASS***
		---------- % ----------				
1	Catuaí SH_3-968	94.0	4.7	1.30	12.0	17.0
2	CatuaíSH_3-987	86.6	10.1	3.30	12.4	17.2
3	CatuaíSH_3-989	79.1	16.7	4.10	12.1	17.1
4	CatuaíSH_3-993	87.6	11.1	1.30	12.5	17.3
5	Catuaí Amarelo IAC62	89.9	8.1	2.10	12.0	16.9
6	Mundo Novo SH_3-106	86.0	8.5	5.50	11.3	16.5
7	Mundo Novo SH_3-112	80.0	15.8	4.20	13.4	17.3
8	Mundo Novo SH_3-122	84.6	12.1	3.30	14.4	17.3
9	Mundo Novo SH_3-136	78.3	18.0	3.70	11.2	17.2
10	Mundo Novo IAC376-4	92.0	3.9	4.00	14.4	16.7

**ASS= Average size sieve*

Analyzing the given data, the F6 generation progenies of Catuaí SH_3 and Mundo Novo SH_3, descendants of BA 10 access, were normal (elephant beans below 5%) and the percentages of peaberry seeds were equal to or slightly lower compared to commercial cultivars. These coffee plants have been highly productive with resistance to others diseases. Nowadays there are others progenies with more resistance factors (SH_3 and others) derived from cultivars originated from crosses with coffee plants carrying the SH_3 alleles, Híbrido de Timor, Icatu and others with the aim to obtain cultivars with more complex and durable rust resistance. This study allowed showing that Catuaí and Mundo Novo cultivars carrying the SH_3 gene may have durable rust resistance with great technological qualities of beans.

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Identification of miRNAs as Mediators of *Coffea arabica* Resistance to *Hemileia vastatrix*

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SUMMARY

Coffee leaf rust, caused by the biotrophic fungus *Hemileia vastatrix* is the most widespread disease of *Coffea arabica*. Resistance of coffee to *H. vastatrix* is mediated by gene-for-gene interactions that are conditioned by at least nine major dominant genes (S_H1 – S_H9) singly or associated.

Plants have evolved sophisticated adaptive responses to biotic stress involving reprogramming gene expression at the transcriptional, post-transcriptional and post-translational levels. miRNAs regulate gene expression by blocking translation or marking the RNA for degradation. In this study, deep-sequencing of the small-RNA fraction obtained from coffee leaves at early stages of the infection process (18/24 h and 48 h) of incompatible and compatible interactions was performed.

RNA sequences with lengths of 19-25 nucleotides derived from six libraries (control and fungal interactions) were analyzed, leading to the identification of 28 families of conserved miRNAs. The miRNAs families' miR156, miR397, miR398 and miR399 are candidates for resistance mediators, since their abundance was increased in the incompatible interaction as compared to the compatible interaction. Their involvement in this pathossystem will be further studied.

INTRODUCTION

MiRNAs are a class of small RNAs (sRNAs) of about 22 nucleotides (nt) that bind to their complementary mRNAs, leading to their degradation or regulation of translation, thus leading to silencing of specific genes (Yan *et al.* 2011; Zhang *et al.* 2006). These miRNAs play a critical role in a large number of cellular processes, such as differentiation, senescence, autophagy, proliferation, apoptosis and response to stress (Huang *et al.* 2013).

In plant-pathogen interactions several miRNAs have been identified as a component of the defence mechanisms in plants (Lu *et al.* 2007; Lu *et al.* 2005). These studies have shown how miRNAs contribute to the rapid reprogramming of gene expression after infection, enhance the basal and specific resistance, and / or help minimize the fitness cost associated with the expression of defence genes (Voinnet 2008).

Deep-sequencing has enabled the discovery of several hundred miRNAs in various plant species using different experimental and computational approaches but this kind of work only

recently has emerged in *Coffea* species. Molecular information through the Coffee Genome Project, of expressed sequence tags (ESTs) were already obtained from various tissues at different developmental stages or exposed to biotic and abiotic stresses. So, the identification of the conserved and non-conserved miRNAs in the transcriptome of coffee, as well as their targets, is timely and has the potential to provide new tools to dissect mechanisms relevant during operational regulation. Our work contributes to increase the knowledge about the non-coding transcriptome of coffee leaves through the sequencing of small non-coding RNAs and, in the future, the molecular basis of coffee-rust interactions.

MATERIALS AND METHODS

Small RNA isolation and sequencing

C. arabica plants of the S4 Agaro (genotype S_H4S_H5) variety grown in greenhouse conditions were inoculated with fresh urediospores of *H. vastatrix*, races II (v_5) or XV ($v_{4,5}$) establishing an incompatible (resistant) and a compatible (susceptible) interaction, respectively. The infected leaves were collected at 18, 24 and 48 hours after inoculation (hai). All samples were immediately frozen in liquid nitrogen and stored at -80ºC until RNA isolation.

Small RNAs from the coffee leaves samples were isolated using the Plant/Fungi Total RNA Purification Kit (NORGEN). The extracted samples were treated with DNAse Turbo (Ambion® TURBO™ DNase, Invitrogen-Life Technologies).

RNA quality and yield was checked in all samples by measuring the UV absorbance (A) at 230, 260 and 280 nm using the ND-1000 Spectrophotometer (NanoDrop Technologies Inc., USA). The integrity of the total RNA was also evaluated on the Agilent 2100 Bioanalyzer.

The purified RNA fraction was prepared for sequencing by Illumina HiSeq 2000 (Fasteris, Switzerland).

Small RNA sequence processing

After removal of the sequencing adapter, reads were further processed using the UEA sRNA toolkit (Moxon *et al.* 2008). Using 'Filter' function only read length ranging from 19 to 25 nt with no match with the plant rRNA/tRNA were kept for further analysis. Conserved mature miRNAs were searched against the miRBase database-release 20 using the miRProf tool and allowing up to two mismatches. The identified conserved miRNA were further grouped by match signature and combined by the organism name and the miRNA family members. The number of reads of the conserved miRNAs was normalized with the total number of sequenced reads in each library.

Target prediction was performed using the *C. canephora* genomic information with the software psRNATARGET (http://plantgrn.noble.org/psRNATarget/).

RESULTS AND DISCUSSION

Deep-sequencing of the small-RNA fraction obtained from coffee leaves at early stages of the infection process (18/24 and 48 hai) of incompatible and compatible interactions was performed. Light microscopic (LM) studies revealed that the first changes in the resistant samples corresponded to hypersensitive host cell death, observed in more than 50% of infection sites, at 48hai (Guerra-Guimarães *et al*. 2012).

An average of 20,972,700 sequence reads were obtained from the coffee leaves (Table 1). After removing reads shorter than 19-nt and longer than 25-nt and with an abundance of 10 or lower, about 70 % of the reads remained for subsequent analysis.
The distribution by sequence length of the reads between 19 and 25 nt showed identical levels of the 21-nt and the 24-nt size classes in leaves (Fig.1A). The filtering of the rRNA/tRNA resulted in a decreasing of the number of reads in the 24-nt class (more than 2-folds) while the 21-nt class remained constant (Fig.1B).

Table 1. General statistics of small RNA sequence reads from coffee leaves.

	18/24H Untreated	18/24H Resistant	18/24H Susceptible	48H Untreated	48H Resistant	48H Susceptible
Total raw reads	19 654 328	23 270 432	19 797 907	21 699 351	20 981 276	20 432 907
reads with 19<seq lenght<25	12 530 773	18 309 692	13 443 391	16 344 278	14 220 892	15 948 184
non-redundant reads with 19<seq lenght<25	2 436 799	3 957 720	2 978 671	3 293 484	3 222 244	3 346 334
reads after filter (>10 & rRNA/tRNA excluded)	6 119 921	10 645 322	7 080 307	9 232 059	7 166 465	9 356 487
non-redundant reads after filter (>10 & rRNA/tRNA excluded)	54 367	100 093	66 547	83 404	70 109	81 698
conserved miRNAs	1 848 983	4 483 082	2 590 286	3 798 366	2 542 212	4 348 786

Nevertheless, when comparing the levels of the unique/non-redundant sequences, according to size, the 24-nt class was more diverse (Fig.1C).

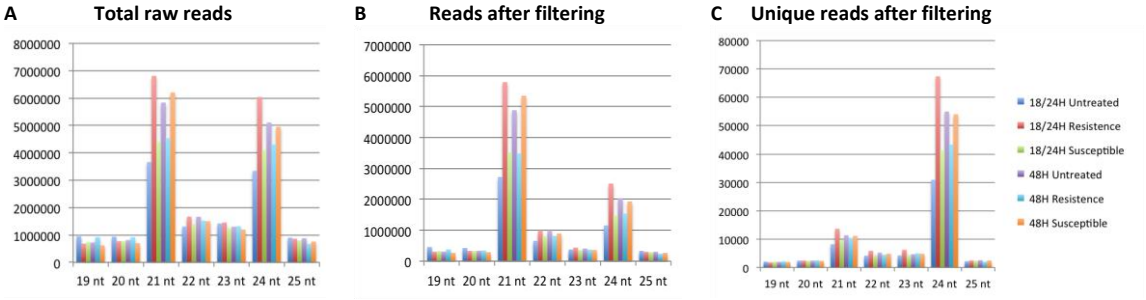

Figure 1. Size distribution of small RNA sequences obtained from coffee leaves.

After filtering the reads according to size, abundance and rRNA/tRNA, as described above, the conserved miRNAs in the six coffee leaf libraries (control - C, resistance - R and susceptible - S samples for the two time periods) were identified by BLAST searches of the small RNA reads against miRBase-release 20. The search for the conserved mature miRNAs led to the identification of members of 28 conserved miRNAs in leaves, and the expression level was assessed. The most highly expressed families in all the samples were miR165/166, miR159 and miR396 with the targets: HD-ZIPIII (class III homeodomain-leucine zipper) protein, MYB (myeloblastosis)-transcription factor and GRF (growth regulating factor), respectively. After the analysis of the expression level of the miRNAs some families were found to be putative candidates involved in the mediation of the coffee-leaf rust interactions, namely, miR156, miR399, miR397 and miR398 (for resistance) and mir160, miR384 and miR394 (for susceptibility). Putative miRNA targets were predicted using *C. canephora* genomic information complementing the information already available in the literature: a Squamosa Binding Protein (SBP) as target of miR156; an ubiquitin-conjugating E2 enzyme (UBC24) for miR399; a laccase for miR397; a copper-binding protein domains for miR398; an Auxin Related Factor (ARF) as target of the miR160; and unnamed proteins for miR384

and miR394. These targets require further experimental validation using the transcriptomic information of *C. arabica* leaves infected by *H. vastatrix* (Fernandez *et al.* 2012).

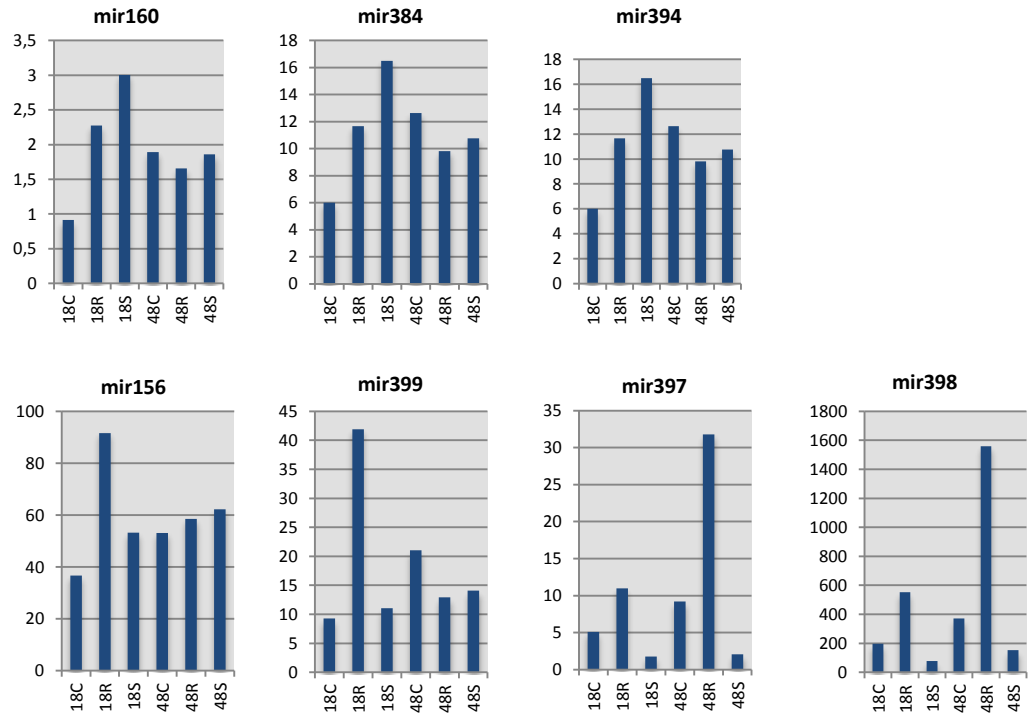

Figure 2. Expression level of the candidate miRNAs families involved in the mediation of the coffee-leaf rust interactions (nº of reads).

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Highly Divergent Alleles in a Candidate Gene of *Hemileia vastatrix* Suggests a Putative Role of Adaptive Significance

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SUMMARY

More than a century after its emergence, coffee leaf rust (CLR), caused by the complex biotrophic fungal pathogen *Hemileia vastatrix*, remains the main threat to Arabica coffee production worldwide. Engaged in a dynamic system of host-pathogen co-evolution with the consequent occurrence of frequent shifts in pathotypes, *H. vastatrix* has been able to maintain its competitive edge over host plants challenging the long-lasting effectiveness of resistant commercial varieties for years. Given the evident high variability and adaptability of the pathogen, a better understanding of the mechanisms underlying adaptation in *H. vastatrix* populations across large geographic areas, different coffee hosts and periods of time is an immediate priority. Since genes involved in coffee-rust interaction are expected to evolve under strong selection, the analysis of genetic differences in putative candidate genes could provide insights on the pathogen virulence evolution. In this study, we analyzed a candidate gene, retrieved from a 454-transcriptomic dataset and identified as activated during early stages of *H. vastatrix* infection, under a population framework. Sequence data from 80 coffee rust isolates revealed a total of 30 polymorphic sites, corresponding to a majority of non-synonymous SNPs (57%), from which 82% lead to a non-conservative amino acid replacement. Very divergent haplotypes/alleles were found, particularly related with coffee host. Within the 11 alleles identified, half were unique to rusts from diploid coffee species, while all other rust samples isolated from the tetraploid *Coffea arabica* and related intra and inter-specific hybrids shared the most common and ancestral allele. Moreover, within this latter coffee host group, specific haplotypes were predominantly present. Interestingly, different protein sequences comprising amino acids with different physicochemical properties are predicted for most haplotypes, suggesting that a relevant adaptive change could have been acquired through protein functional modification. Follow-up gene expression studies are in progress to assess if this variability found at the protein-coding level is also associated with differential expression profiles. Our results provide a first insight on *H. vastatrix* adaptive molecular variation, highlighting the relevance of coffee host as a major selective pressure and suggesting an important adaptive role for this candidate gene, ultimately leading us through the first steps to start unveiling rust adaptation.

INTRODUCTION

Hemileia vastatrix is a dikaryotic biotrophic fungal pathogen that causes coffee leaf rust (CLR), a disease that has been a permanent threat to Arabica coffee production for more than a century. Frequent shifts in pathotypes, driven by the dynamic system of host-pathogen co-evolution, have proved to be a critical limitation for achieving durable CLR resistance, resulting in a gradual breakdown of many of the successfully improved varieties in several countries. Given the evident high variability and adaptability of the pathogen, and the constant threat of new evolving pathotypes emerging under a strong selective pressure and becoming epidemically spread on a continental scale, a better understanding of the mechanisms underlying adaptation in *H. vastatrix* populations across large geographic areas, different coffee hosts and periods of time is an immediate priority. Since genes involved in coffee-rust interaction are expected to evolve under strong selection, the analysis of genetic differences in putative candidate genes could provide insights on the pathogen virulence evolution. In this study, we analyzed a candidate gene, retrieved from a 454-transcriptomic dataset and identified as activated during early stages of *H. vastatrix* infection, under a population framework.

MATERIALS AND METHODS

Rust isolates and DNA extraction

80 isolates of *H. vastatrix* (Hv) from the collection of CIFC/IICT, comprising 19 geographical origins from Asia, Latin America and Africa, different virulence profiles, collection years and coffee hosts. DNA was extracted using a CTAB-based protocol modified from Kolmer et al. (1995) [4].

Sequence data analysis

Sequence data was generated for a candidate gene (Hv00162) with unknown function retrieved from a 454 transcriptomic analysis of three *H. vastatrix* differentiation/ infection stages [3], following primer design with PerlPrimer v1.1.17. and fragment amplification. Sequences were aligned using ClustalW and the heterozygous phase was determined using PHASE v2.1.1 [5]. Polymorphism data was assessed with DnaSP v5 [6] and the haplotype network was generated using the Median Joining method in Network v4.6.1.2 [7]. Correlations between SNP allele or haplotype frequencies and coffee hosts were tested usingMatSAMv2 through a series of univariate logistic regressions [8].

RESULTS AND DISCUSSION

Analysis of Hv00162 sequence data from 80 coffee rust isolates revealed a total of 30 polymorphic sites in a 778 bp fragment, corresponding to a majority of non-synonymous SNPs (57%), from which 82% lead to a non-conservative amino acid replacement (the original amino acid is replaced by another with different physicochemical properties) (Table 1). Although an overall low level of genetic diversity was found, haplotype diversity was high and particularly related with coffee host, mainly concentrated on the rust samples retrieved from diploid coffee species (Table 1, Fig. 1).

Table 1. Genetic diversity indexes and neutrality tests estimated for HV00162. Syn : Number of synonymous SNPs. NonSyn: Number of non-synonymous mutations – Conserved and Non-conserved.

	Population grouping	Hv isolates	Polymorphic sites	Haplotypes	Syn	NonSyn			Mis sense	Nucleotide diversity (π)	Haplotype diversity (H)
						Total	Conserv	Non Conserv			
	Total	80	30	11	13	17	2	14	1	0,00587	0,701
Coffee hosts	*C. arabica*	34	7	4	3	4	-	4	-	0.00389	0.654
	Timor hybrids and derivates	26	7	4	3	4	-	4	-	0.00397	0.676
	C. liberica x *C. arabica*	5	6	3	3	3	-	3	-	0.00388	0.644
	Diploid coffee species	9	27	7	12	15	2	12	1	0.01521	0.830

A pattern of very divergent haplotypes/alelles was found, where the most distinct belong to rusts isolated from diploid coffees, revealing a clear structuring of genetic variation with a probable adaptive significance. In fact, half of the haplotypes identified were unique to rusts from diploid coffee species (H2, H5, H8-11), two of them homozygous, while all other rust samples isolated from the tetraploid *Coffea arabica* and related intra and inter-specific hybrids were heterozygous, sharing the most common and ancestral haplotype (H1), and other of the remaining closer related haplotypes (H3, H4, H6, H7) (Fig. 1A). Also, the predominant presence of specific haplotypes within this latter coffee host group was detected, for instance H6 is only found in rusts from *C. arabica* (30%) and from Timor hybrid (HDT) and respective derivatives (70%), and H4 is more common in rusts from *C. arabica* (61.5%) (Fig.1B.).

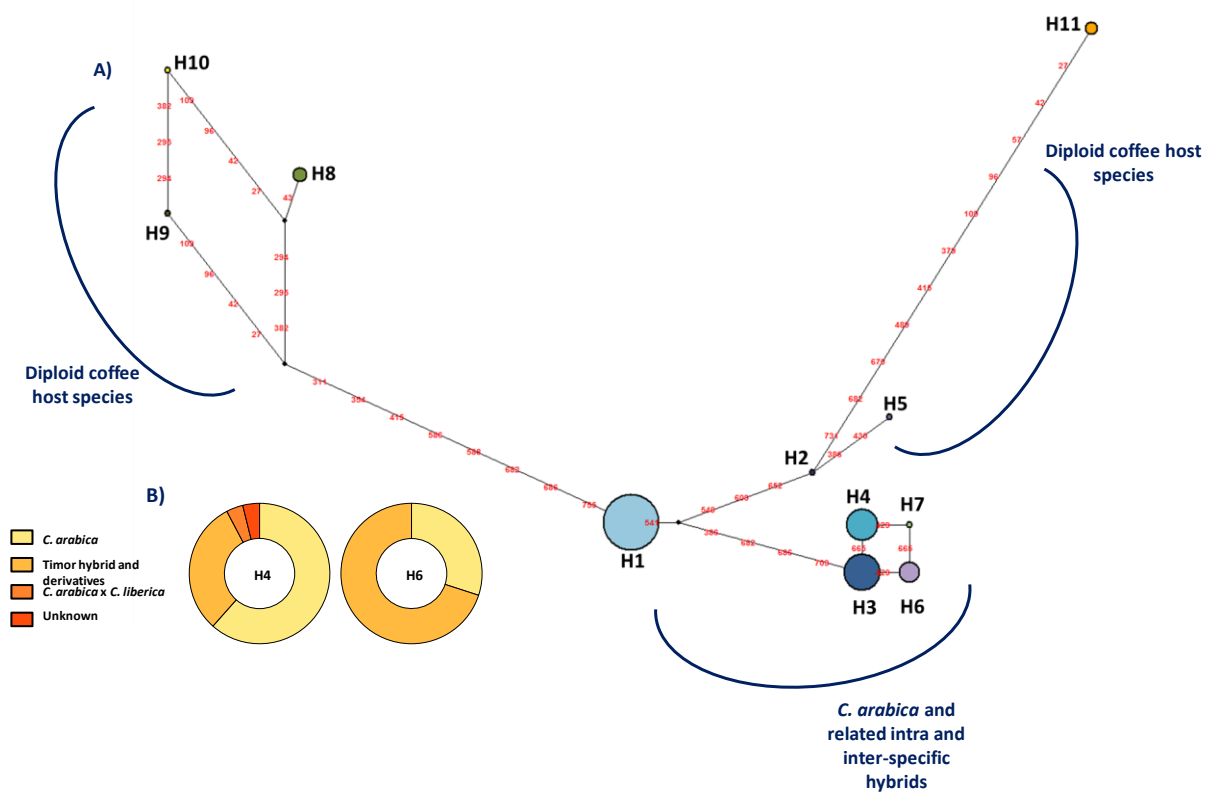

Figure 1. (A) Median Joining haplotype network for Hv00162. Circle size reflects the respective frequency of each haplotype across Hv samples. Each number in the network indicates the sequence polymorphic site of the mutations occurring between haplotypes; (B) Doughnut charts representing the frequency of rust samples isolated from different types of coffee hosts which present a particular haplotype.

Prediction of the amino-acid sequence for each haplotype showed that a different protein sequence, comprising amino-acids with different physicochemical properties, is putatively codified by each of them (except H6 and H7, data not shown), thus a relevant adaptive change could have been acquired through protein functional modification.

Given the evident strong correlation of rust genetic variation with coffee hosts, suggesting the interaction with the host as a major selective pressure, molecular imprints of natural selection were searched by testing associations of SNP alleles with coffee hosts. Twenty SNPs were found to be significantly associated (p<0.01, Wald or G significance tests) with coffee hosts, from which 18 were exclusive of rusts from diploid species, 7 of them corresponding to non-conservative amino acid changes. Since these mutations are likely to alter at some degree the structure and function of the protein, these rust samples seem not only to be very genetically distinct from those associated to tetraploid species, but also present a high variability among each other. For the two other SNPs (386T/C and 709G/C), the allele T from SNP386 tends to be more frequent in rusts from *C. arabica* and related intra and inter-specific hybrids, while allele 709 G is only found in rusts from the tetraploid host group (Fig. 2B.). When performing the association analysis excluding the private SNPs of rusts from coffee diploid species, a significant association was also detected for SNP 665A/G (p<0.01, Wald or G significance tests), for which rusts from coffee diploid species only present allele G and, on the other hand, within rust samples from tetraploid coffees, allele A is more frequent in *C. arabica*- derived rusts (Fig. 2A).

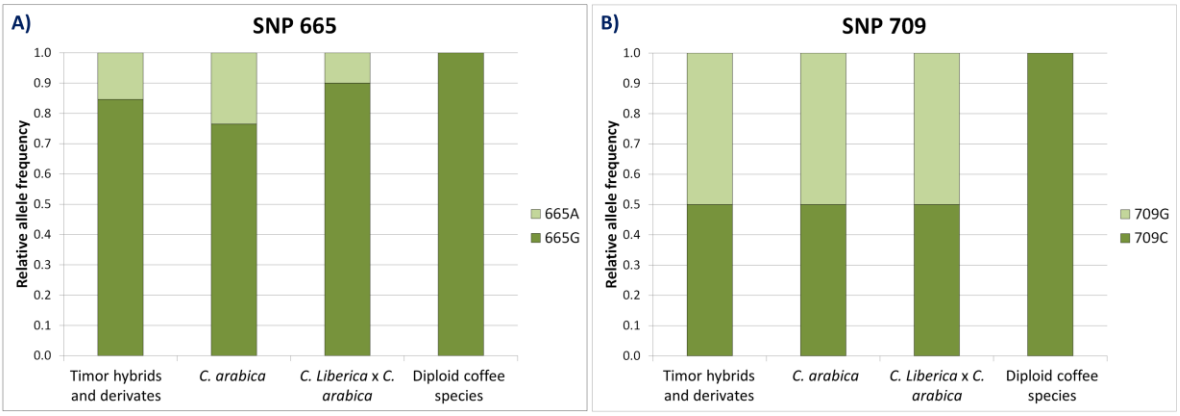

Figure 2. Allele frequency of SNPs significantly associated with coffee hosts as found by MatSAM logistic regressions (p<0.01,Wald or G significance tests): (A) SNP709G/C, showing that allele G is only present in rusts from tetraploid coffee hosts; (B) SNP665A/G, showing that allele A is only present in rusts from tetraploid coffee hosts and is more common in *C. arabica*-derived rusts.

This study provides the first molecular clues on *H. vastatrix* adaptive genetic variation, highlighting the relevance of coffee host as a major selective pressure. The high population genetic distinction and variability found at the protein-coding level suggest a potential adaptive role for this candidate gene, which could be related with host adaptation, also with a promising potential to bring relevant information on gene evolution. Further tracing of insights on the functional activity of this candidate gene is currently in progress through gene expression studies.

ACKNOWLEDGMENTS

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Unveiling the Involvement of Oxidases in the Resistance of *Coffea* spp. to *Colletotrichum kahawae*

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SUMMARY

Cytological, biochemical and molecular studies were undertaken to elucidate the role of oxidases in coffee resistance to *Colletotrichum kahawae* (Ck). Hypocotyls of the coffee variety Catimor 88, resistant to Ck isolate Que2 (from Kenya), were used and compared with the susceptible variety Caturra. Coffee resistance was characterized by a restricted fungal growth associated with hypersensitive-like cell death (HR), monitored by cell autofluorescence and/or browning. The activity of the oxidative enzymes peroxidase (POD) and polyphenol oxidase (PPO) was evaluated. For both genotypes the activity of POD and PPO measured in the infected tissues was, on average, higher than in control samples. Moreover, in the resistant genotype, POD activity started to increase at 24hai which was coincident with the beginning of the observation of HR.

At the molecular level, 33 unigenes with oxidative-related function were identified in an Illumina RNA-seq coffee - Ck database as differentially expressed in Catimor 88 and Caturra infected by *C.kahawae* comparatively with their controls, and grouped into six main classes: multicopper, peroxidase, polyphenoloxidase, germin-like, redoxin domain and isoflavone reductase-like protein. For 20 unigenes, a predominant expression profile showing an increase of activation at 48 and/or 72hai in Catimor 88 when compared to Caturra was detected. For the other 13 unigenes, the main expression profile revealed repression at all time points, for both genotypes. Gene validation and expression profiling is being performed through qPCR during key stages of the infection process.

INTRODUCTION

Colletotrichum kahawae (Ck), the causal agent of Coffee Berry Disease (CBD), is responsible for the most devastating Arabica coffee disease in Africa at high altitude, and represents an imminent threat for coffee cultivation in America and Asia. It has long been recognized that increased knowledge on the key mechanisms of plant resistance is necessary to breed efficiently for durable resistance. Coffee resistance to Ck is characterized by restricted fungal

growth associated with rapid hypersensitive-like cell death (HR). In different pathosystems, the rapid loss of cell integrity during the HR, has been associated with the production of reactive oxygen species (ROS) and an increase in oxidizing enzymes. This work aims to elucidate the role of oxidases in coffee resistance to Ck. Based on field resistance to Ck in Kenya, Catimor 88 (Timor Hybrid derivative) was selected as resistant genotype to the isolate Que2 from Kenya, comparatively with the susceptible variety Caturra.

MATERIALS AND METHODS

Hypocotyl inoculation

Coffee hypocotyls from the resistant genotype Catimor 88 (from Kenya) and the susceptible genotype Caturra (CIFC 19/1) were inoculated with the *C. kahawae* isolate Que2, from Kenya according to the technique previously described. For the different studies, samples were collected at 12, 24, 48 and 72 hours after inoculation (hai).

Light microscopy

For time-course studies of fungal growth and plant cell responses, cross sections of infected hypocotyl fragments, made with a freezing microtome, were submitted to cotton blue lactophenol staining and epifluorescence test. Observations were made with a microscope Leica DM-2500 equipped with a mercury bulb HB 100W, blue light.

POD extraction and activity evaluation

Proteins were extracted from hypocotyls of both genotypes and protein content was measured using a modified Bradford assay. The activity of guaiacol peroxidase (POD) and catechol polyphenol oxidase (PPO) was determined by the increase in absorbance at 480 and 410nm, respectively.

Differential expression analysis from RNA-Seq data

Differential expressed unigenes previously identified and annotated as bearing an oxidative-related function were retrieved from an Illumina RNA-Seq database. Identification of those genes resulted from a previous analysis of RNA-seq data generated from Catimor 88 and Caturra hypocotyls, inoculated with Ck Que2, at 24, 48 and 72hai. Only unigenes with a posterior probability of being differentially expressed (PPDE) > 0.95 and a $-1.0 \geq \log2$ fold change ≥ 1.0 were considered as such.

RESULTS AND DISCUSSION

In both genotypes, the fungus began to penetrate the hypocotyl tissues by 48hai and the hyphal length was significantly higher in the susceptible genotype than in the resistant one, at 72hai (Fig.1 and Fig.2). As shown in Fig. 2 in the resistant genotype, the first cytological changes were displayed at 24hai in 4% of infection sites and corresponded to the hypersensitive-like cell death – HR (associated with the presence of autofluorescent and/or browning cells). At 48hai and 72hai, HR spread to adjacent cells of the epidermis and of the first layer of cortex, being observed in 16% and 36% of infection sites, respectively. In the susceptible genotype this response was also observed, but in a significantly lower percentage of infection sites (1%- 12%, at 24hai and 72hai, respectively) (Fig.2, Fig.3A-C). The analysis of post-penetration fungal growth stages and host responses were similar to those previously described for the same coffee-Ck interactions

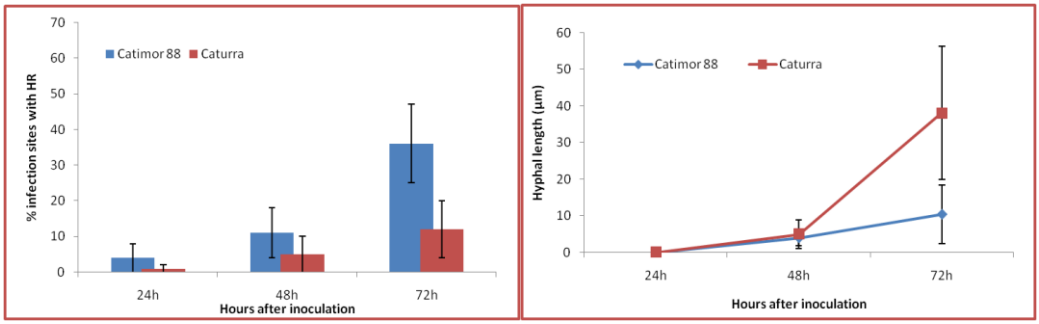

Figure 1. (left) Hyphal length in resistant (Catimor 88) and susceptible (Caturra) hypocotyls, at different time points. The mean values of hyphal length did not differ significantly at 48hai (t= 1.32) but were significantly higher in the susceptible that in the resistant hypocotyls at 72hai (t= 5.05; P≤0.001). Figure 2. (right) Percentage of infection sites with HR. The mean values did not differ significantly at 24hai (t= 1.33) but were significantly higher in the resistant than in the susceptible hypocotyls at 48hai (t= 2.22; P≤0.05) and 72hai (t= 5.76; P≤0.01). Each value (Figs 1 and 2) is the mean±standard deviation of 2 different experiences (100-150 infection sites were observed per experiment per time).

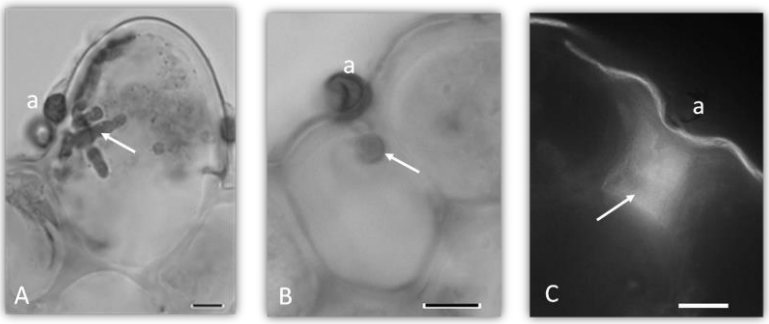

Figure 3. Fungal post-penetration growth stages and plant responses. Light microscope observations, cotton blue lactophenol staining (A and B), epifluorescence test under blue light (C). (A) Infection site showing a melanised appressorium (a) and hyphae (arrow) inside an epidermal cell of the susceptible hypocotyl, 48hai. (B) Infection site showing a melanised appressorium (a) and a vesicle (arrow) inside the epidermal cell of the resistant hypocotyl, 72hai. (C) Infection site showing an apressorium (a) associated with HR-like in one epidermal cell (arrow) of the resistant hypocotyls, 72hai (bars = 10µm)

For both genotypes the activity of PPO measured in the infected tissues was, on average, higher than in control samples (data not shown). In the resistant genotype Catimor 88 (Fig.4A) POD activity started to increase by 24hai reaching the highest value at 72hai in the infected tissues, when compared to the control (non-inoculated hypocotyls). In the susceptible genotype Caturra (Fig.4B), no differences in POD activity were detected between samples (inoculated vs control). These results suggest the involvement of POD in the resistance mechanism of *Coffea* spp. to Ck.

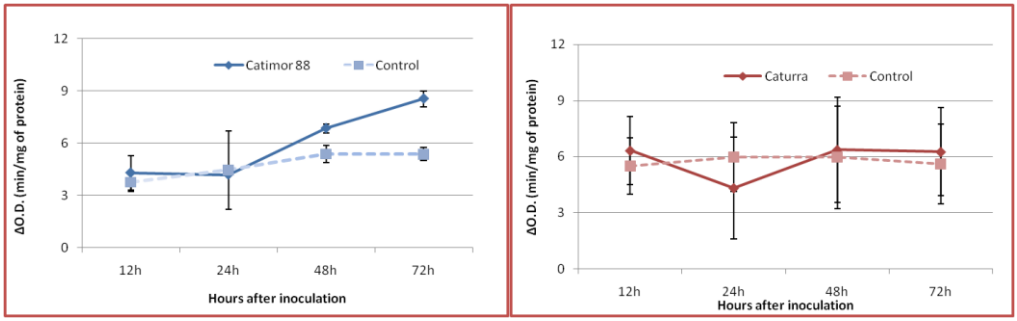

Figure 4. POD activity in healthy (control) and infected hypocotyls of the resistant genotype Catimor 88 (A) and the susceptible genotype Caturra (B). POD activity was expressed as O.D.480nm min^{-1} g^{-1} dry weight and hypocotyls were harvested at different times after inoculation.

Based on an Illumina RNA-seq coffee-Ck database previously generated [10], 33 unigenes with annotation as oxidative-related function were identified as differentially expressed in Catimor 88 and Caturra infected by Ck comparatively with their controls. These unigenes can be grouped into six main classes of oxidases: multicopper, peroxidase, polyphenoloxidase, germin-like, redoxin domain and isoflavone reductase-like protein. An integrative *in silico* analysis of their expression profiles revealed that, in general for 20 unigenes, Catimor 88 presents a higher expression in genes that are activated at 48 and/or 72hai, being normally repressed at 24hai (Fig.5A). Also, for the other 13 unigenes for both genotypes, the predominant expression profile found is repression in all time points (Fig.5B). To the best of our knowledge, this study represents the first integrative attempt to understand the involvement of oxidative enzymes in coffee resistance to Ck.

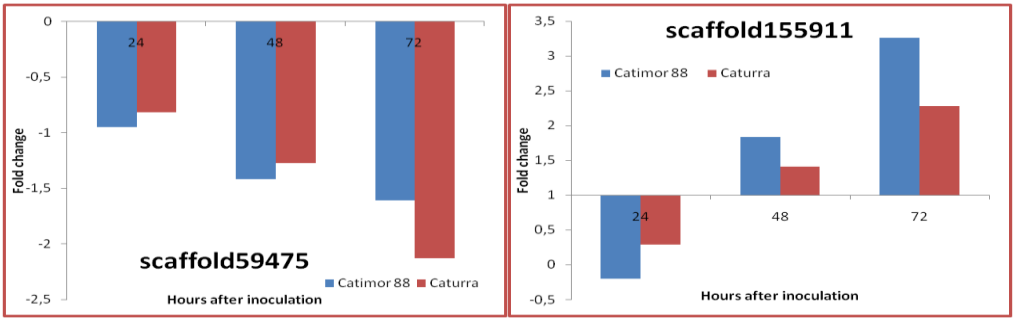

Figure 5. Predominant expression profiles found in the oxidase classes studied, peroxidase (A) and isoflavone reductase-like protein (B), based on a differential expression analysis previously performed with Illumina RNA-seq data from coffee genotypes susceptible (Caturra) and resistant (Catimor 88) to Ck.

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Transcriptional Profiling of Compatible and Incompatible Coffee - *Colletotrichum kahawae* Interactions through RNA-Seq Analysis

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SUMMARY

Coffee berry disease (CBD), caused by the fungus Colletotrichum kahawae, is considered the biggest threat to Arabica coffee production in Africa at high altitude. In C. arabica plantations, CBD can cause up to 20-50% of crop losses, reaching 80% in years of severe epidemics if chemical control is not applied. Several coffee improvement strategies for disease control were developed particularly in Kenya, where variety Ruiru 11 was launched several years ago, and recently some lines of Catimor were selected to release new coffee cultivars with resistance to CBD and leaf rust. One such line is Catimor 88, which is still being used in breeding programmes. Therefore, breeding for coffee resistance remains a powerful strategy to fight CBD, in an economic and sustainable manner. With the purpose of gaining some insights on coffee resistance process, a RNA Illumina sequencing approach was used to characterize the transcriptional profile of two coffee genotypes, respectively resistant (Catimor 88) and susceptible (Caturra) to C. kahawae, during the early stages of the infection process. Three time points were selected (24, 48, 72 hours post inoculation), and two experimental replicates collected. Sequencing of these twenty four libraries provided a total of 1,552,057,070 paired-end reads of 100bp. The data was analyzed to recover genetic information of the fungus and assess differential gene expression when comparing inoculated vs control samples, inoculated susceptible and resistant samples and different time point samples. The data was trimmed and two assemblies were made: one with the infected libraries and another with the control libraries. The first assembly was used to identify plant and fungus sequences, based on triplet nucleotide frequencies (MIPS-EST3) and a blast-based pipeline. From the 209580 contigs, 198036 were classified as plant, 653 as fungus, 8564 and 119 as potentially plant/fungus, respectively, while 2208 remained unclassified. From the assembly of the control libraries, a total of 65759 unigenes with an average length of 1398.64 bp were used as reference for the differential expression analysis. The reads of each library were mapped and their expression quantified using the software RSEM. The differential expression software EBSeq identified 2344 unigenes in the total of comparisons. After KOG and KEGG annotation of the differential expressed unigenes, it was possible to identify two main domains: plant development and defense response. Within plant development, categories involved in the production of energy, such as starch and sucrose metabolism, are enriched, while defense response is majorly represented by categories such as phenylalanine metabolism and phenylpropanoid biosyntesis.

INTRODUCTION

Colletotrichum kahawae, the causal agent of Coffee berry disease (CBD) is the most devastating threat to *Coffea arabica* production in Africa at high altitude, and its dispersal to Latin America and Asia represents a serious concern. The socio-economic repercussions of CBD in Africa and the threat that it represents to other coffee producing countries, led to the need of better understanding the molecular bases of coffee resistance, which will provide useful information to breeding programmes. In this work, a RNA Illumina sequencing approach was used to study coffee defense response to *C. kahawae* by comparing reactions of resistance and susceptibility, and to identify differential expressed genes.

MATERIALS AND METHODS

Inoculation of coffee hypocotyls and sampling

Resistant hypocotyls of Catimor 88 (from Kenya) as well as susceptible hypocotyls of cultivar Caturra (CIFC 19/1) were inoculated with the *C. kahawae* isolate Que2, from Kenya. Hypocotyls at the soldier stage were inoculated as previously described. Hypocotyls were harvested at 24, 48 and 72 hours post inoculation (hpi), corresponding to different stages of the infection process. Two independent experiments were conducted and 40 hypocotyls were collected for each coffee genotype and time points, both at control and inoculated conditions.

Extraction and sequencing

Total RNA was isolated from hypocotyls of all samples with SpectrumTM Plant Total RNA Kit (Sigma-Aldrich, USA), according to the manufacturer's instructions. mRNA-seq libraries for each independent sample, in a total of 24, were constructed at ARKs Genomics (UK) and subsequently sequenced on a Illumina HiSeq2000.

Bioinformatics analysis

The library-derived reads were properly clean, taking into account the need to recover *C. kahawae* genetic information, using TrimGalore!, and for the steps of expression analysis, using SeqTrimNext. Two transcriptomes were assembled, one for fungus-plant sequence separation and other to use as reference for all the downstream analysis. The assemblies were made using the softwares Velvet/Oases with a k-mer of 31. The contigs were clustered with CD-HIT-EST and scaffolded using SSPACE. A custom blast-based pipeline was used for redundancy cleaning. The reference transcriptome was annotated using Blast2Go and RAPSEARCH, against the KOG and KEGG databases. Using the plant-fungus sequence separation transcriptome, two methods were applied to classify contigs as fungus and plant: MIPS-EST3 and a blast pipeline adapted from Fernandez et al. (2012). For the expression analysis, the reads were mapped back to the reference transcriptome, and their expression quantified using RSEM. The differential expression analysis was performed by the R package EBseq. Only unigenes with a posterior probability of being differentially expressed (PPDE) > 0.95 and a $-1.0 \geq \log2$ fold change ≥ 1.0 were considered as such.

RESULTS AND DISCUSSION

Transcriptome assembly and annotation

After the steps of cleaning, assembly, clustering, scaffolding and redundancy removal, the coffee reference transcriptome resulted in 65759 scaffolds with an average length of 1398.64bp and N50 of 2623. On the other hand, the plant-fungus sequence separation transcriptome, skipping the steps of scaffolding and redundancy cleaning, resulted in 209580 contigs with an average length of 1410.75bp. The reference transcriptome annotation using the KOG database revealed 23252 annotations (35.36% of the transcriptome), where the categories better represented were "metabolism" (6.91%) and "celular processes and signalling" (9.49%). The KEGG annotation was also performed and 5.85% of the transcriptome (3850 unigenes) was successfully identified in 136 metabolic pathways.

Plant-Fungus sequence identification

Combining the results from the two methods used for plant-fungus identification, the contigs were assigned to 5 different categories. From the positive identification provided by both methods, 198036 contigs were considered as "plant" and 653 as "fungus". From the classification only by one of the methods, 8564 and 119 were respectively considered as "Potentially plant" or "Potentially fungus", while 2208 contigs were not classified due to contradictory results of both methods. The unclassified category may include not only contigs that failed to be properly classified, but also contaminant sequences, which are neither from coffee nor from *C. kahawae*.

Differential expression analysis

To get some insights on coffee reaction to the infection, two different data comparisons were made: control vs infected and time-point vs time-point.

Table 1. Control vs Inoculated DE unigenes. Shared indicates the number of unigenes present in both genotypes. Values inside brackets correspond to unigenes only expressed in a respective condition and time-point.

	24h	48h	72h	Total
Upregulated				
Resistant	228(18)	671 (0)	1169 (610)	1320
Susceptible	22(8)	517 (0)	520 (235)	761
Shared	3	371	433	
Downregulated				
Resistant	10 (9)	52 (0)	254 (235)	297
Susceptible	3 (2)	38 (0)	102 (94)	135
Shared	0	3	31	

From the comparisons between control and infected libraries, 1841 unigenes were identified as differential expressed. A predominance of unigenes being differentially expressed (DE) in the resistant genotype (1617 vs 895 unigenes) was observed, as well as a predominance of upregulated unigenes (Table 1). At 48 hpi, both susceptible and resistant genotypes did not show unigenes expressed uniquely at that time-point. Furthermore, a predominance of differentially expressed unigenes was detected at 72 hpi for both genotypes when comparing not only time-points, but also control-inoculated samples. A consistent, higher number of

differentially expressed unigenes was found in the resistant genotype, relatively to the susceptible genotype, in both situations. Also, 72hpi seems to be a very active step during the infection process, corresponding to the switch from the biotrophic to the necrotrophic phase in the susceptible genotype, and to the accumulation of phenols and display of HR in 50% of infection sites in the resistant genotype.

Annotation and expression profiles of the differential expressed unigenes

To obtain a general perspective of the biological processes involved in fungal infection, the KOG and KEGG annotations of the DE unigenes between control and inoculated in both genotypes, were compared. The KOG annotation revealed approximately the same categories in both resistant and susceptible genotype. Signal transduction mechanisms and post-translational modification, protein turnover, chaperones were consistently the functional categories better represented. This supports an expected high biological transcriptomic activity of both genotypes during the fungal infection, triggering several signaling mechanisms and increasing protein biosynthesis. KEGG annotation of the same unigenes showed that the pathways involved with phenylalanine and phenylpropaniod biosynthesis and metabolism, are the most representative, in the resistant genotype, while, in the susceptible genotype, "starch and sucrose metabolism" is the most representative pathway, independently of the time-point (Fig. 1). Phenylalanine and phenylpropanoid pathways are known to be related with the defense response in different pathosystems, namely in coffee-*H. vastatrix*, and enzymes like phenylalanine ammonia-lyase (PAL) are activated in the early stages of these interactions and have been implicated with the production of several compounds associated with fungal invasion. With this study, a further step was taken for the understanding of the functional genomics of coffee resistance to *C. kahawae* through the identification of putatively involved unigenes. Such knowledge may be potentially applicable to similar pathosystems.

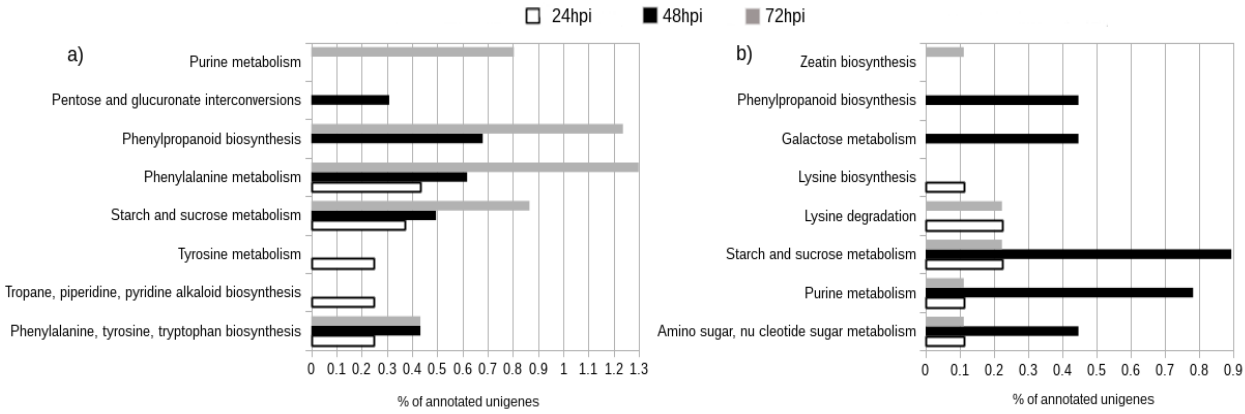

Figure 1. Top 5 KEGG annotations of the unigenes identified as differentially expressed in the resistant (a) and susceptible (b) genotypes comparisons between control and inoculated at 24, 48 and 72 hpi. The percentage of annotated unigenes is relatively to the total of differentially expressed unigenes of each genotype.

ACKNOWLEDGMENTS

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Validation of RT-qPCR Reference Genes for Expression Data Analysis in *Colletotrichum kahawae*

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SUMMARY

Colletotrichum kahawae is an emergent pathogen causing severe epidemics of Coffee Berry Disease on Arabica coffee crops in Africa. Currently, the molecular processes involved on *Coffea arabica* - *C. kahawae* interactions are still poorly understood, as well as the differences in pathogen aggressiveness, making crucial the development of functional studies. Quantitative real time PCR (RT-qPCR) has been one of the most promising approaches to perform gene expression analyses in candidate genes. However, proper data normalization with reference genes is an absolute requirement for qPCR correct measurement of gene expression. In this study, a set of 12 candidate reference genes were selected based on two different approaches for the validation of suitable *C. kahawae´*s reference genes. Six candidate genes were selected from the literature as the most promising reference genes for other related species (actin (ACT), cyclophilin type peptidyl-prolyl cis-trans isomerase/CLD (CYP1), translation elongation factor (EF-1), 6-phosphogluconate dehydrogenase (G6PDH), serine threonine-protein phosphatase (PP1), ubiquitin carboxyl-terminal hydrolase (UBH)) and the remaining were selected from an Illumina RNA-seq dataset based on gene expression stability during the first hours of coffee interaction (24, 48 and 72 hpi) with *C. kahawae*. Gene expression of the 12 candidate reference genes was evaluated at three key time points corresponding to the main stages of pathogenesis (differentiation of melanized appressoria, fungal penetration and establishment of the biotrophic phase, and switch to necrotrophy) for three different interactions with *C. kahawae* isolates bearing different aggressiveness. Since *C. kahawae* is a hemibiotrophic pathogen with substantial biomass variations during the infection process, an additional normalization step was performed. Gene expression stability was assessed using the statistical algorithms incorporated in GeNorm and NormFinder softwares to select the appropriate reference genes. To our knowledge this is the first study on validation of *C. kahawae* reference genes. Taken together this results will provide guidelines for reference gene selection towards a more accurate and widespread use of RT-qPCR to study *C. kahawae*.

INTRODUCTION

Colletotrichum kahawae, the causal agent of Coffee Berry Disease (CBD), is a highly aggressive and specialized fungal pathogen of Arabica coffee (*Coffea arabica*), generating yield losses up to 80%. Though still restricted to the African continent, the eminent threat of *C. kahawae* dispersal to other continents makes crucial performing molecular and functional studies to unveil *Coffea arabica* - *C. kahawae* interactions. Real-time RT-qPCR has become

the gold standard approach for gene expression analysis, but its accuracy depends on the use stably expressed genes as internal controls for data normalization. Despite their wide-spread use, the suitability of reference genes for any type of experiment is not given *"a priori"*, making crucial the validation of the best set of reference genes. So far, most studies have focused on validating a subset of commonly used reference genes for specific contexts. However, the search for new reference genes from a genome-wide background within the experimental context under study, such as Illumina RNA-seq dataset, is a promising approach that has not been extensively explored.

In this work, candidate reference genes were selected using both strategies and tested for RT-qPCR normalization at three key time points corresponding to the main stages of pathogenesis, for three different interactions with *C. kahawae* isolates bearing different aggressiveness. The gene expression stability was assessed using the statistical algorithms incorporated in GeNorm and NormFinder softwares.

MATERIALS AND METHODS

Biological material

in planta - Conidia of *C. kahawae* isolates Ang29, Ang67 and Z12 were produced on extract malt agar after 7 days at 22°C. Hypocotyls of cultivar Caturra (CIFC 19/1) susceptible to the *C. kahawae* isolates were inoculated according to the technique previously described. Plant material was collected according to microscopic observations at key points of pathogenesis (differentiation of melanized appressoria, fungal penetration and establishment of the biotrophic phase, and switch to necrotrophy). *in vitro* - Fresh conidia of *C. kahawae* isolates were collected and spread in sterile distilled water in petri dishes to germinate (18-22 h) with mature melanised appressoria(AP) and spread on liquid medium (Malt peptone) to produce saprophytic mycelia after 72h(M).

RNA extraction and cDNA synthesis

Total RNA was isolated from hypocotyls with the Spectrum™Plant Total RNA Kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. Residual genomic DNA was digested with DNase I (On-Column DNase I Digestion Set, Sigma-Aldrich, USA). RNA purity and concentration were measured at 260/280 nm and 260/230 nm using a spectrophotometer (NanoDrop-1000, Thermo Scientific), while RNA integrity was verified by agarose gel electrophoresis. First-strand cDNAs were synthesized from 1.7 µg of total RNA in 20 µl final volume, using Omniscript RT kit (Qiagen) and oligo (dT)18 primer (MBI Fermentas, Lithuania) following the manufacturer's instructions. For each sample, a final volume of 400 µl of cDNA was obtained and stored at -20ºC.

Candidate gene selection

Six candidate genes were selected from the literature as the most promising reference genes for other related species (actin (ACT2), cyclophilin type peptidyl-prolyl cis-trans isomerase/CLD (CYP1), translation elongation factor (EF-1), 6-phosphogluconate dehydrogenase (G6PDH), serine threonine-protein phosphatase (PP1) , ubiquitin carboxyl-terminal hydrolase (UBH)) and the remaining were selected from an Illumina RNA-seq dataset based on gene expression stability during the first hours of coffee interaction (24, 48 and 72 hpi) with *C. kahawae*.

Quantitative real-time PCR

The RT-qPCR were performed using an iQ5 real-time thermalcycler (BioRad, USA), based on EvaGreen® Supermix (BioRad) as described in [2].

Assessment of expression stability

The biomass normalization with DNA was performed as previously described.The expression stability of each candidate reference gene and the best combination of reference genes were obtained using a pairwise method by GeNorm and a model-based method by NormFinder software. The analysis was performed considering three *in planta* groups (Ang 29, Ang 67 and Z12) and one *in vitro* group. Each group has three collection points from two different assays (experimental replicates), each one with three biological replicates.

RESULTS AND DISCUSSION

Based on searches of the literature a group of previously used reference genes in fungi was selected (ACT2, CYP1, EF-1, G6PDH, PP1, UBH) and the remaining were selected from an Illumina RNA-seq dataset (ck20430, ck28444, ck36020, ck39066, ck48742, ck34620). Specificity of real-time PCR products was confirmed by the presence of a single peak in the melting curve and the presence of a single band with the expected size. The amplification efficiencies range from 1.910-1.975. However some candidate reference genes (ck39066, EF-1, G6PDH, UBH) were not expressed in all *in planta* samples, being excluded from the analysis (data not shown).
As expected for a hemibiotrophic pathogen, substantial biomass variations during the infection process were observed. For some reference genes, the Ct values of candidate genes span from 34 to 24 cycles along the infection process (Fig. 1).This variation in Ct values reflects the variations in the amount of fungal biomass (and therefore RNA) present *in planta* at each moment. This is clearly illustrated by similar variations in *C. kahawae* DNA content in these samples (Fig. 1).

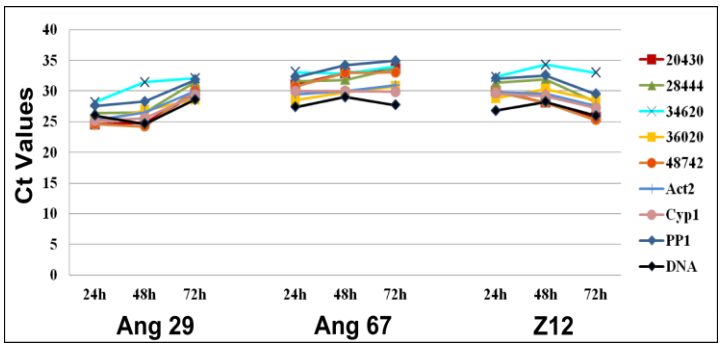

Figure 1. RNA transcription levels of reference genes tested, presented as Ct mean value in the different samples, and respective biomass quantification (DNA of gene ck39066)

However, the statistical tools like GeNorm and NormFinder used to validate the best set of reference genes were developed to assess the stability of reference genes when the amount of RNA is similar between samples [3,4]. To achieve this in *in planta* samples, the approach previously devised for the coffee leaf rust pathogen, *Hemileia vastatrix*, was implemented. The stability of eight candidate genes was evaluated using GeNorm and NormFinder algorithms for several datasets: A- *in planta* Ang 29; B- *in planta* Ang 67; C- *in planta* Z12; D- *in vitro*. As shown in Fig. 2, the results of both programs were consistent, with only slight differences in the ranking order, except for the *in vitro* development stages. Based on these

results, two reference genes were consistently chosen for all *C. arabica* - *C. kahawae* interactions as being the best set of reference genes (Act2, Cyp1), changing only the third most stable gene (A- ck28444; B- ck34620; C-PP1). For the *in vitro* dataset slight differences were found between the best three reference genes chosen from GeNorm (ck48742; ck20430; PP1) and NormFinder (ck20430; PP1; Cyp1) software. The difference observed between the two algorithms could be due to the fact that they use very different methods to assess gene stability. The worst reference gene was ck48742 in all *in planta* samples and ck36020 for the *in vitro* samples.

The analysis carried out by geNorm also enables the determination of the optimal number of reference genes, through the calculation of pairwise variation (Vn/Vn+1) between two sequential candidate genes. High values indicate the need for the inclusion of another gene to obtain a reliable normalization factor, which should contain at least two internal controls. Thus, extra reference genes can be included until the Vn/Vn+1 value is smaller than a threshold of 0.15 as recommended by Vandesompele et al.. In this work, the pairwise variation values, for the three *C. arabica* - *C. kahawae* interactions, are quite below of the recommended cut-off value (data not shown). Therefore an additional step, using a set of genes of interest, will be necessary to test the impact of using two or more genes in the normalization process. For the *in vitro* samples the pairwise variation values are higher than the recommendation value of 0.15, however the lowest value correspond to V3. This work provides the first reported assessment of reference genes for use in expression studies of *C. kahawae* with different aggressiveness and provides, for the first time, the most suitable set of genes for *in planta* and *in vitro* situations.

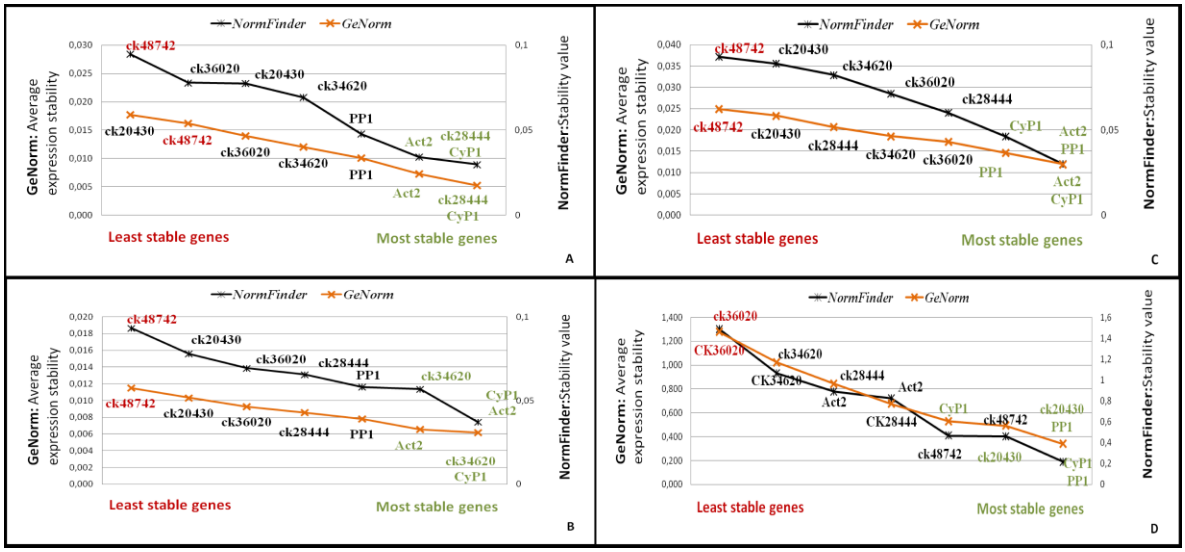

Figure 2. Gene expression stability values of the eight potential reference genes. The stability values on the right axis were calculated with NormFinder (black line) and the average expression stability values M (orange line) on the left axis were calculated with geNorm. Genes are plotted from the least (red colored) to the most stable expressed genes (green colored). A- *in planta* Ang 29; B- *in planta* Ang 67; C- *in planta* Z12; D- *in vitro*.

ACKNOWLEDGEMENTS

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SFRH/BPD/656 86/2009; SFRH/BPD/88994/2012, SFRH/BPD/84508/2012, attributed to AV, ASP, PT and AC, respectively)

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Applicability of Real-time PCR as a Tool for Detection of Rice (*Oryza sativa*) in Ground Roasted Coffee

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SUMMARY

The aim of the present study was to use a Real-Time PCR system for detection of rice as adulterant of ground roasted coffee. The specificity of primer pair ARROZ1 was determined by running reactions with genomic DNA from rice as well as other foods such as corn, wheat, coffee, soybeans and barley. The dissociation curve for ARROZ1 demonstrated that this primer pair was specific for rice detection. The absorbance ratio 260:230 in the *in natura* samples was = 1.6, which indicates a good quality isolate. The method was sensitive and specific to detect and quantify down to 0.5% of rice in roasted coffee.

INTRODUCTION

Coffee is a commodity with high commercial value. This makes it a target of fraudulent mixtures with a diversity of cheaper materials, such as rice, among others. Because of that, many methods using different techniques have been developed in order to ensure the quality and authenticity of coffee [4-8]. However, most of these methods rely on the experience of the analyst, are passive of human error, unspecific or have low sensibility. Currently, Real-Time Polymerase Chain Reaction (PCR) has been applied for food species identification due to its fastness, simplicity, sensitivity and specificity [9]. DNA molecules are highly stable and are present in most biological tissues, what makes of them a good choice for genetic differentiation and identification [10]. While recombinant DNA technology has shown to be a promising tool to determine the authenticity of various processed foods, it has not been used to detect coffee adulteration. Recently, we developed methods for identification of barley and corn in ground roasted coffee [11]. In the present study, we used a Real-Time PCR system for detection of another food product used for coffee adulteration: rice.

MATERIALS AND METHODS

Samples

Fresh samples of rice (*Oryza sativa*) and coffee (*Coffea arabica*) leaves were used as specific target and, barley (*Hordeum vulgare*), corn (*Zea mays*), wheat (*Triticum aestivum*) and soy (*Glycine max*), were used as non-specific targets. All grains were purchased at a local market and were not genetically modified. Ten green coffee beans samples (four *C. arabica* and six *C. canephora*) were obtained directly from producers in São Paulo, Espírito Santo and Minas Gerais, Brazil, and were used as control samples. Coffee was roasted in a fluidized bed roaster (I-roast, USA) at 230°C to give dark color degree (# 35 Agtrom-SCAA); barley and corn were

roasted in a microwave oven to reach the same color as coffee. Samples were ground in a mill (IKA A11basic to pass a 500μm sieve).

To build the five point curve for adulterants quantification, a blend containing 80% of arabica and 20% of robusta roasted beans was used and 0.5%; 1%; 2%; 5% and 10% of rice were added to the coffee blend.

DNA Extraction

Coffee leaves and raw rice were submitted to DNA extraction with CTAB protocol. For roasted beans and rice, DNeasy kit/ buffer CTAB were used. DNA concentrations were determined in all samples by spectrophotometer (Shimadzu UV-1800 Japan) at 260nm.

Primers design

DNA sequences corresponding to the endogenous genes for coffee and rice were surveyed from Genbank (accession number NC008590.1, NM_001049010.2, respectively). Sequences were submitted to the program Basic Local Alignment Search Tool (BLAST) to analyze the similarity with other species. The primer pairs were designed using Genefisher software setting up the size amplicon of 100 pb. Primers were synthesized by Eurofins MWG Operon and their amplification was confirmed using *in silico* PCR runs at BIOINFX (http://www.bioinfx.net/). Coffee primer pair (CAFÉ1): Forward-TTC CGA AGT CCT GGA GAG; Reverse-CGG AGG ATA TCT CAA TCG with a amplicon length of 114 bp. Rice primer pair (ARROZ1): Forward- GTG GAA ATC AGC TCA CTG; Reverse- AAG GCC TAA CTC TGA AGG with an amplicon length 116 bp.

PCR parameters

PCR runs were achieved using SDS-ABIPRISM 7000 (Applied Biosystems). The reaction mixture contained 1 x Power SYBR Green Master Mix (Applied Biosystems) 240nM primers and 50ng DNA in 25μl final volume. Thermal conditions were as follows: 10 min at 95º C, 45 cycles of 15 s at 95 º C and 1 min at annealing temperature (Tm) of primer CAFE1 and ARROZ1.

Limit of detection (LOD) and limit of quantification (LOQ)

The following serial dilutions of DNA template were assayed for each primer pair to obtain standard curves for coffee: 0.0002%, 0.001%, 0.005%, 0.01%, 0.05%, 0.1%, 0.5%, 1%, 5%, 10%, 50%, 100% (= 50 ng). For rice, the dilutions were: 0.01%, 0.05%, 0.1%, 0.5%, 1%, 5%, 10%, 50%, 100% (= 50 ng) Ten replicates for each dilution point were used. The amplification efficiency was calculated based on the standard curves slope, using the following equation: E[%] = [10(-1/(slope)) - 1] x 100. All samples were tested positively with their respective primers, confirming the absence of false negative results that might occur due to PCR inhibition. LOD was considered to be the analyte concentration at which the method detected its presence in at least 95% of the assays (<5% false negative results). LOQ was calculated as follows: MCt-(2 * SD), where MCt = Mean Ct value and SD = corresponding standard deviation [14].

RESULTS AND DISCUSSION

Specifity of primers and their LOD and LOQ

Regarding the specifity of primers, melting curves from specific (G) and non-specific (H) targets are shown in Figure 1. The melting curve for the specific targets demonstra ted that these primer pairs were specific for rice and coffee detection. The nonspecific amplification peaks were attenuated by increasing annealing temperature.

The method was sensitive and specific to identify and quantify levels down to 0.2 and 0.4pg pg/µL extract of coffee (control) DNA, respectively, and 9.0 and 17.0 pg/µL extract of rice DNA, respectively. The amplification reaction efficiency was 107%.

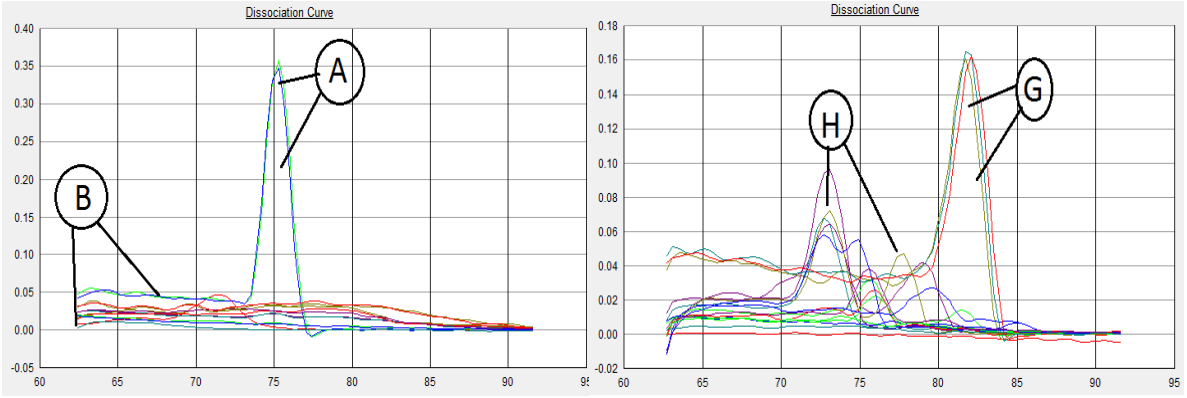

Figure1. Primer Café1 (AB), Arroz1 (GH). Each primer pair was tested with genomic DNA of target (*arabica* / *canephora* coffees and rice) and non-target (wheat, soy, corn, barley).The letters A and G show specific amplifications and letters B and H demonstrate nonspecific amplifications.

Target detection in control samples

Figure 2 shows the Ct values obtained in Real-time PCR analysis from spiked samples (intentionally adulterated) used to build the laboratory adulteration standard curves as well as the equations for percentage estimation. Correlation coefficient (R^2) was 0.998 and the slope -2.9271, indicating amplification efficiencies of 119%. These results are comparable to the parameters of performance data reported in established protocols for validation of analysis for detection and quantification of DNA sequences [12-14]. The method was sensitive and specific to detect and quantify down to 0.5% of dark roasted rice in dark roasted coffee, which was the lowest percentage tested in the present work. Lower percentages of the adulterant will be tested.

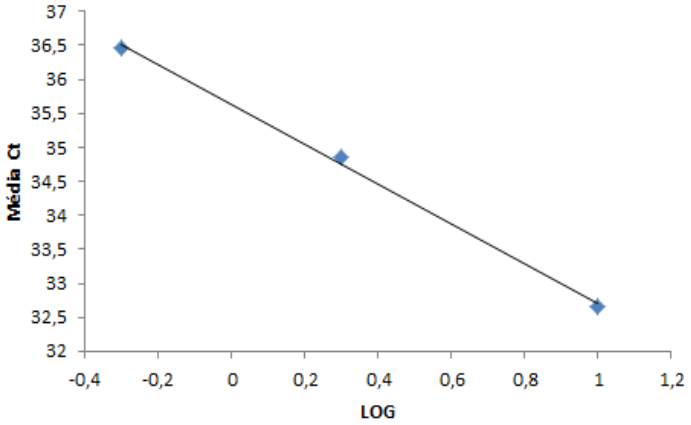

Figure 2. Coffee blends spiked with roasted rice. Value Ct = 8 replicate. Slope: -2.9271 with R2: 0.998.

CONCLUSION

A method was developed for detection of roasted rice in ground roasted coffee. The methodology based on real-time PCR showed to be sensitive and specific to detect small amounts of adulterants, and can be considered a promising tool to be used for adulterants detection by regulatory agencies.

FINANCIAL SUPPORT

FAPERJ and CNPq (Brazil)

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Joint FAO/WHO food standards programme codex alimentarius commission. (2010). Thirty-third Session. Geneva, Switzerland, 5-9 July 2010 Report of the thirty-first session of the codex committee on methods of analysis and sampling. ANNEX II: Validation of a quantitative PCR method.

Cytological Evaluation of the Infection Process of *Hemileia vastatrix* (race XXXIII) in Resistant and Susceptible Coffee

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SUMMARY

The race XXXIII of *Hemileia vastatrix* was recently identified in Brazil, infecting some cultivars that had been released as resistant to coffee leaf rust. To gain new insights into the interaction between coffee plants and this race of the fungus, we evaluated the fungal growth and the plant defence responses in coffee genotypes resistant (Híbrido de Timor – natural *Coffea arabica* x *C. canephora* hybrid) and susceptible (*C. arabica* cv. Caturra) to race XXXIII. This analysis was performed by light microscopy. In the leaves of resistant genotype, the fungus stopped its growth with higher frequency at pre-haustorial stages, inducing the hypersensitive-like response (HR) of the stomatal cells, monitored by cell autofluorescence, which also indicated the accumulation of phenolic-like compounds. Reversely, in the majority of the infection sites (70%) of the susceptible genotype, the fungus was able to colonize the host tissues, produced a large number of haustoria and sporulated around 20 days post-inoculation. The results reported here will improve the knowledge of the interaction between coffee and the race XXXIII of *H. vastatrix*. In addition, the data will be used to define key time points of the infection process for construction of cDNA libraries, aiming at the study of differentially expressed genes in the interaction.

INTRODUCTION

Coffee leaf rust caused by *Hemileia vastatrix* Berk. & Broome is the most important disease of *Coffea arabica* L. and *C. canephora* Pierre ex Frohener in Brazil. The fungus is widely distributed in all coffee producing areas of the country, causing severe economical damages. Although the use of resistant cultivars is one of the main strategies for disease control, the emergence of new races of the fungus has led to the breakdown of resistance in the field. Recently, the race XXXIII was identified in Brazil, infecting some cultivars that had been released as resistant to coffee leaf rust, including the cultivar 'Oeiras MG 6851' [1]. This cultivar is derived from a cross between *C. arabica* cv Caturra Vermelho CIFC 19/1 and Híbrido de Timor (HDT) CIFC 832/1, the main source of resistance in coffee breeding programs. Coffee – rust interactions are governed by the gene-for-gene relationship and the resistance of coffee plants is conditioned at least by nine major dominant genes ($S_H1 – S_H9$) singly or associated [2,3], although other major and minor genes may also be involved [3,4]. During the infection process, *H. vastatrix* differentiates several specialized infection structures, such as germtubes, appressoria, infection hyphae and haustoria. In coffee susceptible genotypes, this process culminates with the colonization of mesophyll cells and sporulation as early as 20 days after infection [5]. For a number of coffee genotypes, the resistance to *H. vastatrix* is frequently expressed after the formation of the first haustorium. Cytological studies have shown that this post-haustorial resistance is associated with the rapid plant cell death,

haustoria encasement with callose and β-1,4-glucans, deposition of phenolic-like compounds, plant cell wall lignification, accumulation of intercellular pectin-like material containing polysaccharides and phenols, and plant cell hypertrophy [6,7,9]. Although these events are well described in the pathosystem coffee - *H. vastatrix*, temporal differences in the fungal development and in the plant responses may occur, depending on the coffee genotype and the race of the pathogen involved. Since, to our knowledge, there is no information about the interaction between coffee and the race XXXIII of *H. vastatrix*, the goal of this study was to evaluate the early stages of the infection process in resistant and susceptible coffee plants, at cytological level. The results reported here will improve the knowledge of this interaction. In addition, the data will be useful to define the cDNA libraries in the RNAseq analysis, aiming at the study of differentially expressed genes in these interactions.

MATERIALS AND METHODS

Coffee plants and rust fungus

Plants of *Coffea arabica* 'cv. Caturra' (CIFC 19/1, genotype S_H5) and of Híbrido de Timor (CIFC 832/1, genotype $S_H5,6,7,8,9,?$) were inoculated with fresh uredospores of *H. vastatrix* race XXXIII (genotype v_5v_7 or $v_5v_7v_9$) to establish a compatible and an incompatible interaction, respectively.

Light microscope observations of fresh tissues

Pre-penetration fungal growth stages (germinated uredospores and appressoria formation over stomata) were visualized on leaf pieces, as previously described [5]. For time course studies of fungal growth and plant cell responses, cross sections of infected leaf fragments, made with a freezing microtome, were submitted to blue lactophenol staining and epifluorescence test [7,8,9]. Autofluorescence is thought to indicate the presence of phenolic-like compounds and cytoplasmic autofluorescence and/or browning is frequently associated with plant cell death [10,11]. Observations were made with a microscope Olympus BX-41 equipped with a mercury bulb HB 100W, UV light (excitation filter 330-385 nm).

RESULTS AND DISCUSSION

By 24 hours after inoculation (hai), in both susceptible and resistant leaves, the uredospores presented a high percentage of germination (around 75%) and 50% of uredospores differentiated appressoria on stomata. No differences were found on susceptible and resistant plants, as also observed by Silva et al. [6,9]. These initial events depend on the specific host surface recognition. Topographical features of the plant cuticle like the dimension of the outer lip of stomatal guard cells act as a signal for differentiation of infection structures. The tip of germ tube is able to recognize the topographical features of the plant surface, increasing the probability of finding a stomatal opening [12]. After appressorium formation over stomata, *H. vastatrix* develops a penetration hypha that grows into the substomatal chamber, producing at the advancing tip two thick lateral branches resembling an anchor. From each lateral branch of the anchor is formed a hypha (haustorial mother cell – HMC) with the subsidiary cells being the first invaded by haustoria. In susceptible leaves, in the majority of the infection sites, the fungus pursues its growth with formation of more intercellular hyphae, including HMCs, and a large number of haustoria in the cells of the spongy and the palisade parenchyma and even of the upper epidermis. On the other hand, in resistant genotypes, the fungal growth inside leaf tissues is stopped at different stages of the infection, with higher frequency in the stage of HMC with formation of few haustoria [6 and references therein, 9].

In the present study, the penetration hypha stage was the one that occurred with higher frequency (about 70%) in the resistant as well as in the susceptible leaves, by 24hai. However, at 96hai, the fungal growth was significantly higher in susceptible than in resistant leaves. In the susceptible leaves, HMC with haustoria and intercellular hyphae were found, whereas in resistant ones the majority (65%) of the infection sites remained as penetration hypha (Figure 1A). By 17 days after inoculation (dai), many intercellular hyphae and haustoria were present in susceptible leaf sections and at 21dai were also found uredinia that represents the final stage of the infection process (Figure 1B).

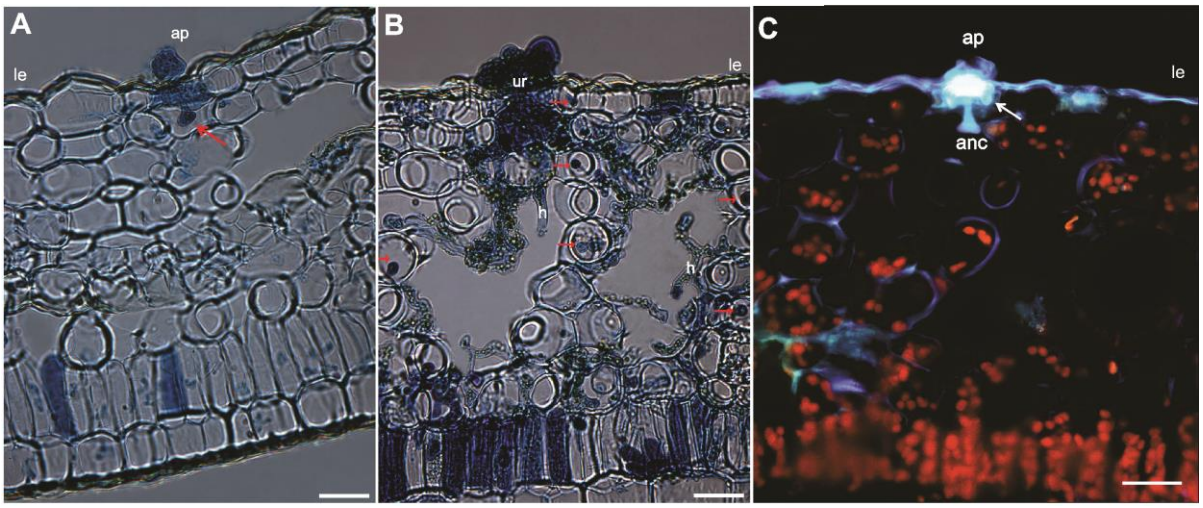

Figure 1. Light microscope observations. A and B. Colonization of leaf tissues by the race XXXIII of *H. vastatrix* in resistant (A) and susceptible (B) leaf tissues, at 96hai and 21dai, respectively. Cotton blue lactophenol staining: Fig. A. Apressorium over stomata and a penetration hypha into the substomatal chamber (arrow). Fig. B. Hyphae (h) and haustoria (arrows) within the cells of the lower epidermis (le) and mesophyll. C. Cytological responses induced by the fungus in the resistant genotype, 24hai. Epifluorescence test (UV light): an anchor (anc) associated with autofluorescence of guard and subsidiary cells (arrow), indicating plant cell death (HR) and accumulation of phenolic-like compounds. Bar=20 µm

Table 1. Percentage of infection sites with hypersensitive cell death and accumulation of phenolic-like compounds (monitored by the presence of autofluorescent and/or browning cells) in leaves of the coffee genotypes resistant and susceptible to *H. vastatrix* (race XXXIII).

Hours after inoculation	% of infection sites with autofluorescent and/or browning cells in leaves of the coffee genotypes	
	HDT832/1 (R) x ± SD	**cv. Caturra (S)** x ± SD
10	0	0
17	18 ± 3	30 ± 5
24	65 ± 13	30 ± 5
48	73 ± 8	30 ± 5
72	83 ± 8	28 ± 6
96	93 ± 3	28 ± 3

R = resistant genotype; S = susceptible genotype; x ± SD = Mean ± Standard Deviation
Data recorded from 60 infection sites in each time point were presented as the combined values of two experiments because no significant differences were found between them.

In resistant genotypes, many defence responses are activated in order to prevent the fungal growth inside host tissues. In the present study, the first cytological responses induced by the fungus (Table 1) were observed particularly in stomatal cells (Fig. 1C) of both coffee genotypes by 17hai, and corresponded to hypersensitive-like cell death and accumulation of phenolic-like compounds. In the resistant coffee genotype, these responses were observed in 18% of infection sites at 17hai, reaching 65% and 93% at 24 and 96hai, respectively (Table 1). In the susceptible coffee genotype, these responses were observed in about 30% of infection sites, from 17 to 96hai (Table 1). Although these values are higher than those referred in other compatible coffee-*H. vastatrix* interactions [6 and references therein, 7], the fungus was able to grow and colonize the host tissues successfully (Figure 1B). Typically, the HR is associated with race-specific resistance involving gene-for-gene interactions [11,13]. On the other hand, as in the present work, several studies agree that accumulation of phenols may be associated with cell death in host resistance, thus being one of the first lines of plant defence against infection [8,9]. These cytological observations indicate that the resistance of HDT832/1 to race XXXIII of *H. vastatrix* is pre-haustorial, contrary to the post-haustorial resistance that is generally described for coffee - *H. vastatrix* interactions [9]. This finding is consistent with the results of Diniz et al. [8] that suggest that the rapid resistance response preventing formation of haustoria may be the basis for the longer durability of the resistance of HDT832/2 to *H. vastatrix* races. However, further studies are required to improve the knowledge of the interaction between coffee and the race XXXIII of *H. vastatrix*, once this race was able to overcome the resistance of derivatives of HDT under field conditions [1]. The informations generated in this investigation will increase the knowledge about this pathosystem. Furthermore, they will be used to define key time points of the infection process for construction of cDNA libraries, aiming to the study of differentially expressed genes in the interaction between coffee and the race XXXIII of *H. vastatrix*.

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Functional Analysis of *CcDREB1D* Promoter Region from Haplotypes of *Coffea canephora* through Genetic Transformation of *Nicotiana tabacum*

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SUMMARY

In many higher plants, DREB genes were shown to be involved in the transduction pathways of abiotic stress. Previous results showed that *CcDREB1D* gene expression increased under drought stress in leaves of drought-tolerant clone 14 of *Coffea canephora* Conilon but not in those of the drought-susceptible clone 22. By sequencing the *DREB1D* promoter regions of these two clones, several nucleic polymorphisms ("single nucleotide polymorphism" [SNP] and insertion/deletion [INDELs]) were found. In order to know if these polymorphisms could explain the differences of *DREB1D* gene expression observed between the clones 14 and 22, 5' deletions of several alleles of the *CcDREB1D* promoter regions were made and cloned independently into the binary vector pBI101 in order to analyze their ability to control the expression of the *uidA* reporter gene in transgenic tobacco (*Nicotiana tabacum*) plants. This work shows preliminary results of *DREB1D* promoter constructs analyzed in leaves of *N. tabacum*.

INTRODUCTION

Coffee is one of the commodities most exchanged in international markets, with Brazil being a major producing country. It is cultivated in more than 80 countries and 25 million people depend on coffee for their livelihoods in Latin America, Africa, and Asia. The annual world production is always subjected to regular oscillations mainly explained by the natural biennial cycle and adverse effects of climatic conditions. Periods of drought and high temperature are key factors affecting coffee plant development and production. In the case of severe drought, flowering can be affected, leading to abortion of developing fruits, and under extreme conditions it may even cause plant death. As a consequence of global warming, coffee-growing geographical regions could also suffer delocalization, leading to important environmental, economic, and social problems. In such a context, the generation of drought-tolerant coffee varieties has now turned into one of the priorities of many coffee research institutes.

Recent studies resulted in the identification of many candidate genes for drought tolerance presenting contrasted differential expression profiles between genotypes. Among those, genes involved in the water stress response pathway, such as the DREB transcription factors were also identified. Results of *CcDREB1D* gene expression in the leaves of clones 14 (tolerant) and 22 (sensitive) of *C. canephora* Conilon, showed higher expression for clone 14 than clone 22 under water stress conditions [4]. After sequencing, the polymorphisms identified in the

promoter regions of *CcDREB1D* genes in clones 14 and 22, may indicate the participation of three haplotypes (15, 16 and 17) in the genetic control for drought tolerance. With the aim of studying the participation of these polymorphisms in the response to drought, several genetic constructions of the *CcDREB1D* promoter regions were made in the pBI101 binary vector and tested by genetic transformation of *Nicotiana tabacum* cv. SRI through their capacity in controlling the expression of the *uidA* reporter gene coding for the *β*-glucoronidase (GUS) enzyme.

MATERIALS AND METHODS

Recombinant (pBI-derivatives) plasmids and controls (pB121-positive and pB101-negative) were amplified into *E. coli* and introduced into *A. tumefaciens* EHA105 for genetic transformation of tobacco. Histochemical GUS assays were performed with leaf explants of control and transformed T0 plants grown *in vitro* and subjected to a dehydration assay as previously described. In that case, a single T0 plant was randomly selected plants for each construction, transferred onto filter paper and leaf samples were regularly collected (2, 4 and 6h of drying). For gene expression, leaf explants were frozen in liquid N_2, total RNAs were extracted and treated as previously described. The qPCR was performed with SYBR green fluorochrom (SYBRGreen qPCR Mix-UDG/ROX, Invitrogen) according to the manufacturer and using a FAST7500 apparatus (Applied Biosystems). For each sample, *uidA* expression levels were standardized with the expression of *NtACT* gene (endogenous control of constitutive expression) coding for the actin. Data were treated by SDS 2.0.1 program (Applied Biosystems). Expression levels were calculated with the ΔC_T (C_T [*uidA*] - C_T [*NtACT*]) method and expression levels were expressed in relative quantification by calculating the values of $2^{-\Delta\Delta CT}$. The primer pairs used were:

•	*uidA gene*	*GUS-F*	*5'-GCACTAGCGGGACTTTGCAA-3'*
•		*GUS-R*	*5'-CGCGAAGCGGGTAGATATCA-3'*
•	*NtACT gene*	*NtACT F*	*5'-CCACTGCTGAACGGGAAATT-3'*
•		*NtACT R*	*5'-GCTGCTCTTGGCAGTGTCAA-3'*

RESULTS AND DISCUSSION

Histochemical tests indicated a strong GUS activity (high coloration) for the positive control (pBI121) while no activity was detected for the negative control (pBI101) (Fig. 1). For the transformed T0 plants containing the pDREB1D::*uidA*, different results were obtained with the allelic isoforms tested. For example, no GUS activities were detected in leaves of tobacco plants transformed by pD14-hp15D and pD14-hp16D constructions. For plants transformed by the pD22-hp17D construction, GUS activities were weakly detected mainly in midrib and lateral veins of unstressed plants (Fig. 1 - sample A3). However, GUS activities increased in lateral veins under dehydration (at 2h [sample B3] and 4h [sample C3]) and decreased at 6h of treatment (sample D3). These results showed that when allelic forms of DREB1D coffee promoters were able to function in tobacco, they can be considered as weak promoters compared to the strong and constitutive CaMV35S promoter. They also suggested that the coffee pD22-hp17D promoter was activated by dehydration in tobacco.

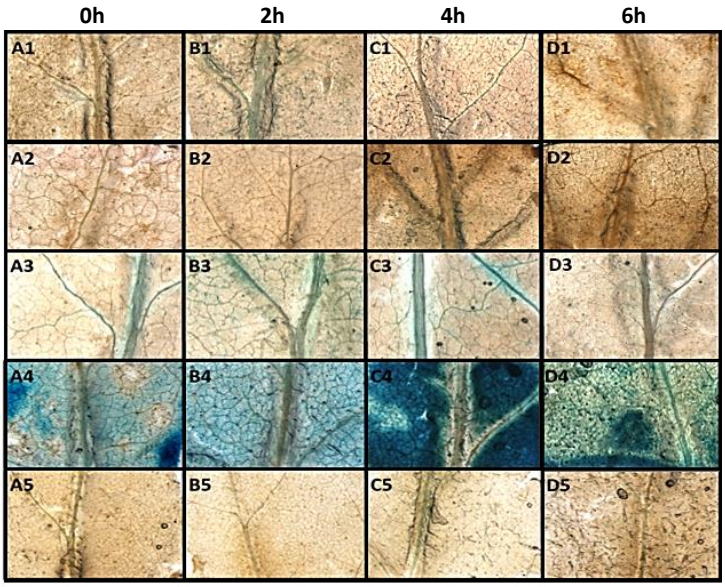

Figure 1. Histochemical test for detection of *β*-glucuronidase activity (GUS) in leaves of *N. tabacum* plants transformed with the pDREB1D::*uidA* constructions and subjected to dehydration. pDREB1D constructions were pD14-hp15D (A1-D1), pD14-hp16D (A2-D2), pD22-hp17D (A3-D3), pBI121 (A4-D4) and pBI101 (A5-D5) [5,6] subjected to dehydration (A = control [0h]; B = 2 h; C = 4 h; D = 6 h).

In order to check if expression of *uidA* reporter gene really induced under dehydration in T0 tobacco plants transformed by pD22-hp17D, its expression was tested by RT-q PCR (qPCR) (Fig. 2). As expected, high *uidA* gene expression levels were observed in leaves transformed by the pBI121 vector, confirming the high strength of the CaMV35S promoter in tobacco.

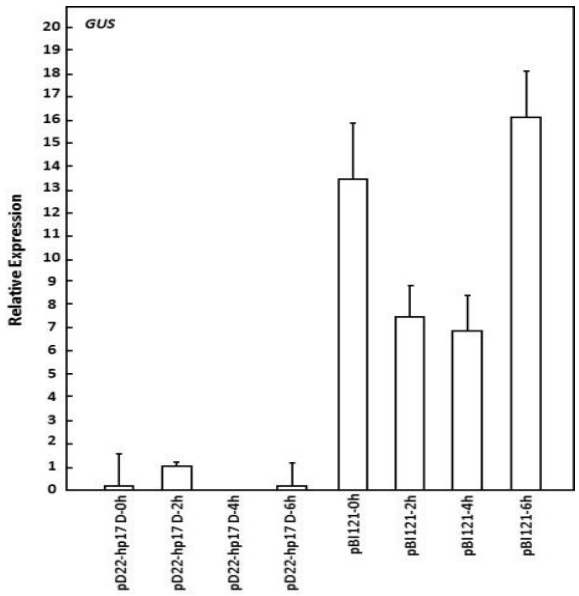

Figure 2. Expression of *uidA* reporter gene analyzed by RT-qPCR in leaves of transformed (T0) plants of *N. tabacum* subjected to dehydration. The expression was tested in a single T0 transformed plant randomly selected for the pD22hp17D and pBI121 (positive control) constructions. For both constructions, leaf explants were collected before (0h) the dehydration test, as well as at 2h, 4h and 6h of drying. Transcript abundances were standardized with the expression of the reference gene

***NtACT*. Relative expression values (expressed in arbitrary units) are the mean of at least three technical repetitions ± SD. Results are expressed using the sample pD22hp17D-2h as an internal calibrator (relative expression = 1).**

In leaves of plants transformed by pD22-hp17D, *uidA* gene was barely detected without (0h) stress. Even weak, this basal expression confirmed the existence of the GUS enzymatic background previously observed by colorimetric assays in unstressed leaves. A slight induction of the *uidA* gene expression was also observed after 2h of dehydration. However, *uidA* gene expression was undetectable at 4h of dehydration. At 6h of dehydration, *uidA* gene expression was equal to that detected at the beginning (0h) of the experiment. It is also worth noting that the expression of *uidA* the reporter gene was not induced by dehydration in tobacco plants transformed with the different constructions of haplotypes (15 and 16) of the *CcDREB1D* promoter (data not shown).

Altogether, these results showed that:

- not all DREB1D promoter-sequences of *C. canephora* were able to function in transgenic tobacco. This suggests that (i) the nucleic polymorphisms pre-identified in these sequences play an important role in controlling *CcDREB1D* gene expression and that (ii) the functioning of the allelic forms of coffee pDREB1D promoters is different in tobacco, suggesting that the polymorphisms identified in these promoters participate in the regulation of these sequences,
- when functioning in tobacco, these coffee pDREB1D promoters are weak compared to the CaMV35S promoter,
- the haplotype 17 of the pDREB1D (pD22-hp17D), derived from *C. canephora* clone 22, was induced by dehydration in the tobacco leaves. This shows that the molecular mechanisms implicated in the regulation of the gene expression in response this stress are (at least partially) conserved between coffee and tobacco plants.

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Phenotyping and Genotyping a *Coffea canephora* Population, Cultivated at High Altitude, Aiming at a GWS Program for Coffee

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SUMMARY

Significant advances recently occurred in *Coffea canephora* research, such as the completion of its full genome sequencing. The information generated can be used in advanced molecular approaches for genetic improvement, like genome-wide selection programs (GWS). The purpose of this study was the phenotyping and genotyping of 403 *C. canephora* individuals cultivated at *Cerrado* climatic conditions characterized by a relatively high altitude (>1000m) and a long dry season. Phenotypic evaluations started in 2012 with the measurement of several agronomical traits such as vigor, secondary branching, leaf-rust susceptibility and precocity of fruit maturation. Production (evaluated in fruit liters) of each plant was also measured during two consecutive years (2012 and 2013), along with a detailed classification of fruits harvested in 2012 (bean size, weight, defects and sieve). In addition, predawn-leaf water potentials (Ψ_{pd}) were evaluated during the dry season of 2013. Furthermore, genotyping by sequencing (GBS) of 403 plants was performed using the nextRAD methodology, while genetic analyzes were done through Cervus and Adegenet softwares. Results obtained allowed to conclude there is a great potential for cultivation of *C. canephora* selected clones under irrigated conditions at high altitudes. The phenotypic and genotypic diversities studied with our population also seems suitable for GWS studies in coffee.

INTRODUCTION

There are approximately 120 *Coffea* species, among which two are economically important: the tetraploid *Coffea arabica* L. (2n = 4x = 44) and the diploid *C. canephora* (2n = 2x = 22). *C. canephora* is an allogamous species that displays genetic self-incompatibility. Due to its cross-fertilization properties, it has greater genetic variability than *C. arabica*, being more resistant to diseases and pests and able to adapt to different climatic conditions. It is commonly grown at low and medium altitudes, even in drought-prone areas, as it shows greater tolerance to water deficit.

Significant advances in *C. canephora* genomics have occurred in recent years resulting in new tools to help accelerate the genetic improvement of this species. As an example, one can cite the recent conclusion of the genome sequencing of *C. canephora*, a future reference for

advanced molecular genetics to be applied directly on the improvement of this species, such as genome-wide selection programs (GWS). The goal of the present study is to genotyping and phenotyping 403 individuals of a *C. canephora* population cultivated in *Cerrado* climatic conditions characterized by a relatively high altitude (>1175 m) and a long dry season under.

MATERIALS AND METHODS

Plants corresponded to a population of *C. canephora* Conilon planted in 2009 at the experimental field of Embrapa Cerrados research center (Planaltina, DF, Brazil). Several parameters such as production, predawn-leaf water potential (Ψ_{pd}), sieve, 100 grains weight, classification as to defects and fruit morphology, were evaluated for the 403 cultivated individuals. In addition, genomic DNA from leaves of these individuals was extracted to perform genotyping by means of the nextRAD approach. Ψ_{pd} were measured the 4th september 2013 after 50 days of water suspension and before the return of irrigation. Using the genotyping data, the values of genetic diversity such as, observed heterozygosity (Ho), expected heterozygosity (He), null-allele frequencies (F) and polymorphism information content (PIC), were calculated by Cervus and adegenet software.

RESULTS AND DISCUSSION

The frequency distribution of the average production data were obtained during the 2011-2012 and 2012-2013 harvests (Fig. 1). For a great part of the population (approximately 170 plants), the productivity ranged between 11.32 to 14.71 L.

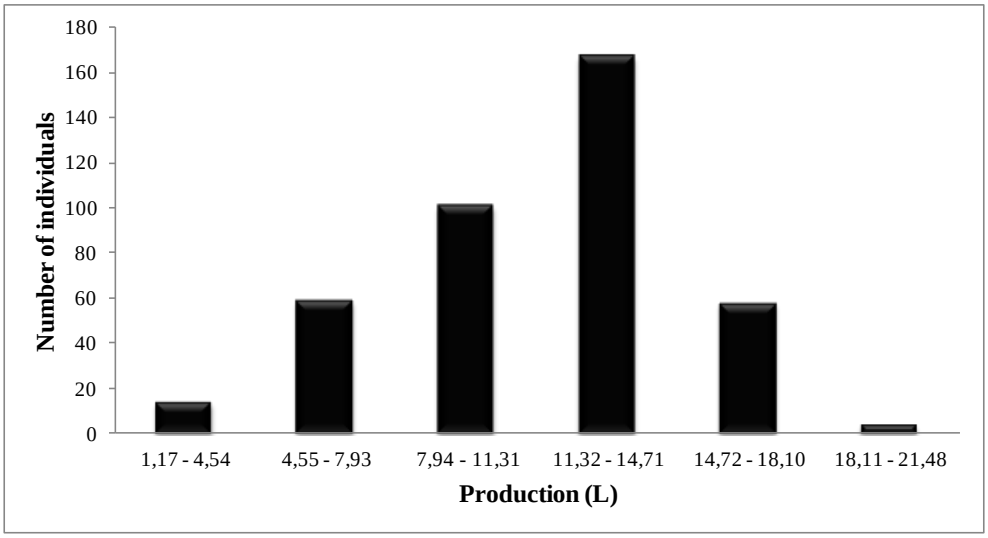

Figure 1. Frequency distribution of average production in liters (L) of two years (2011-2012 and 2012-2013) for a population of 403 selected individuals of *C. canephora*.

Figure 2 shows the Ψ_{pd} for 403 individuals selected in the *C. canephora* population. Most of them had values ranging from -1.0 to -0.5 MPa. As weather conditions were the same for the whole population, significant variation of Ψ_{pd} values, characterized a great diversity for drought tolerance within the studied population, with the presence of drought-tolerant (-1.0 MPa > Ψ_{pd}) and drought-susceptible (-2.0 MPa > Ψ_{pd}) plants.

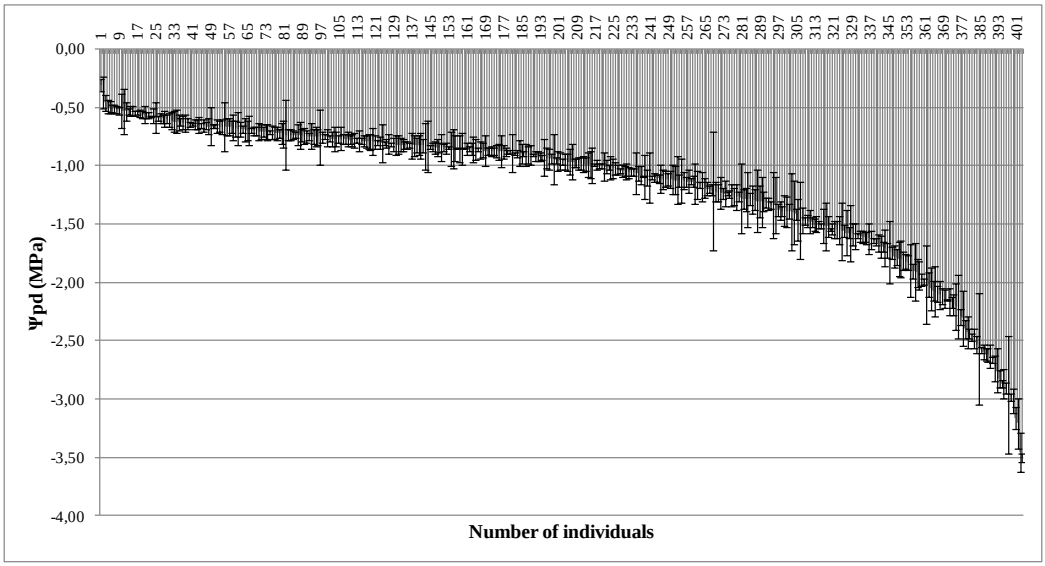

Figure 2. Predawn-leaf water potential (Ψ_{pd}) for 403 selected individuals of a *C. canephora* population.

Averages of production (2011-2012 and 2012-2013 crop/year) and Ψ_{pd} were compared for 17 plants (Figure 3). This allowed the identification of highly productive plants and also drought-tolerant (Ψ_{pd} ± -0.5 MPa), like L12P8 and L8P42. It is worth noting that the plant L5P47 showing the lowest Ψ_{pd} value had an average production above 12L.

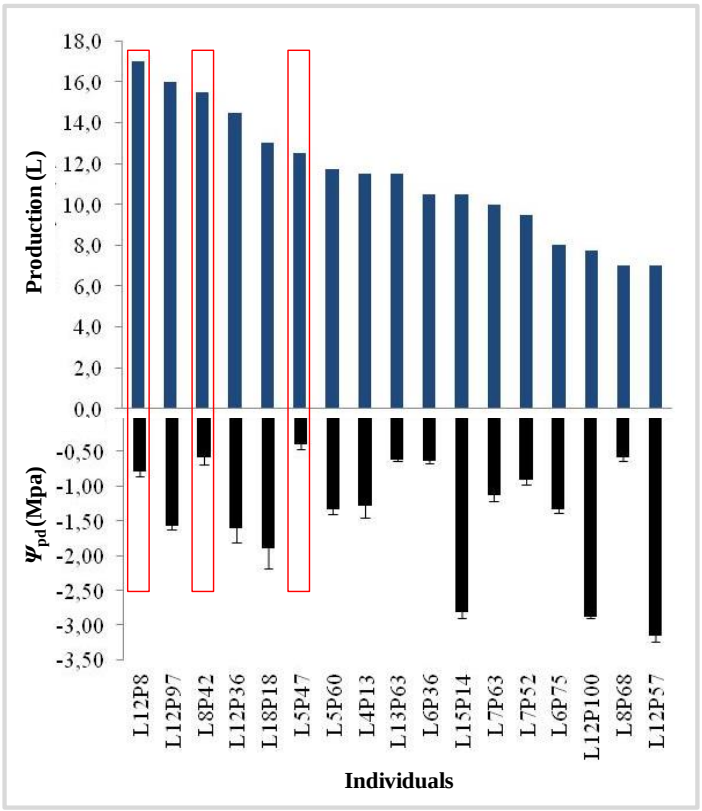

Figure 3. Comparative graph of average production (top) and average Ψ_{pd} (bottom) of 17 plants in a population of *C. canephora*. Red outlines indicate plants that stood out for both indices.

Through GBS approach, a total of 5,412 SNPs was identified. These SNPs were distributed along the 11 chromosomes of *C. canephora*, some of them in genes and others in intergenic regions. The mean Ho for all loci was 0.343, ranging from 0.006 to 0.993; for 1,413 loci, Ho was greater than He, presenting negative values for (F). Such behavior is expected at loci with excess of observed heterozygous genotypes. The lowest PIC, 0.063, was registered for SNP 4722, while the greatest, 0.5, related to SNP 525 (Figure 4). The average PIC was of 0.28 and 53% of SNPs had values greater than or equal to 0.3 PIC, indicating a good level of information content.

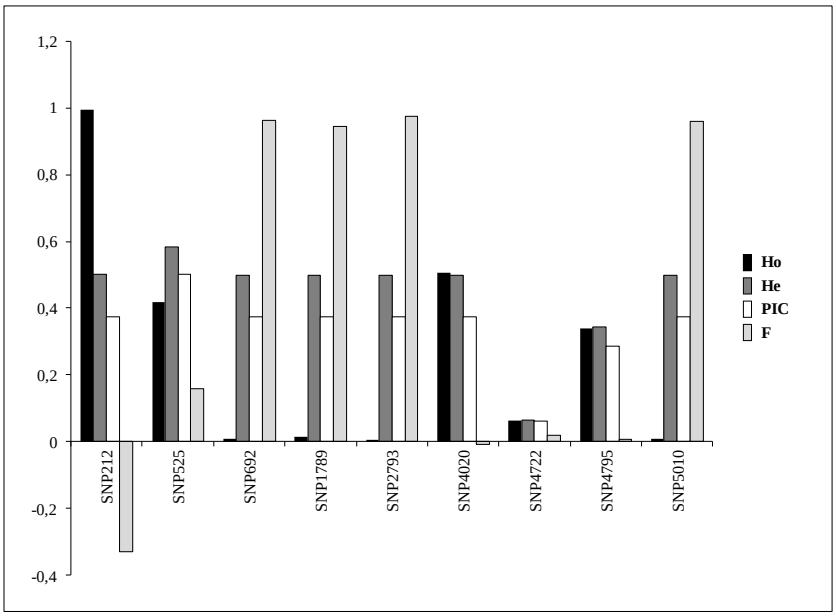

Figure 4. Frequency distribution of Ho, He, PIC and F for SNPs markers in *C. canephora*. Results show that genotype diversity of the studied population seems suitable for genome-wide association studies in coffee.

Results obtained so far, indicates that the population under study is adequate for further association studies as the analyzed individuals display sufficient phenotypic and genotypic diversity.

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Denoeud, F. et al. The coffee genome provides insight into the convergent evolution of caffeine biosynthesis. *Science* 2014, 345, 1181-1184.

Drought Effects on Expression of Genes Involved in ABA Signaling Paythway in Roots of Susceptible and Tolerant Clones of *C. canephora*

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SUMMARY

Our study aims to evaluate the expression of genes encoding elements responsible for the ABA biosynthesis, perception and transduction pathway such as *CcNCED3*, *CcPYL7* and *CcPP2C-1* in roots of clones tolerant (n° 14, 73 and 120) and susceptible (n° 22) to drought of *C. canephora* Conilon grown under controlled conditions (greenhouse) with or without irrigation. For each clone and water regime, total RNA was extracted from roots and the transcriptome profiles was evaluated using 454 sequencing. For these three genes, expression were also analyzed by real-time qPCR to validate the gene expression levels obtained by the RNA-seq *in silico*. In roots of all clones analyzed, results showed that the expression of the genes *CcNCED3. CcPP2C-1* and *CcPYL7* increased with water suspension particularly for the drought-tolerant clone 14.

INTRODUCTION

Coffee is a major agricultural *commodity* in the world and Brazil is the largest producer and second largest consumer. Drought is one of the main limiting factors to the national coffee production, so the selection of tolerant cultivars is of great importance, especially given the expansion of Brazilian coffee to areas subject to water stress [1]. Of the two species of higher marketing, *Coffea canephora* is best suited to the drier regions. Plant growth under drought conditions is influenced by changes in photosynthesis, respiration, translocation, ion absorption, metabolism of nutrients and hormones [2]. The ABA is a very important hormone in response to abiotic stresses, being synthetized in the roots and leaves. The aim of this study was to evaluate the expression of genes directly related to ABA biosynthesis. The results presented here concerned expression studies in roots of *C. canephora* Conilon submitted to drought of genes *CcNCED3*, *CcPYL7* and *CcPP2C-1*, known to encode for key proteins implicated in biosynthesis, perception and transduction of ABA signaling pathway.

MATERIALS AND METHODS

Plant material

Roots were collected from drought susceptible (22) and drought-tolerante (14, 73 and 120) clones of *C. canephora* Conilon grown under controlled conditions (greenhouse) with (I) and without (NI) irrigation as previously described [3]. Under NI condition, predawn leaf water potential (Ψ_{PD}) corresponded to -3.0 MPa for all clones.

RNA isolation, sequencing and in silico analysis

For all clones and irrigation conditions, total RNAs were extracted using TRIzol® reagent Reagent (Life Technologies) according to manufacturer's instructions and treated with RQ1 RNase-Free Kit DNase (Promega) to remove traces of contaminating genomic DNA. cDNAs were synthesized (ImProm II, Promega) and the sequencing was performed by pirosequencing-454 (Roche). *In silico* gene expression analysis was performed using the Q-software package DNAStar (Lasergene), based on the quantification of reads. For both, the cDNA sequences 25.574 resulting from the Structural Genome Project of *Coffea canephora* were used as a reference in the analysis [4]. The data were normalized by RPKM [5].

Expression analysis by RT-qPCR

The qPCR was performed with SYBR green fluorochrom (SYBRGreen qPCR Mix-UDG/ROX, Invitrogen) according to the manufacturer and using a FAST7500 apparatus (Applied Biosystems). For each sample, gene expression levels were standardized with the expression of *CcUBQ10* gene (endogenous control of constitutive expression) coding for the ubiquitin protein. Data were treated by SDS 2.0.1 program (Applied Biosystems). Expression levels were calculated with the ΔC_T (C_T [*gene*] - C_T [*CcUBQ10*]) method and expression levels were expressed in relative quantification by calculating the values of $2^{-\Delta\Delta CT}$.
The primer pairs used were:

*CcNCED3*gene	*CcNCED3*-F	5'- GCCTGGGAAGAGCCTGAAAC -3'
	CcNCED3-R	5'- CCCCTCGTCACATTCATTGAA -3'
*CcPP2C-1*gene	*CcPP2C-1*-F	5'- ATGGCTTGTGGGATGTCATGA -3'
	CcPP2C-1-R	5'- CGTTCTTCTTGTGCCAAAGCA -3'
*CcPYL7*gene	*CcPYL7*-F	5'-GAGAAGCACATTCTTGGGGATCAA-3'
	CcPYL7-R	5'- GGATGCACGGTAAGGATGGA -3'

RESULTS AND DISCUSSION

Study of CcNCED3 gene expression

In roots of all clones analyzed, *in silico* and qPCR experiments showed that *CcNCED* gene expression increased under drought compared to control (irrigation) condition. It is worth noting that this increase was particularly high for the drought-tolerant clone 14 (Fig. 1A and 1B).

Study of CcPP2C-1 gene expression

Once again, a relatively good correlation was observed for this gene between *in silico* (Fig. 1C) and qPCR results (Fig. 1D), demonstrating an increase of *CcPP2C-1* gene expression under drought condition in roots of all clones analyzed. However, this increase under NI condition was higher for the clone 14 than for the others.

Study of CcPYL7 gene expression

For *CcPYL7* gene coding for the ABA receptor PYL 7, no great differences of expression were observed *in silico* between clones and irrigation conditions. However, qPCR

experiments clearly revealed higher expression in clone 14 than in others, as well as an over-expression of this gene under NI condition (Fig. 1E and 1F).

Altogether, our results showed that:

- it exits a relatively good correlation between *in silico* analyses and results of qPCR experiments for the three genes presented,
- the drought-tolerant clone 14 was the only one presenting highest levels of gene induction under water deficit (NI), highlighting the fact that abscisic acid signaling system is of great importance for the mechanisms of drought tolerance in coffee,
- *CcNCED3* gene expression increased in roots of *C. canephora* Conilon under drought stress. This observation, also reported before in other plants [6, 7].

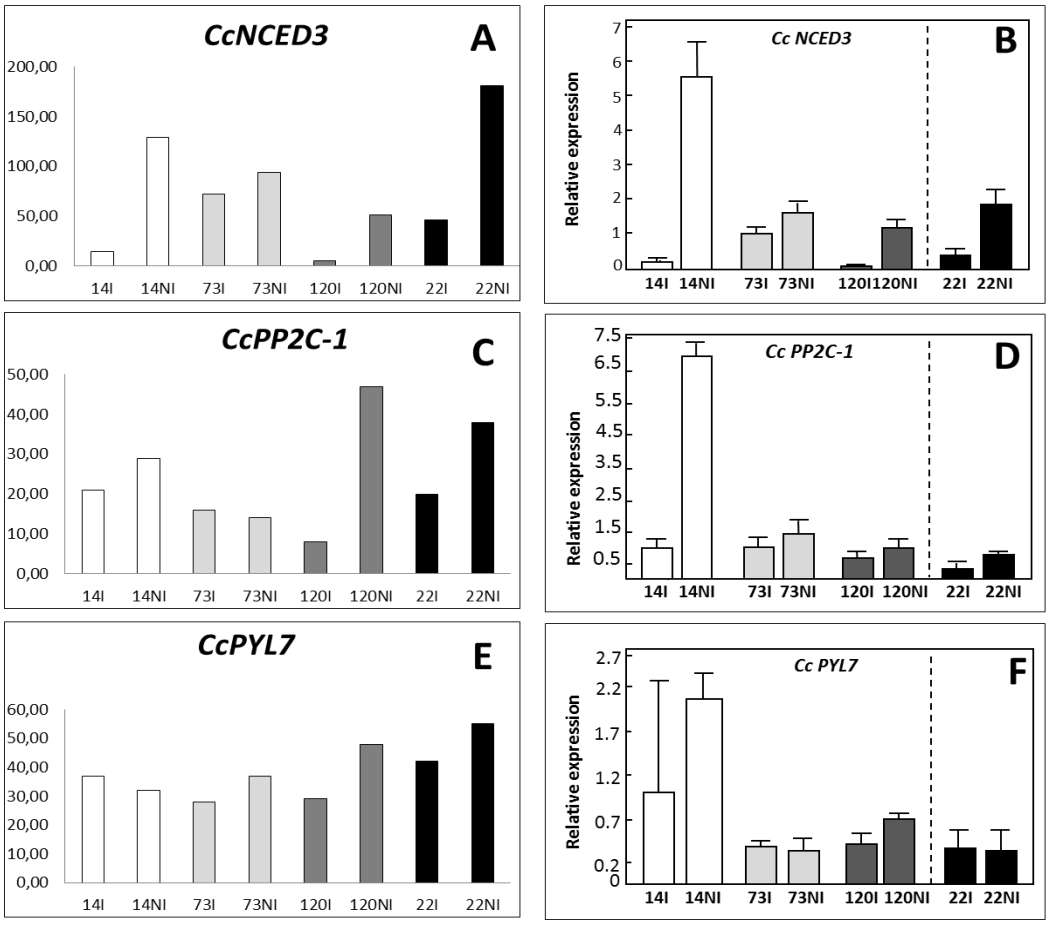

Figure 1. Expression profiles of transcripts that encode 9-cis-epoxycarotenoid dioxygenase 3 (A and B), Phosphatase 2C (C and D) and receiver ABA PYL (E and F) for *in silico* (A, C and E) and qPCR analyses (B, D and F) in roots of clones of *C. canephora* 14, 73 and 120 (drought tolerant) and 22 (sensitive to drought) subjected to water stress (NI: no irrigated) and without stress (I: Irrigated). For qPCR the relative expression (ER evaluated in arbitrary units) was compared to reference gene expression (constitutive expression) *CcUBQ10*. The calibrator used for normalization of qPCR data was the sample 14I (RE = 1).

For ABA signaling in cells, recent studies indicate direct interaction between intracellular receptors of ABA (PYR/Pyl/RCARS) and type 2C protein phosphatases, inducing a cascade of signals that ensure plant tolerance to water stress [8]. From the results presented here for *CcNCED3*, *CcPYL7* and *CcPP2C-1*genes analyzed, it is worth noting that highest induction

of expression were observed in roots of 14 clone under drought stress. This suggested the participation of ABA-dependent pathways in controlling drought tolerance in this clone of *C. canephora* and also highlighted the fact different molecular mechanisms account for drought tolerance in *C. canephora* Conilon [9].

ACKNOWLEDGEMENTS

This work was supported by the CONSÓRCIO PESQUISA CAFÉ and INCT-Café (FAPEMIG/CNPq).

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Genetic Transformation of *Setaria viridis* with *CcUNK8* for Enhanced Drought Tolerance

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SUMMARY

Our work is focused on the identification and functional characterization of orphan genes from coffee, which may have a high potential for innovation and biotechnological applications. This study presents data obtained for one of these orphan genes, called *CcUNK8* (*UNK* for *Unknown*), previously identified as a candidate gene for drought tolerance in coffee. Aiming to identify the functions of CcUNK8 protein, the cDNA of this gene was cloned in an expression vector used to transformed embryogenic callus of *Setaria viridis* by *Agrobacterium tumefaciens*. Thirteen T_0 transformed plants of *S. viridis* were selected and the presence of the inserted T-DNA was confirmed by PCR. Leaf *CcUNK8* gene expression was analyzed by RT-qPCR and presented a variation between transformed plants. Physiological and phenological analyses (with or without drought) were performed in order to see if *CcUNK8* expression enhanced drought tolerance in *S. viridis*.

INTRODUCTION

Coffee is a perennial crop considered one of the most important agricultural commodities in the world. By consequence, aiming at the establishment of tools to help accelerating the genetic improvement of this species, significant advances in coffee genomics have occurred in recent years [1]. As an example, one can cite the recent completion of the complete genome sequencing of *Coffea canephora* [2]. Recent bioinformatics analyses of complete plant genomes indicate that about 20-30% of the total complete set of genes is novel and specific to each species. These genomic sequences do not exhibit any similarity with those already deposited in global databases and are commonly called "no hits" [3]. Recent concepts, called these "no hits" as "orphan genes" and postulate that their emergence resulted of adaptive responses specific to each species as a function of stresses and adverse conditions faced by these plants during the evolutionary process [3]. Large scale coffee EST sequencing also showed the presence of "no hits" in both *C. arabica* and *C. canephora* species. Some of these “no hits” were also recently identified as candidate genes for drought tolerance in coffee [4]. This was for example the case of *CcUNK8* which showed higher over-expression under drought in leaves of drought-tolerant clones of *C. canephora* than in those of the drought-susceptible clone.

MATERIALS AND METHODS

We used the vector pUBI:CcUNK8 containing in the T-DNA region containing the selective gene hpt II that confers resistance to hygromycin and the CcUNK8 full-length cDNA under

the control of the constitutive promoter (pUBI) of maize ubiquitin. The genetic transformation of Setaria viridis by Agrobacterium tumefaciens was performed according to previously reported protocols [5]. For gene expression, leaf explants were frozen in liquid N_2, total RNAs were extracted and treated as previously described [4]. The qPCR was performed with SYBR green fluorochrom (SYBRGreen qPCR Mix-UDG/ROX, Invitrogen) according to the manufacturer and using a FAST7500 apparatus (Applied Biosystems). For each sample, CcUNK8 expression levels were standardized with the expression of SiUBI gene (endogenous control of constitutive expression) coding for the ubiquitin. Data were treated by SDS 2.0.1 program (Applied Biosystems). Expression levels were calculated with the ΔC_T (C_T [CcUNK8] – C_T [SiUBI]) method and expression levels were expressed in relative quantification by calculating the values of $2^{-\Delta\Delta CT}$. The primer pairs used were:

SiUBI	UBI-F	5′-CCGGCGAAACCTACCAGTT-3′
	UBI-R	5′-GAGCCTCCATGGGATAATGC-3′

Ten wild-type and T_2 plants of 32 days old were cultivated in controlled greenhouse conditions (T 24°C, RH 54 %), separated in 2 blocks of 5 plants and then subjected (NI: non irrigated) or not (I: irrigated) to water deficit by suspension of irrigation during 7 additional days. At the end of the treatment, all plants were subjected to destructive tests to measure the fresh weigh (FW) of both shoot and root biomass.

RESULTS AND DISCUSSION

Using the pUBI:*CcUNK8* vector as the construction to transform *S. viridis*, it was possible to obtained thirteen independent T_0 events of transformation. All of them were check by conventional PCR experiment to confirm the presence of T-DNA region of pUBI:*CcUNK8* vector (data not shown). For all these events, *CcUNK8* gene expression was checked in leaves by RT-qPCR (Figure 1).

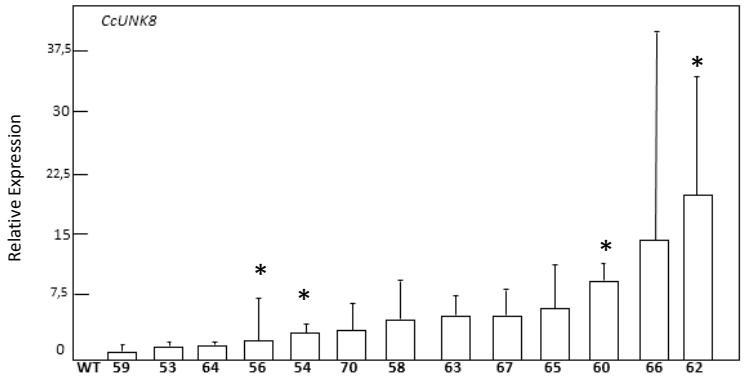

Figure 1. Expression of *CcUNK8* cDNA analyzed by RT-qPCR in leaves of transformed (T_0) plants of *S. viridis*. The expression was tested the thirteen events obtained (53, 54, 56, 59, 60, 62, 63, 64, 65, 66, 67 and 70) and a negative control (WT: wild-type of *S. viridis*). Transcript abundances were standardized with the expression of the reference gene *SiUBI*. Relative expression values (expressed in arbitrary units) are the mean of at least three technical repetitions ± SD. Results are expressed using the sample n°59 as an internal calibrator (relative expression = 1). * events selected to experiment of water suspension.

As expected, no expression was observed in leaves of the wild-type plant (WT: not transformed = negative control). For all T_0 transformed plants of *S. viridis*, *CcUNK8* gene expression was detected and ranged from 1 (lowest expression for the event n°59) to

(highest expression for the event n°62). Based on this experiment, it was decided to select only four events (n°54 and 56 with low *CcUNK8* expression; n°60 and 62 for medium/high expression) that were further analysed in details during the experiment of water suspension (Fig. 2).

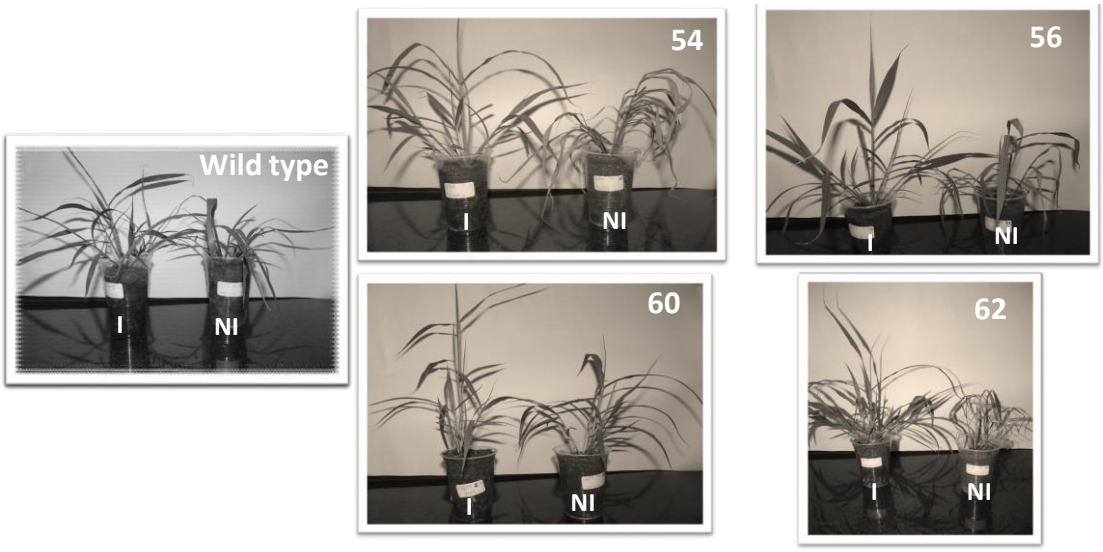

Figure 2. Wild-type and T_2 transformed (events n°54, 56, 60 and 62) plants of *S. viridis* grown under controlled condition with irrigation during 32 days after germination. and subjected (NI: not irrigated) or not (I: irrigated) to drougth stress during 7 additional days. Teffects of drought can be seen for NI treaments by leaf yellowing and drying.

In order to evaluate the effects of water suspension on phenotype and plant development, the fresh biomass in roots and shoots was measured for all of them (Fig. 3). The classic symptoms of drought stress, like wilting, leaf winding and reduced growth, became visible around the 5th day after water suspension, and appeared more pronounced for the WT and transformed events n°56 and 62 of *S. viridis* than for transformed event n°54 and 60.

Under irrigated (I) condition, we also observed that accumulation of fresh biomass in roots and shoot was higher for all transformed events of *S. viridis* in comparison to WT (Fig. 3A and B). This was particularly observed and confirmed statistically for the event n° 62.Under non-irrigated (NI) condition, amount of fresh biomass varied between organs (root and shoot) and plants analysed. For example, root FW appeared similar for WT and transformed events n°54, 56 and 60 but was higher for transformed events n°62. Regarding shoot FW, no significant differences were observed between all plants analysed.

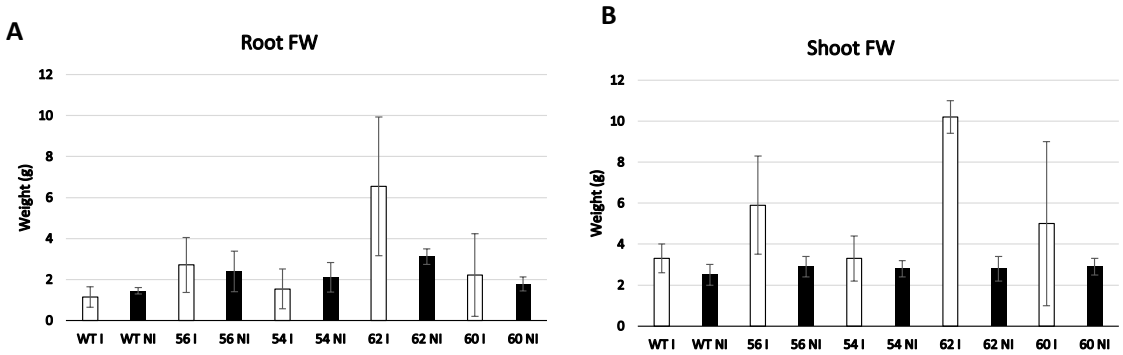

Figure 3. Average fresh weights (FW: expressed in gr.) of roots (A) and shoots (B) of 39 days old plants of wild-type (WT) and T_2 events (54, 56, 60 and 62) of *S. viridis* transformed by pUBI:*CcUNK8* irrigated (I: white isobars) or not irrigated (NI: black isobars) during the last 7 days of the treatment.

Altogether, these preliminary results:

- showed that the *CcUNK8* gene was expressed to different levels in all T_0 events of *S. viridis*,
- identified T_2 transformed events (*e.g.* n°62) of *S. viridis* displayed higher FW biomass compared to untransformed (WT) plants.

This phenotype could be directly related to *CcUNK8* gene expression levels measured in transformed *S. viridis*. In order to verify such a possibility, new experiments of water suspension using more uniform plant materials are currently ongoing.

ACKNOWLEDGEMENTS

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Discrimination of Fragment Linked to S_H3 Rust Resistant Gene in Coffee by Melting Temperature Analysis

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SUMMARY

Coffee Leaf Rust (CLR) is a fungal disease caused by *Hemileia vastatrix* and is one the major diseases of coffee. Resistant to CLR is conferred by S_H3, a major dominant gene that has been introgressed from a wild coffee species *Coffea liberica*. The two DNA markers, BA-124-12K-f and Sat244, reported to be closely linked to S_H3 gene of *C. liberica*, were introduced in this experiment for validation by the real-time PCR. The amplification protocol was defined and 'Tm calling' application in real-time PCR was used for analysis. The amplified product of *C. liberica* by BA-124-12K-f revealed a sharp and specific peak with a melting temperature at 80.94 °C while no product were amplified in *C. canephora* (Robusta)and *C.arabica* var. typica. Melting temperature of *C. liberica* by Sat244 that reported to indicate zygosity status revealed two peaks at 82.28°C and 86.63°C while those of *C. canephora* (Robusta) were at different temperatures. Thus, this method provided a simple, fast, cost effective and high-throughput protocol for screening of S_H3 related Coffee Leaf Rust (CLR) resistant population.

INTRODUCTION

Coffee Leaf Rust (CLR) caused by the obligate parasitic fungus *H.vastatrix* Berk & Br. is a major disease which greatly limits arabica coffee (*C. Arabica*) production in almost all growing countries around the world. Presently, there is information of the gene that participates in the resistance mechanism. Coffee rust resistant is conferred by nine genes, S_H1 to S_H9. S_H1, S_H2, S_H4 and S_H5 genes have been identified in *C.arabica* germplasm. S_H3 gene supposedly is derived from *C. liberica* and S_H6, S_H7 S_H8 S_H9 genes from *C. canephora*. Classical genetic studies determined that the introgressed CLR from *C. liberica* is controlled by a single, dominant gene (S_H3). Amplified fragment length polymorphism markers that are linked to the resistance locus have been identified. A total of ten sequence-characterized genetic markers, included simple sequence repeat (SSR) markers, sequence-characterized amplified region (SCAR) markers were developed to indentify the S_H3 leaf rust resistant gene. Two sequence characterized DNA markers (Sat244 and BA-124-12K-f) were found to be closely linked to S_H3 gene, one of the highly effective genes for rust resistance. It was reported that the Sat244 marker is more efficient for distinguishing the homozygous and heterozygous status of S_H3 gene while the BA-124-12K-f marker is perfectly linked with S_H3 rust resistant gene. However, the adoption of the above results for screening of coffee rust resistant gene in our laboratory by both agarose gel electrophoresis and PAGE proved problematic in both handling and result interpretation. Although, Polymerase Chain Reaction (PCR) amplification and agarose gel or PAGE are common techniques used for genotyping, discrimination of the bandings can become problematic when fragment sizes are very close in addition to aberration of banding caused by inappropriate electrophoresis handling. This error could lead to misinterpretation and misleading of the result that impact greatly on the breeding program.

Presently, Real-time PCR has been applied widely due to its accurate, sensitive and fast quantification. At the end of the amplification by the conventional PCR, the product has to be run on a gel for detection of the amplified product. In real-time PCR, this step can be avoided since the technology combines the DNA amplification with the immediate detection of the products in a single tube. Melting temperature analysis which is an assessment of the dissociation-characteristics of doubles-stranded DNA during heating can be applied for discrimination of the amplified products. This approach equipped in the Real-time PCR can be adapted for analysis of the target of interest. Specific identification is achieved by the melting temperature peak profiles. Due to the above mentioned benefits, this study thus aimed to develop a real-time PCR protocol for a fast, reliable and effective screening of S_H3 gene in our coffee population for CLR resistant screening.

MATERIALS AND METHODS

Plant materials

Twenty-eight trees including pure *C.arabica* var Typica and HDT derivatives (Arabica x Robusta) collected from Chiang Mai Royal Agricultural Research Center were used for this study. *C. liberica* was used as a positive control and *C. canephora* (Robusta) and *C.arabica* var typica were used as negative control samples.

DNA Extraction

Total genomic DNA from fresh leaves was extracted by the method of Li and Midmore (1999) with some modifications. Each sample was ground in liquid nitrogen in a mortar. The powder was transferred to 800 µl of DNA extraction buffer [2% cetyl trimethylammonium bromide (CTAB), 1.4 M NaCl, 0.1 M Tris-HCl (pH 8.0), 0.02 M EDTA (pH 8.0), 1% polyvinylpyrrolidone (PVP)]. The samples were incubated at 60°C for 30 min and then centrifuged at 12,000 rpm for 10 min. The supernatant was mixed with 500 µl of chloroform: isoamyl alcohol (24:1 v/v). After centrifugation at 12,000 rpm for 10 min, the DNA was precipitated by adding 500 µl of ice-cold isopropanol, incubated at -20°C for 15 min and then centrifuged at 12,000 rpm for 2 min. The pellet was washed two times with ice-cold 70% ethanol and then centrifuged at 12,000 rpm for 2 min, dried at room temperature and dissolved in 180 µl of distilled sterile water, 20 µl of 5 M NaCl, 200 µl of 95% ethanol and gently mixed. This mixture was centrifuged at 12,000 rpm for 2 min; the pellet was washed with ice-cold 70% ethanol and then centrifuged at 12,000 rpm for 2 min, dried at room temperature and finally resuspended in 40 µl of TE buffer. The DNA was quantified using UV-Vis spectrophotometer and 2-µl aliquots of genomic DNA were verified by 1% agarose gel electrophoresis in 0.5X TBE buffer. SYBR®Gold (Invitrogen) was used as a fluorescent nucleic acid gel stain.

Real-time PCR amplification and Melting curve profile analysis

Two DNA markers closely linked to S_H3 gene BA-124-12K-f and Sat244 were subjected for the amplification [1]. Real-time PCR amplification and analysis were performed using a LightCycler®480 Instrument equipped with software version 1.5 (Roche Diagnostic GmbH, Germany). All real-time PCR reactions were performed in triplicates. Each reaction mixture contained 1 µl of the above prepared 100 ng/µl DNA, 3 µl of 0.1 µmol each of a forward and a reverse primer, 0.2 µM each of dATP, dCTP, dGTP and dTTP, 1X *Taq* buffer with 25$(NH_4)_2SO_4$, 0.2 mM $MgCl_2$, 0.06 units of Taq DNA polymaerase (Fermentas, USA) and 3.33 µM SyTo9 Green Fluorescent Nucleic acid Stain (Invitrogen, USA), in a total volume of 20µl. The amplification was performed as following: initial denature program for 5 min at

94°C followed by 35 cycles of : (i) 40 sec at 94°C for denature, (ii) 40 sec at 66°C for annealing, (ii) 45 sec 72°C for extension with a single fluorescence measurement. After the amplification, a melting curve analysis with a temperature gradient of 0.11°C/s from 65 to 95°C was applied for confirmation of specific amplification. Finally, the samples were cooled down to 40°C for 30 sec. The melting curve profile was determined by the 'Tm calling' analysis equipped in the software.

RESULTS AND DISCUSSION

S_H3 gene was reported as one of the highly effective genes for rust resistance that supposedly is derived from *C. liberica*. In the present study, the two markers BA-124-12K-f and Sat244 reported to be closely linked to S_H3 leaf rust resistant gene were validated. By our defined protocol, the markers showed clear melting peaks that could identify the plant samples for presence or absence of S_H3 gene. The amplified product of *C. liberica* by BA-124-12K-f which was reported to confer resistant of S_H3 gene revealed a sharp and specific peak with the melting temperature at 80.94 °C while no product were amplified in *C. canephora* (Robusta) and *C.arabica* var. typica (Fig 1a). This result supported the finding that this resistant gene was not present in susceptible species as reported in other studies. This marker product was in fact amplified in both field tolerance and moderately susceptible to rust. Accordingly, the HDT derivatives (Arabica X Robusta) subjected for the testing in this study showed no amplified product.

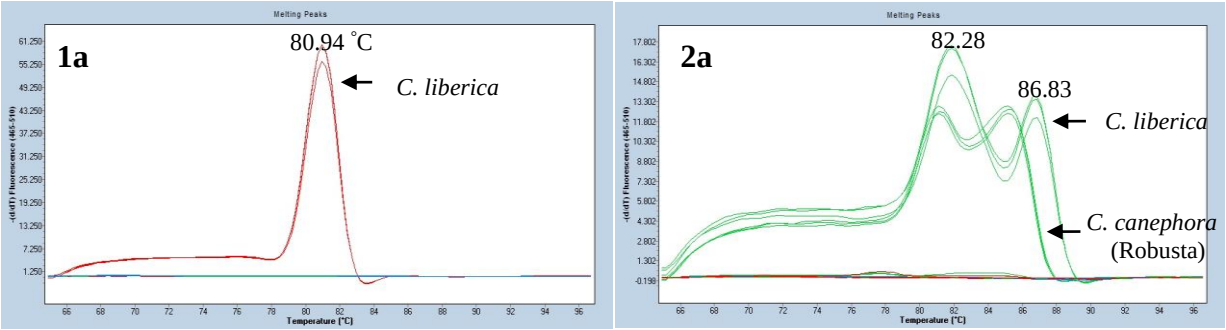

Figure 1. The Tm analysis of coffee samples amplified by BA-124-12K-f and Sat244 markers. a) The melting temperature of *C. liberica* products amplified by BA-124-12K-f, revealed one sharp peak at 80.94 °C. b) The melting temperature of *C. liberica* and *C. canephora* (Robusta) PCR product amplified by Sat244 revealed two different peak patterns.

The melting temperature of *C. liberica* by Sat244 marker that reported to indicate zygosity status revealed two peaks at 82.28 °C and 86.63 °C while those of *C. canephora* (Robusta) were at 81.65°C and 85.30 °C that were of different melting temperature to *C. liberica* (Fig. 1b). The melting temperature of the amplicon depends mainly on its nucleotide composition. It is the temperature at which the two strands of a double helix separates. This temperature depends greatly on its sequence, length, and GC content. The melting profiles can be easily visualized and compared which simplify discrimination of the test and unknown samples. Thus it is possible to identify the profile obtained from the product with similar nucleotide composition. By this principle, the melting temperature peak of *C. canephora* (Robusta) that was reported to conferred S_H6, S_H7 S_H8 S_H9, thus revealed different profiles compared to those of *C. liberica*. These two peaks by Sat244 product visualized by Real time PCR revealed two closely DNA bands by agarose gel electrophoresis (Fig. 2).

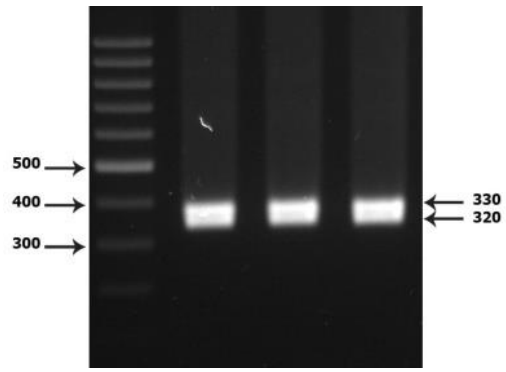

Figure 2. Agarose gel amplification pattern of Sat244 maker associated with S_H3 leaf rust resistance gene in *C. liberica.*

Though the amplified products of Sat244 marker could reveal zygosity status of S_H3gene in the test samples, however, the two melting temperature peaks produced by real-time PCR is yet to be identified. S_H3 homozygous dominant and heterozygous including homozyzous recessive accessions have to be introduced from other collection centers for identification of their melting temperature profiles. These profiles can be used as core patterns for zygosity status identification by other unknown testings.

In summary, the results presented support the report that the two DNA markers, BA-124-12K-f and Sat244 are closely linked to S_H3 gene and can be generally used for screening of this gene in coffee populations. With this reported modification, the manipulation can be more effective and reliable when melting curve profiles are applied to identify DNA products.

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Coffee Genetic Diversity Analysis of Coffee in Yunnan

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SUMMARY

The genetic diversity is studied using ISSR (Inter-simple sequence repeat PCR) markers among 76 coffee (*C. arabica*) individuals representing of 16 accessions derived from different hybrids obtained from 8 different countries. ISSR is based microsatellite repeat sequences as primers to detect polymorphic DNA markers, and its operation is simple, fast and efficient. DNA extraction is used coffee leaf as materials followed by a reliable, stable and fast and efficient PVP-CTAB method. Ten out of 33 screened polymorphic primers are selected for genetic diversity analysis, 116 genetic loci are obtained with polymorphic allele frequency of 57.8%. UPGMA dendrogram of the genetic similarity coefficient are compared by using NTSYS software based on 76 individual ISSR fingerprints data. The results show that the genetic diversity is narrow in the tested materials with the coefficient similarities ranging from 0.66 to 0.97.

INTRODUCTION

China has commercially planted coffee since 1950s, more than 100 000 ha and the total yield is 82 000 tons until 2013/2014. Coffee germplasm resources in China is mainly collected by Dehong Tropical Agricultural Research Institute of Yunnan (DTARI), Spice and Beverage Research Institute of Chinese Academy of Tropical Agricultural Science (SBRI/CATAS), Tropical and Subtropical Economical Crops Institute of Yunnan Academy of Agriculture science (TSECI/YAAS). By the end of 2013, DTARI has collected and conserved 5 species 352 accessions, including 331 accessions of *Coffea arabica* and hybrids, 13 accessions of *Coffea canephora*, 3 accessions of *Coffea liberica*, 1 accession of *Coffea eugenioides*, 1 accession of *Coffea racemosa*. SBRI/CATAS has conserved more than 100 accessions, including 60% of *Coffea canephora*. TSECI/YAAS has also conserved almost 200 accessions .

ISSR is used to detect variation between DNA sequences, and is more repeatability and stability than RAPD. SBRI/CATAS has study on the DNA extraction and PCR preliminary test of *C. canephora* and *C. arabic*a. To analyze the genetic diversity on C. *arabica* of Yunnan, and it has not been reported at domestic and abroad. The use of molecular marker technology in hybrid progenies, identifies false hybrid offspring, reduces the population to decrease the work intensity and expenses, and shortens the breeding period. The diversity analysis revealed the similarity and complexity of coffee genetic background and provided identification and germplasm preservation with a theoretical basis.

MATERIALS AND METHODS

The study materials were collected from Germplasm Repository of coffee in Ruili City, Ministry of Agriculture in China (E 97 ° 51 ', N 24 ° 01', altitude 800m) at April to May, 2013. 74 individuals of 14 hybrid parents and 2 individuals of cultivated species were collected (Table 1). 5-6 full parietal buds without pests were collected from each individual coffee tree, put into 20×15cm sample bag and sealed after being tagged, conserved in 4 ℃ refrigerator.

Table 1. 74 individuals of 14 parents used in hybrid and two coffee cultivars.

No.	No. of Parental	Variety Name	Origin	Characteristic Description	No. of Individual
1-4	11	Caurai140	Burundi	High yield, no-resistant CLR, is hybrid of between Mudo Novo and Caturra.	1.2.3.5
5-7	12	Catimor 7963	Portugal	Commercial cultivars in Yunnan, China	1.2.3
8-11	26	S288	India	Commercial cultivars in 1980s，natural hybrid of C. arabica and C.liberica.	1.3.4.5
12-20	40	Rume sudan	Kenya	Semi wild variety, resistant to CBD, collected in Boma Plateau of Sudan 1942	1.2.4.5.6.7.8.9.10
21-29	41	K7	Kenya	A choice of French Mission in Kenya with the cupping character of Bourbon	1.3.4.5.6.7.8.9.10
30-39	47	HDT1343	Kenya	Discovered natural hybrid of C. arabica and C. canephora in 1940, with resistance to all known rust.	1.2.3.4.5.6.7.8.9.10
40-44	51	SL28	Kenya	A variety of resistant to drought in Tanzania, with good cup quality and vigorous growth.	1.2.3.4.5
45-48	52	SL34	Kenya	With best cup quality than SL28, without resistance to CBD、CLR, vigorous growth.	1.2.3.5
49-55	59	Mexico 11	Mexico	High yield, incomplete resistance to rust.	1.2.4.5.6.7.9
56-59	63	MA2	Malaysia	High yield, with resistance to CLR.	1.2.4.5
60-65	78	Catimor 7963	Portugal	Artificial hybrids of Caturra and HDT832/1, main commercial cultivar in Yunnan.	3.4.5.6.7.8
66-67	7-1	Bourbon139	Burundi	With vigorous growth and good cup quality.	1.3
68-69	8-1	Mibiuzi49/1448	Burundi	With vigorous growth and good cup quality.	1.5
70-74	105	Blue Mountain	Kenya	Low yield, resistance to CBD, with good cup quality when grown in high altitude.	1.5.8.9.11
75	200	Aika	Yunnan	High yield, resistance to rust.	5
76	296	Purple mutant	Yunnan	High yield, resistance to rust.	2

Genomic DNA extraction: Extract coffee leaves genomic DNA with the improved Mo Rao's method.

ISSR amplification reaction system: Used improved Combes's method. 20μL reaction liquid contains 2μL 10 × Buffer (Mg2+ Plus), 1.6 μL dNTP Mixture (2.5 times), 2 μL Primer (Table 2), 0.2 μL Taq DNA polymerase, 1 μL Template DNA, 13.2 μL ddH_2O. Add a drop of mineral oil on the top of the reaction liquid to prevent water evaporation in reaction process. Amplification procedure: Pre-denaturation at 94 ℃ for 5 min, 36 cycles at 94 ℃ for 45s, 52 ℃ for 45s and 72 ℃ 1.5min, followed by the final extension at 72 ℃ for 8min. Reaction products were stored at 16 ℃ for 99min.

Table 2. ISSR Primers for coffee genetic diversity analysis and its amplification results.

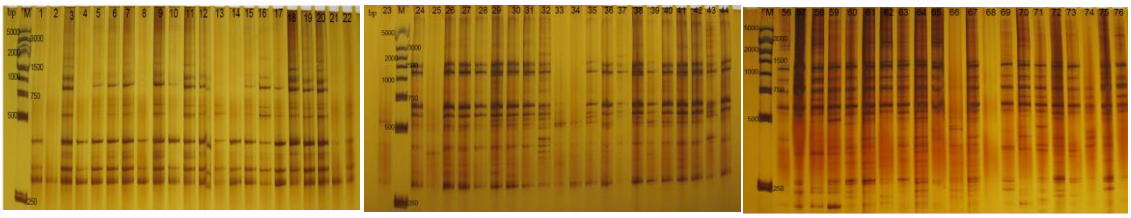
Primer	Primer sequences	Bands of amplification	Bands of polymorphism
ISSR809	5'-GAGAGAGAGAGAGAGAG -3	14	5
ISSR812	5'-GAGAGAGAGAGAGAGAA -3	8	4
ISSR816	5'-CACACACACACACACAT-3'	9	7
ISSR825	5'-ACACACACACACACACT-3'	11	4
ISSR851	5'-GTGTGTGTGTGTGTGTYG-3'	12	7
ISSR855	5'-ACACACACACACACACYT-3	12	8
ISSR856	5'-ACACACACACACACACYA-3	12	7
ISSR862	5'-AGCAGCAGCAGCAGCAGC-3	18	10
ISSR880	5'-GGAGAGGAGAGGAGA-3'	8	5
ISSR888	5'-DBDCACACACACACACA-3'	12	10

Electrophoresis: Detected degeneration product with 6.0% PAGE electrophoresis. The reaction products were observed by staining with 0.0075% $AgNO_3$ solution, being developed with 1.5%NaOH and 0.4% Formaldehyde solution.

Cluster analysis on Coffee parent materials is carried out with NTSYS-PC[6]. The genetic differences between each two materials according to each of the two root well for standard genetic distance. The genetic distance between each two samples according to D : D=-ln [2Mxy/(Mx+My)]. Mx and My separately are the total bands number of X and Y. Mxy are the bands both of X and Y. Base on the genetic distance obtained, UPGMA cluster analysis was used to research the genetic relationship and diversity.

RESULTS AND DISCUSSION

In this paper, the improved PVP-CTAB method for DNA extraction was used. The method of Mo Rao's is adding the PVP when grinding, then added chloroform and extracted liquid, and afterward, subpackage. For large quantities of samples, it is easily to become confusion in this method. After improvement, the PVP is added to the extracted liquid to make the mixture solution, after grinding, directly add mixture. After the improvement, when we extract DNA on amount of large quantities of samples, the time and manpower were saved, and it not easy to confuse samples.10 primers with good diversity and clear band were screened out to amplify the PCR products of 76 individuals with 14 parents and 2 cultivars. Polyacrylamide gel detection was carried out after 1% agarose gel detection (Fig. 1).



a . b. c.

Figure 1. ISSR-PCR profile based on partial primers and coffee parents and cultivars. Label: a. primer 816, samples 1-22； b. primer 855, samples 23-44; c. primer 880, samples 56-76

An original matrix comprised by 116 loci band data was obtained. A total of 2850 genetic similarity coefficient between each two different individual, of which the maximum similarity coefficient was 0.97 (41/6 and 41/7) and the minimum was 0.66 (11/2, 11/3 and 11/5). According to the genetic similarity coefficient, all the 76 individuals were divided into 2 groups with the threshold value of 0.54 (Fig. 2).

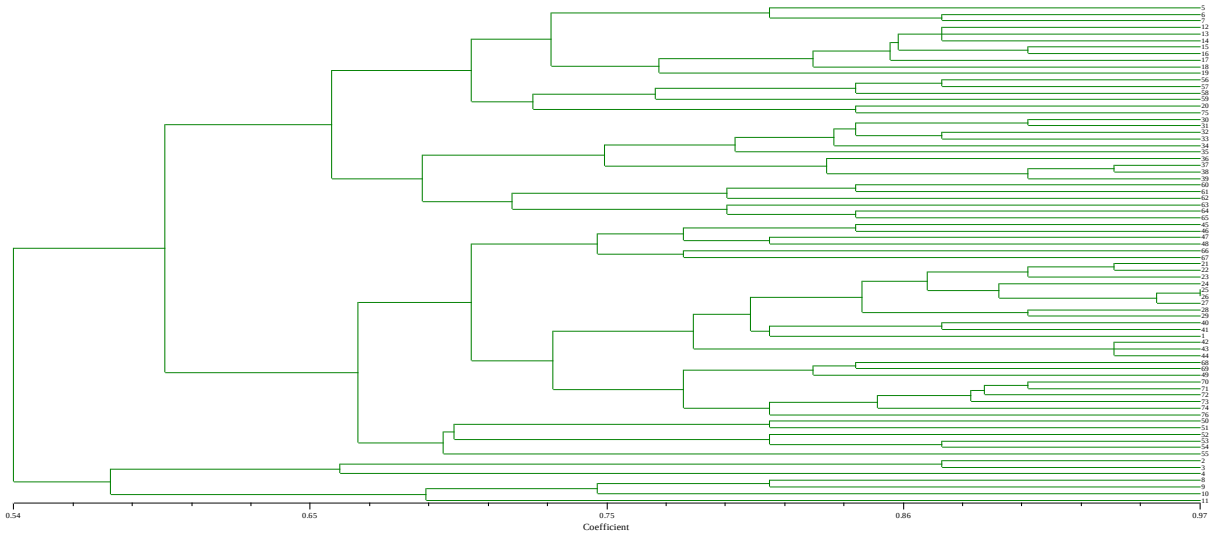


Figure 2. Damdrogram of 74 individuals of 14 parents plants used in hybrid and two cultivars based on ISSR-PCR data

Group I was consists of 2 subgroups, namely A and B. Subgroup A were divided into 2 classes, and class 1 was made of 2 subclasses. In subclass 1, sample 12 Catimor was gathered with sample 40 Rume Sudan, perhaps because they have the similar gene. Sample 63 MA2, sample 40/10 Rume Sudan and sample 200 Aika were gathered in subclass 2. Sample 40/10 showed up here perhaps because the wrong coffee tree was planted by then. And MA2 was gathered with Aika, it confirmed our guess. That is, Aika is the specific phenotype of MA2 in dry and hot environment in Ljiangba of Baoshan. Aika has small and curl leaves, but the leaves are not so curl in moist and hot weather in Dehong, while it's more like MA2. Perhaps MA2 and Aika both gathered in the Catimor's subgroup, just because they all contain Catimor's gene.

Class 2 was made of 2 subclasses, too. Sample 47 HDT1343 and sample 78 Catimor7963 were appeared in class 2, because Catimor7963 was filial generation of between Caturra and HDT1343. HDTA1343 and HDT832/1 were the same origin germplasm with different

resistant to CLR. HDT1343 was introduced into Kenya from Columbia, then be introduced into China, while there are differences between these individuals. In coffee breeding resistant to CLR, its resistant gene was the antibody sources. Catimor7963 and its series type are the main varieties in Yunnan, and there are some differences between individuals.

Subgroups B were divided into 2 classes. In class 1, SL28 and Bourbon139 in subclass 1 were clustered together with K7, SL34, Mibiuzi49/1448 and blue Mountain of subclass 2 because that they are all derived from stalked varieties in African and they are all no-resistant to CLR. This result is consistent with Tran's. SL28 and SL34 are cultivars of Kenya, and K7 breed by Kenya is suit for the environment of Australia. Bourbon139 and Mibiuzi49/1448 are bourbon's variation coming from Burundi, have fairly vigorous growth but without resistant to rust. Blue Mountain with purple leaves has very well cup quality when grown in Jamaica, but shows ordinary when grown in Kenya and DTARI. Sample 296 is a purple variety found in DTARI's planting field, with fairly vigorous growth, bigger bean, resistant to rust, and its leaves curl in the dry weather. We think that sample 296 should be a cultivar of Blue Mountain, based on the discovery in a plantation in Menghai county, Xishuangbanna, Yunnan. And it's why they clustered together. In class 2, Mexico 11 was introduced from Hainan, has a fairly vigorous growth, not normally used in planting for being infect by rust. Its genetic background is not clear, and clustered into a single category.

In group II, Catuai 140 of subgroup C clustered into a single category is because it is the natural hybrid of Mudo Novo×Caturra, and it's the main cultivars in Colombia and Brazil. Sample 26 S288 of subgroup D was clustered into a single category, too. It's derived from a natural hybrid in coffee plant in Balehonnur, India. There are genes from *C. liberica*, and it has already been infected by rust in Dehong Yunnan now.

So far, the main cultivar planted in Yunnan is tetraploid *C. arabica*. They are mostly derived from the variety of Typical's progeny all over the world. A few are the hybrid progeny of C. *arabica* and *C. liberica*, *C. arabica* and *Coffea canephora*. The genetic basis is relatively narrow. This result is consistent with Lashermes with analysis the genetic diversity of cultivars and Semi wild species, and proved the genetic basis of commercial cultivars [8]. The research of Tran suggested that the genetic diversity existed between individuals of each cultivar, however, the genetic basis is very narrow for self pollination of dominance.

Due to the 5% cross pollination rate of *C. arabica* in the field, other individuals and some varieties was clustered together. And when we collect the varieties, it's impossible to bagging before pollination. In addition, some mistakes would be when the germplasm relocated. Therefore, more accurate result would be got when more marking method would be used and more tests would be carried out in sample collecting. Mirian's opinion is that there is no single way in the identification of inbreeding unless combined with botany, agronomy, molecular and so on.

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Molecular Mechanisms in the First Step of ABA-mediated Response to Drought in *Coffea canephora* clones

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SUMMARY

Global climate changes are becoming more unpredictable and abiotic stress, such as drought and high temperatures, are key factors that affect coffee plant development and production. Although the strategies for drought adaptation in perennial plants are complex, there is genetic variability within the *Coffea* genus that could be used to increase tolerance. Drought tolerant clones of *Coffea canephora* have been characterized as vigorous plants with high productivity throughout years under water deficit. Recently, novel intracellular ABA receptors (PYL/RCARs) involved in sensing and signaling of this hormone were identified. A mechanism of ABA transduction was proposed, involving PYR/PYL/RCARs receptors interacting with PP2Cs phosphatases and SnRK2 protein kinases. The goal of this study was to identify and characterize orthologous genes of this tripartite system in *C. canephora*. For this purpose, protein sequences from *Arabidopsis*, citrus, rice, grape, and tomato were chosen as query to search orthologous genes in sequence databases. This approach allowed the identification and characterization of 24 candidate genes (9 PYL/RCAR, 6 PP2Cs and 9 SnRK2s) in *C. canephora* genome. The protein domains identified in the predicted coffee sequences enabled to characterize these genes as family's members of receptors (PYR/PYL/ RCAR), phosphatases (PP2Cs) or kinases (SnRK2) of ABA response pathway. Phylogenetic analysis allowed the classification of coffee-polypeptide sequences between subclasses and subfamilies. The gene structures of these three gene families were functionally annotated in Coffee Genome Hub (http://coffee-genome.org/). Tissue (leaf, seed, root and floral organ) differential expression was verified through *in silico* analyses. Regarding drought conditions, data from root transcriptome showed contrasting gene expression for those genes between the tolerant (Cc14, Cc73 and Cc120) and susceptible (Cc22) clones of *C. canephora*. By comparing gene expression profiles of only drought tolerant (Cc14, Cc73 and Cc120) clones, differential responses were observed suggesting the existence of multiple biological mechanisms for drought tolerance in coffee. All those evidences will help to identify the genetic determinism of drought tolerance essential to obtain molecular markers that could be used in coffee breeding programs.

INTRODUCTION

Abiotic stresses such as drought and high temperatures are key factors that affect coffee plant development and production. During the last decade, previous data showed that coffee-growing geographical regions could suffer important geographical delocalization as a consequence of global warming. Furthermore, it was predicted in marginal regions without irrigation or during dry seasons that coffee yields could decrease as much as 80%. Nowadays, according to the last report of the Intergovernmental Panel on Climate Change, a 3 ºC

increase in temperature would lead to major changes in the distribution of coffee producing zones. Extrapolating the historical tendencies in temperature and precipitation to 2020 in coffee producing areas, the analyses predict that coffee production in Veracruz, Mexico, is likely to decline about 34%. The suitability for coffee crops in Costa Rica, Nicaragua and El Salvador will be reduced by more than 40% while the loss of climatic niches in Colombia will force the migration of coffee crops towards higher altitudes by mid-century. In the same way, specifically for Robusta coffee, data shows similarly deep changes in both distribution and total area suitable for coffee production in Uganda. As for the first world producer, is expected that coffee plants will migrate towards more favorable zones in the South. Regarding production, in the main coffee producing states of Brazil the potential area for production would decline from 70-75% of the states to 20-25%. The damage from drought at the beginning of the year is expected to cause a global supply deficit in crop year 2014/2015, with production in Brazil officially estimated to decrease by 9.3% to 44.57 million bags. Besides the loss of coffee production, the biochemical composition of beans could also be modified by drought, variations in rainfall and temperatures affecting sugar, proteins and caffeine contents and consequently beverage quality.

Altogether, this highlights the importance to develop new coffee cultivars better adapted to climate change. Between the two economical important species in the genus, *C. canephora* stands out regarding abiotic tolerance. Moreover, the Kouillou group (SG1) appears to be more tolerant to drought than Robusta (SG2). Among the strategies displayed by coffee plants to cope with drought stress, leaf folding and inclination were commonly observed for Guinean and SG1 genotypes. Several drought-tolerant clones of *C. canephora* var. Conilon have been characterized as vigorous plants with high productivity throughout years under drought stress. Fingerprint analyses also revealed that these Conilon clones belong mainly to the SG1 group of *C. canephora*. There is evidence that the drought tolerance in some clones of *C. canephora* is mainly associated with rooting depth and stomatal control of water use. At the molecular level, several differentially expressed genes and proteins were investigated in leaves of tolerant and sensitive *C. canephora* clones upon drought acclimation. Genes coding for protein functioning as secondary messengers (*CcNSH1*, *CcEDR1* and *CcEDR2*), related to abscisic acid (ABA) perception and signal transduction (*CcPYL3*, *CcPYL7* and *CcPP2C*), transcription factors (*CcABI5*, *CcAREB1*, *CcRD26*, *CcDREB1*), photosynthesis (*CcPSBP*, *CcPSBQ*, *CcRBCS1*), and drought protection (*CcHSP1*, *CcDH3*, *CcAPX1*), were previously characterized.

The abscisic acid (ABA), discovered in the 1960's, is a vital plant hormone synthesized in roots and leaves, acting as central regulator that protects plants against abiotic stresses such drought. It was characterized as important endogenous small molecule that mediates stress-responsive gene expression, stomatal closure, and vegetative growth modulation. Over the past few decades, lot of work was done elucidating the molecular mechanisms underlying ABA sensing and signaling. More recently, novel intracellular ABA receptors (PYL/RCARs) involved in ABA sensing and signalling via their direct interaction with the type 2C proteins phosphatases (PP2Cs) were discovered in *Arabidopsis thaliana*. The core of the ABA signalling network comprises a subfamily of PP2Cs and three Snfl-related kinases, SnRK2.2, 2.3 and 2.6. The current ABA signal transduction model can be described as follow: in the absence of ABA, SnRK2 kinases are inactivated by PP2Cs which physically interact with SnRK2 and dephosphorylate a serine residue in the kinase activation loop, a phosphorylation essential for kinase activity. On the other hand, when ABA binds to the ABA receptors family PYR/PYL/RCAR, this allows the bounds of the receptors and the catalytic site of PP2Cs to inhibit their enzymatic activity. In that case, ABA-induced inhibition of PP2Cs leads to SnRK2 activation.

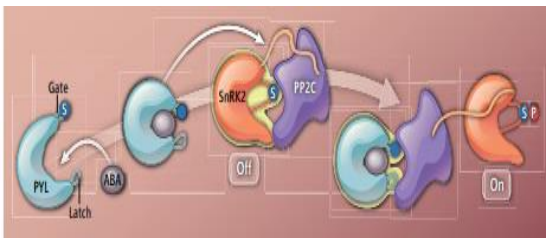

Figure 1. PYL ABA Partner swap. Molecular mimicry between the kinase SnRK2 and the hormone receptor PYL bound to ligand ABA permits alternate binding to the PP2C phosphatase. This change in partners activates (on) or deactivates (off) SnRK2, allowing it to phosphorylate downstream signals.

With prospect of global water crisis, these recent laudable success in deciphering the early steps in the signal transduction of the "stress hormone" ABA has ignited hopes that crops can be engineered with the capacity to maintain productivity while requiring less water input. The goal of this study was to identify and characterize orthologs genes of this tripartite system in *C. canephora*.

MATERIALS AND METHODS

In order to isolate the *PYR/PYL/RCAR-PP2C-SnRK2* orthologs genes from *C. canephora* genome, searches were done in public databases sequences through the identification numbers of pre-selected genes found into literature (ID). Query sequences of species of interest such as *A. thaliana*, *S. lycopersicum*, *V. vinifera*, *C. sinensis* and *O. sativa* were founded using the following databases: NCBI (http://www.ncbi.nlm.nih.gov/), TAIR (http://www.arabidopsis.org/), AtGDB (http://www.plantgdb.org/AtGDB/), Phytozome (http://www.phytozome.net/), Sol Genomics Network (http://solgenomics.net/), SlGDB (http://www.plantgdb.org/SlGDB/), GreenPhyl (http://www.phytozome.net/), The Grape Genome Database (http://www.genoscope.cns.fr/externe/), Gramene (http://www.gramene.org/), Plant Genome Database (http://www.plantgdb.org/), Citrus Genome Database (http://www.citrusgenomedb.org/) and Rice Genome Annotation (http://rice.plantbiology.msu.edu). BLAST search was carried out in the Coffee Genome Database (http://coffee-genome.org/) and Rubiaceae ESTs database (e-value < e-10) using the NCBI query sequences previously identified and selected. The sequences identified in coffee were translated and aligned to protein sequences from the other species using the alignment program MAFFT available at South Green Bioinformatics Platform (SGBP platform - http://southgreen. cirad.fr) into Galaxy interface. The conserved domains of amino acid residues, characteristic of the gene families studied, were highlighted by the GeneDoc (http://www.nrbsc.org/gfx/genedoc/) program. Genes that did not contain specific domains were removed from the analysis. The 454 transcriptome root data was used to *in silico* analyses. Tolerant clones (Cc14, Cc73 and Cc120) of *C. canephora* and the susceptible clone (Cc22) to drought were obtained as rooted stem cuttings from the Institute for Research and Rural Assistance (Incaper, Vitoria, ES, Brazil). The experiment was carried out as described previously.

RESULTS AND DISCUSSION

In *C. canephora* genome, 9 genes codifying for PYR/PYL/RCAR homologs proteins of *A. thaliana*, *S. lycopersicum*, *V. vinifera*, *C. sinensis* and *O. sativa* were found. In order to evaluate the evolutionary conservation of this receptor among these species, and to verify the

characteristic START domain in inferred coffee protein sequences, the amino acid sequences from all species were compared and appeared well conserved among them (Fig. 2). Previous studies indicated also that PYR/PYL/RCAR family is highly conserved in other plants species, with 'gate' and 'latch' regions characterizing the functional domains. Similar analyses conducted for PP2C and SnRK2 inferred protein, led to the identification of 6 phosphatases and 9 kinases.

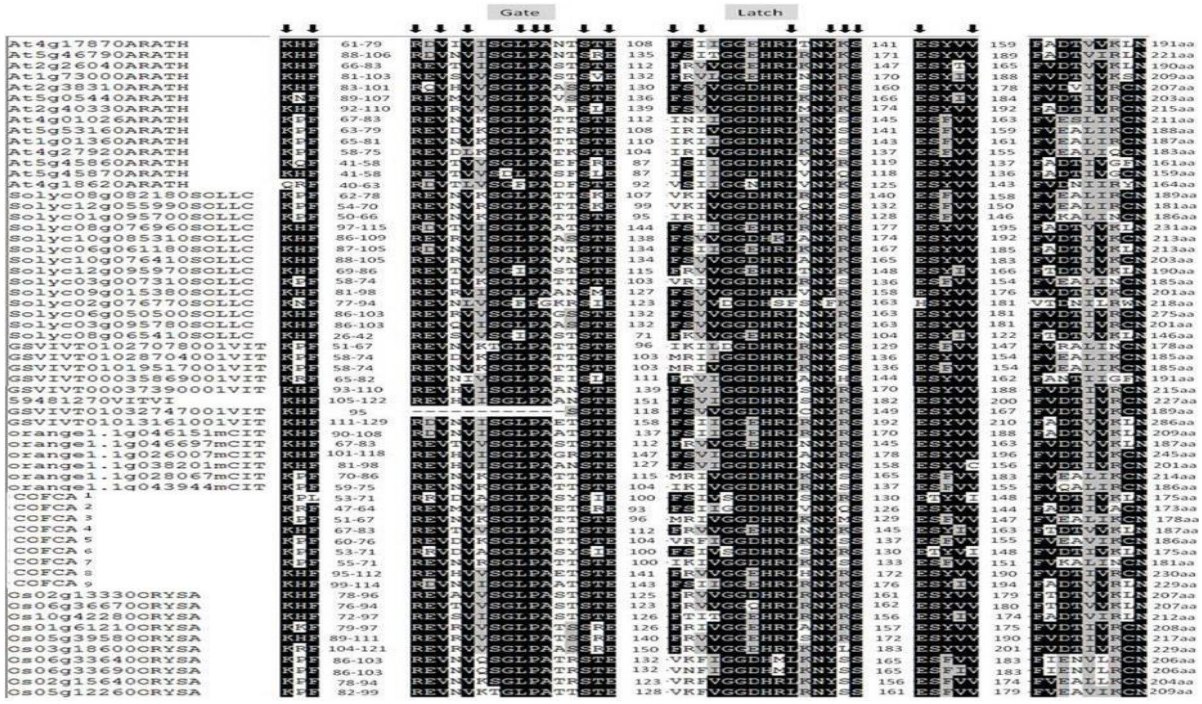

Figure 2. Sequence alignments of the PYR/PYL/RCAR proteins. Amino acid sequences are shown only for functional residues and domains. Residues positions are marked with the numbers nearby. The length of the amino acid sequences are indicated. Conserved residues are marked in black or grey shading. Residues forming the ABA pocket ligand-binding are marked with black arrows. The gate and latch domains are indicated. Functional residues and domains are based on previous reports [40,41]. ARATH: *A. thaliana*; SOLLC: *S. lycopersicum*; VITVI: *V. vinifera*; CITSI: *C. sinensis*; COFCA: *C. canephora*; ORYSA: *O sativa*.

The phylogenetic analysis showed the 9 sequences PYR/PYL/RCAR of *C. canephora* were distributed in three main subfamilies (Fig. 3) of ABA receptors. These subfamilies were also found in other species. Previous work in *C. canephora* showed differential expression for two receptor genes, CcPYL3 and CcPY7, in leaves of tolerant and sensitive clones under drought conditions. On the other hand, *in silico* gene expression analyses in roots (Figure 3D, 3E, 3F) for the three gene families (PYR/PYL-PP2C-SnRK2), also indicate divergent profiles of expression among clones under water deficit. Moreover, *in silico* gene expression analyses in different tissues could suggest the existence of specialized biological function for the coffee proteins into each family (Fig. 3A, 3B, 3C).

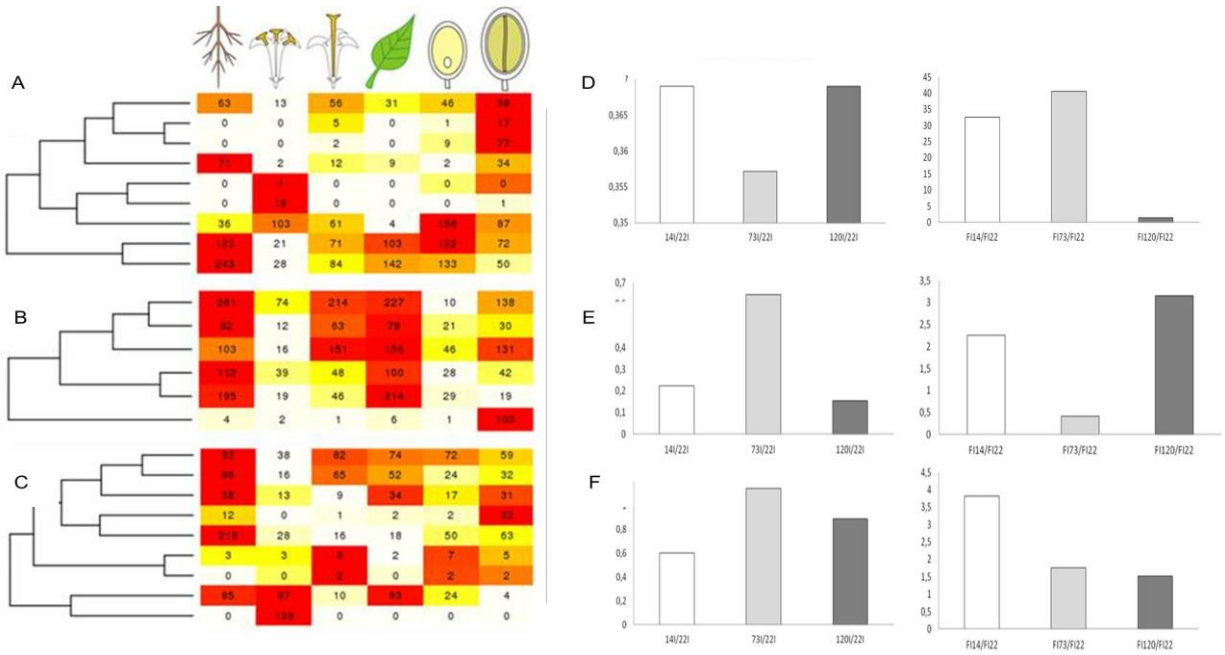

Figure 1. *In silico* gene expression for PYR/PYL-PP2C-SnRK2 in *Coffea canephora*. Heatmap for the ABA response genes in roots, stamen, pistil, leaf, perisperm and endosperm of (A) *PYR-PYL-RCAR* (B) *PP2C* and (C) *SnRK2* gene families. On the right, the candidate genes with differential *in silico* expression according to transcriptomic root data among *C. canephora* clones (D) *CcPYL* (E) *CcPP2C* and (F) *CcSnRK2*. Analyses of Qi value (left) and Q value (right) are considered. The Qi value is calculated using the expression of each tolerant clone (Cc14, Cc73, Cc120) in a controlled situation (under irrigation) dividing the expression of susceptible clone (Cc22) in the same condition. The Q value is calculated using the induction factor (FI) of each tolerant clone dividing the FI of the susceptible clone. FI is calculated dividing the expression value under drought condition by normal condition for each clone. All expressions data were normalized with RPKM. The dendogram was performed using a Euclidean distance method in Coffee Genome Hub (http://coffee-genome.org/).

Once these results are validated through qPCR analyses, the genomic and transcriptomic sequence of each clone could be compared and the identification of polymorphisms could be used as valuable information to understand better the diversity of mechanism to deal with water deficit in these plants. All those evidences will help to identify the genetic determinism of drought tolerance essential to obtain molecular markers that could be used in coffee breeding programs.

CONCLUSION

The tripartite system in *C. canephora* concerning the molecular mechanism in the first step of ABA-mediated response to drought is comprised by 24 orthologs candidate genes: 9 receptors PYR/PYL/RCAR, 6 phosphatases PP2Cs and 9 kinases SnRK2s. The protein sequences identified show conserved motifs which can evidence them as members of families belonging to ABA response pathway. Phylogenetic analyses allowed the classification of these coffee sequences into subfamilies, clades and subclasses of known gene families. The 24 candidate coffee genes are differentially expressed *in silico* in tissues such as, root, leaf, seed and flower. In roots, some analyses also indicate that several mechanisms of gene response among tolerant clones under water deficit conditions are active.

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Population Genomics of *Hemileia vastatrix* Using RAD Sequencing

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SUMMARY

With the advent of next-generation sequencing, it is now possible to undertake population genomic studies of massive scope in both loci and fungal isolates. Several of these studies have already targeted different pathosystems, providing crucial insights on the molecular mechanisms responsible for the pathogenicity and adaptation of fungal pathogens. However, the tremendous informative potential of this approach has yet to be used to investigate the pathogenicity of the most severe disease on Coffee crops, Coffee Leaf Rust, caused by *H. vastatrix*. The specific pathosystem *Coffea* spp. - *H. vastatrix* is an interesting case study because it follows the Flor's gene for gene model and more than 45 pathotypes have been described. Despite its recent emergence, previous studies have reported a high genetic variability and adaptability that has challenged the durability of resistant coffee genotypes for years. Moreover, being a biotrophic pathogen and depending completely on the host to complete its life-cycle, *H. vastatrix* engages a dynamic interaction that has been little explored at the molecular level. It is uncertain whether these dynamics are the results of an "arms race", where novel virulence and resistant alleles are continuously favoured, or a "trench warfare", where balancing selection maintains a high level of polymorphism and virulence genes are continuously recycled. However, knowledge of these dynamics at the molecular level is crucial to understand and predict how the pathogen will respond to the deployment of resistant coffee genotypes. In this work we addressed this issue by using RAD sequencing technology to generate thousands of molecular markers across 24 isolates of *H. vastatrix*, carefully selected based on their pathotype and geographic origin. A total of 1,330,825 genotypes have been uncovered across 53,233 variant loci which provide an incredibly rich data matrix with which hypothesis can be tested. The majority of the genotypes are fixed within fungal isolates (61%), but a non negligible number of heterozygous genotypes are also present (39%), emphasizing the genetic variability not only between *H. vastatrix* isolates but also within the same isolate. This data will initially be used to assess the demographic patterns of *H. vastatrix,* such as the geographical point of origin and dispersion patterns. Then, this information will be used interpret genome-wide associations between loci and pathotypes, that will simultaneously provide a deep insight on the molecular mechanisms and dynamics of *H. vastatrix*'s pathogenicity as well as molecular markers for each pathotype. As a consequence, the results of this work will allow an easy identification of *H. vastatrix* races and provide important information to be used when developing more sustainable resistance strategies.

INTRODUCTION

Hemileia vastatrix, the causal agent of Coffee Leaf Rust (CLR) has been changing the socio-economic and landscape of coffee crops throughout the Tropics. The specific pathosystem *Coffea* spp. - *H. vastatrix* follows the Flor's gene-for-gene model, and more than 45 physiological rust races (pathotypes) have already been identified, based on phenotype, in world surveys of 60 years at the Coffee Rust Research Center (CIFC). This pathogen presents a high genetic variability and adaptability that has challenged the durability of resistant coffee genotypes for years. However, the genetic loci and mechanisms responsible for its virulence remain unknown. In this work we present preliminary data on a project that uses next generation sequencing technology to address this issue.

MATERIALS AND METHODS

Tewnty four *Hemileia vastatrix* samples from the collection of IICT/CIFC comprising 12 geographic locations (Angola, Brazil, Central African Republic, Ethiopia, India, Kenya, Mozambique, Philippines, S. Tomé, Tanzania, Timor and Uganda) and 19 virulence profiles were sequenced using RAD sequencing after restriction with the PstI enzyme in one lane of Illumina HiSeq2000 at Floragenex (Oregon, USA). This sampling also included two sample pairs (178/178a and 292/292a) that represent an original – mutant pair obtained at CIFC, which should differ solely on one inferred virulence gene. RAD loci were pre-processed for de-multiplexing and barcode removal by custom scripts at Floragenex. Assembly of the raw reads was performed using PyRAD v2.13 using the following parameters: minimum coverage for a cluster of 10, a clustering threshold of 0.9, a minimum samples per locus of 12 and a maximum number of individuals with shared heterozygous site of 3. Maximum likelihood phylogenetic analyses of the resulting loci were carried out with RAxML v8.0.24 with 500 bootstrap replicate. A preliminary analysis of natural selection acting on variable loci for the inferred virulence gene V2 was carried out for 496 variable loci using the FST outlier detection software Lositan.

CONCLUSION

In total, 29 088 loci were uncovered across all 24 *H. vastatrix* samples with each sample containing an average of 26 399 ± 2374 loci. These loci were found in at least 12 samples, while 7969 (27% of the data set) were present in all 24 samples. While the majority of the loci were invariable, a total number of 21 246 variable sites and 9537 parsimoniously informative sites (variable sites at least in two or more isolates) were found (Figure 1). This represents a proportionally low number of variable loci relatively to previous studies using RAD sequencing but it is congruent with a putative recent origin of *H. vastatrix*. Nevertheless, the absolute genetic variation found within the 24 *H. vastatrix* samples of broad geographic and virulence spectrum is still large for phylogenetic analysis. Indeed, the data set used for the phylogenetic analyses comprised 2 474 152 nucleotide sites and 58 416 unique alignment patterns and we were able to reconstruct the phylogenetic relationships among the 24 taxa depicted in Figure 2.

Despite the large amount of nucleotide variation in our data set, the inferred phylogenetic tree was mostly poorly supported. Nevertheless, three main clades were uncovered, with a strong relation to coffee hosts, reflecting their respective ploidy. Samples from Clade II and III are derived from diploid coffee hosts in nature, whereas samples from Clade I infect *C. arabica* an HDT (Timor hybrid) derivatives. Inside Clade I, due to the very low bootstrap values, we could not find any genetic structuring of the isolates according to geography or virulence profile.

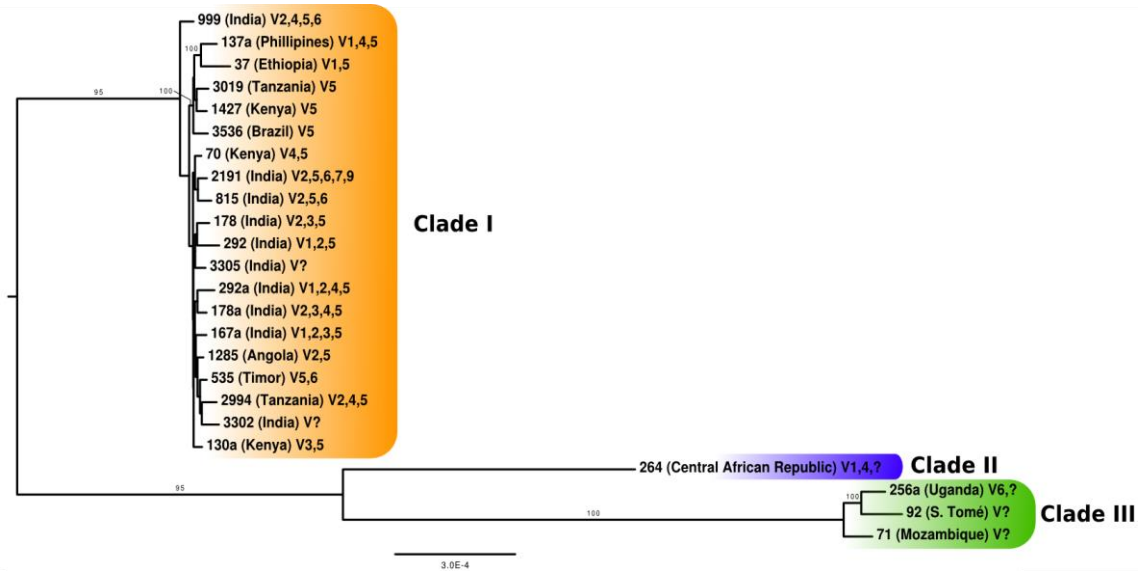

Figure 1. Maximum likelihood phylogenetic reconstruction of the 24 H. vastatrix samples. Only bootstrap values above 70 are shown above branches. Each H. vastatrix isolate is shown with its code, geographic location in parenthesis and the respective virulence profile.

Intriguingly, the absence of any genetic structure in the presence of a large amount of genetic variation is in disagreement with the reports that *H. vastatrix* is an asexual pathogen. Indeed, clonal populations are expected to acquire population differentiation and thus, population structure, much faster than populations undergoing sexual reproduction. Given that our data set comprises neutral loci as well as loci potentially under selective pressures, such as host genotype, the observed phylogenetic pattern among the taxa in Clade I more closely resembles a case of a large panmitic population where isolates freely undergo sexual reproduction or, at least, some form of parasexuality that involves meiosis. In this case, the recombination resultant from such sexual interactions would erase any signature of genetic structure at neutral loci. Natural selection could still maintain differentiation on adaptive loci for a given environmental pressure, but since there is usually a much smaller proportion of these loci on the genome, their signal is largely overwhelmed by neutral loci.

Preliminary analyses of natural selection acting on variable loci were undertaken with an FST outlier detection method that compared isolates containing the virulence gene V2 against isolates without this virulence gene. Loci with an FST value above the average neutral FST estimate may be under divergent selection, whereas loci with FST values below that estimate are possibly under balancing selection. Analysis of 496 genes revealed only five loci (1%) putatively under divergent selection and 86 loci (18%) possibly under balancing selection. This preliminary analysis demonstrates the existence of five loci that may be able to link the virulence phenotype with a corresponding genotype but the relatively high amount loci with very low FST values also suggests that allele frequency, rather than its absence/presence, may also be important to explain virulence in *H. vastatrix*.

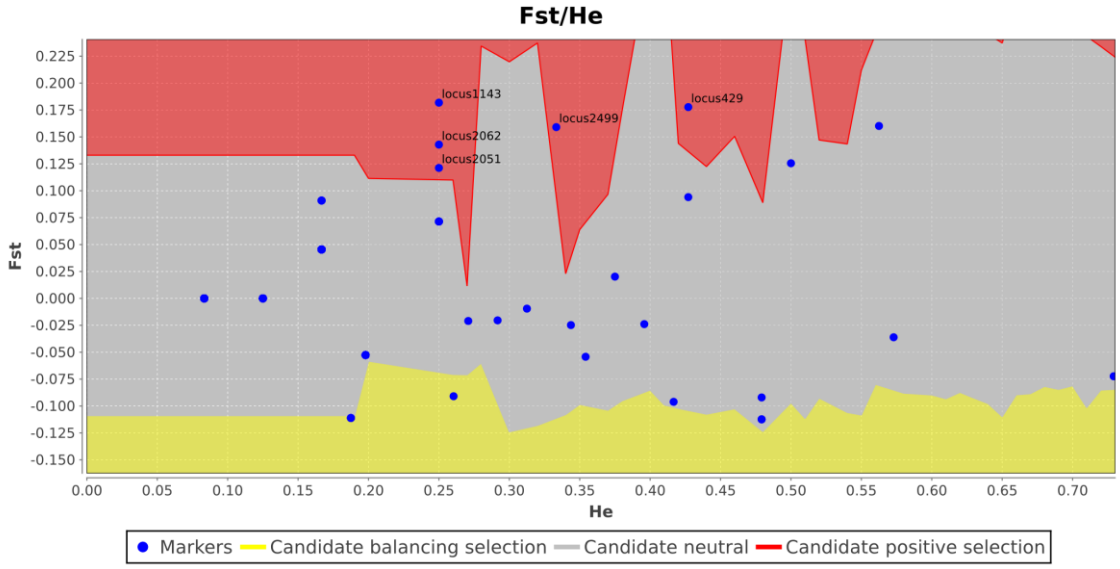

Figure 2. Plot of the FST values for each loci in function of the heterozygosity value. Loci within the red background are possibly under divergent selection, whereas loci within the yellow background are possily under balancing selection.

Therefore, while our results tantalizingly suggest that *H. vastatrix* populations may not be clonal, it will be critical to estimate the recombination rate among isolates as well as to perform further genome-wide scans between populations of different geographic or pathogenic backgrounds in search of potentially adaptive loci that may be responsible for its virulence in *Coffea* spp. These issues are of crucial importance for disease management, since sexual populations are much better suited to overcome resistant host genotypes and require different control strategies. Ultimately, this information will lead to our goal of associating a fungal genotype to a pathotype and the genomic mechanisms underlying the emergence of virulence and resistance overcoming of *H. vastatrix* populations.

ACKNOWLEDGEMENTS

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Assessment of Genomic and Karyological Diversity of Colletotrichum Kahawae

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SUMMARY

Colletotrichum kahawae is the causal agent of Coffee Berry Disease (CBD), which is capable of infecting green berries on Arabica coffee. Although this disease is currently restricted to Africa, it represents a threat to cultivation in America and Asia. The thorough understanding of *Colletotrichum* biology will certainly contribute to better suited disease management approaches. Potential differences in genomic composition and karyological analysis may contribute to deepen the knowledge on the infection process, such as specific pathogenicity factors in extra chromosomes, and to contribute to the taxonomic placement of the CBD pathogens in the *C. kahawae* cluster within the 'gloeosporioides' species complex.

In this work, a combination of karyological and genomic tools were used to further characterize *C. kahawae*. Several isolates representing the genetic and geographical diversity of the species were analyzed using different approaches that include chromosome separation and characterization by pulse field gel electrophoresis, cytogenetic analysis and chromosome number estimation by microscopy and genome size determination by flow cytometry. Differences among *C. kahawae* isolates were identified concerning the genome size, which ranged 74-89 Mb, and the number of minichromosomes (below 2 Mb), from 1 to 5.

INTRODUCTION

Colletotrichum kahawae (Ck), a fungal plant pathogen, infects specifically Arabica coffee crops causing Coffee Berry Disease (CBD). Crop losses occur following infection of green berries with the formation of dark sunken lesions leading to anthracnose symptoms causing their premature dropping and mummification. Although CBD is currently restricted to Africa, the risk of its introduction in America and Asia is currently a major concern. Ck is very closely related to other members of the 'gloeosporioides' species complex, but has unique traits as only Ck is capable of infecting green coffee berries. Also, there is evidence that a recent host-jump under-laid the speciation of Ck from a specific group within the 'gloeosporioides' complex. Studies of the molecular basis of host-parasite interactions in *Colletotrichum* are of utmost importance. Within this scope, the identification of supranumerary chromosomes can be of great relevance, as these may harbor key pathogenicity factors. These small extra chromosomes are usually considered conditionally dispensable chromosomes, and can be associated with virulence. Indeed, in previous works,

genes that contribute to the ability of the fungus to cause disease were located on small sized supranumerary chromosomes. In this work we use a combination of karyological and genomic tools to further characterize the genome of Ck.

MATERIALS AND METHODS

Colletotrichum spp. isolates used in this study were: CBD-isolates obtained from infected green *Coffea arabica* berries in five African countries representing the three groups that were previously differentiated. CBD-isolates were named after their geographical location: Angola (Ang6, Ang21, Ang29, Ang81), Cameroon (Cam1-3) and East Africa (Que2, Zim1, Ug2-7, Ug9). Non CBD-isolates were selected in order to represent two populations closely related to *C. kahawae*, *Colletotrichum* sp. 2 (=*C. aotearoa*; isolate C1282.4) and *Colletotrichum* sp. 2 (=*Ck* ssp. *ciggaro*; C1262.12, C1206.3 and PR432). *C. gloeosporioides s.s.* Ug8 and PT808 and *C. higginsianum* (IMI349063) were also included in the study.

The nuclear DNA content of the fungi was estimated by flow cytometry using a Partec CyFlow Space flow cytometer (Partec GmbH, Görlitz, Germany) using *Arabidopsis thaliana* (cv. Columbia) as reference standard (2C = 0.32 pg DNA). Data analysis was done according to.

Protoplasts were obtained by digesting mycelium with lysing enzymes (12 mg ml^{-1}) (Sigma-Aldrich) according to with slight modifications. Protoplast suspensions were immobilized in low melting agarose plugs. DNA release was done as described in.

For pulse field gel electrophoresis, chromosomes were separated in a 0.8% Pulsed Field Certified Agarose gel in 0.5x TBE at 12°C. Run times were as described in Fig. 1.

Karyology was performed using conidial suspensions (in PDA) incubated in slides at 25ºC until germination. For arresting metaphase in Que2 isolate, 100µl of 0.006% thiabendazole (TBZ) was added and allowed to incubate for 5h. Isolate Ang29 was incubated with 1% TBZ for 1h. After incubation, material was fixed in methanol:acetic acid (9:1) flame dried and stained with 4",6-diamidino-2-phenylindole (DAPI). Slides were then mounted in Vectashield antifade solution. Observations were made by UV-excitation using an epiflourescence microscope.

CONCLUSION

The genome size determined for Ck by flow cytometry ranged 74-89 Mb (Table 1), slightly above that of *C. higginsianum* and *C. gloeosporioides* (65-68 Mb). However values for Ck were close to the values for *Colletotrichum* sp. 1 and 2 (70 and 74 Mb respectively).

Karyological analysis allows the assessment of morphological characters of mitotic chromosomes. Treatment with thiabendazole (a microtubular formation inhibitor) was successful in obtaining fully condensed chromosomes that enabled reliable chromosome counting. Eleven chromosomes were estimated for isolate Que2 (Fig. 1B) and 11 to 14 for isolate Ang29 (Fig. 1A). This potential difference may be linked to the different number of minichromossomes as revealed by the PFGE analysis.

Table 1. Genome size estimations (Mb) among *Colletotrichum kahawae* isolates, including reference *C. higginsianum* and *C. gloeosporioides* isolates.

Phylogeny		Isolate	Genome size (Mb)					
			Mean	SD	CV	Min.	Max.	n
Colletotrichum kahawae (CBD-causing isolates)	Angola populations	**Ang6**	78.44	2.54	3.24	75.79	80.85	3
		Ang21	76.54	0.52	5.71	75.95	76.86	3
		Ang29	77.60	4.43	4.61	73.67	82.40	3
	Cameroon populations	**Cam1**	89.28	2.19	2.46	87.10	91.49	3
		Cam2	74.41	4.18	5.61	69.64	77.44	3
		Cam3	79.68	3.06	3.84	77.52	81.85	2
	East Africa populations	**Que2**	79.12	4.62	5.84	72.31	86.32	6
		Ug2	78.52					1
		Ug3	76.18					1
		Ug4	74.18					1
		Ug5	74.15	5.67	7.65	69.45	80.46	3
		Ug6	74.07					1
		Ug7	75.69					1
		Ug9	74.20					1
Colletotrichum sp. 1		**PR432**	73.87	4.17	5.80	68.39	80.12	7
		C1206.3	65.81	3.56	6.41	63.16	70.82	4
Colletotrichum sp. 2		**C1282.4**	73.51	2.14	5.93	71.06	75.01	3
Colletotrichum gloeosporioides		**PT808**	68.23	3.59	5.26	71.98	64.83	3
		Ug8	65.14	2.11	3.25	68.23	63.63	4
Colletotrichum higginsianum		**IMI 349063**	68.34	1.77	2.59	67.09	69.59	2

Phylogeny adapted from [2]. Weir and collaborators [7] have described *Colletotrichum* sp. 1 as *C. kahawae* ssp. *ciggaro* and *Colletotrichum* sp. 1 as *C. aotearoa*.

A,C - isolate Ang29. B - isolate Que2. Germinated conidia on the slide were incubated with thiabendazole for metaphase arrest previously to staining with DAPI. Well separated metaphase chromosomes (A,B); Yellow arrows indicate potential minichromosomes (C).

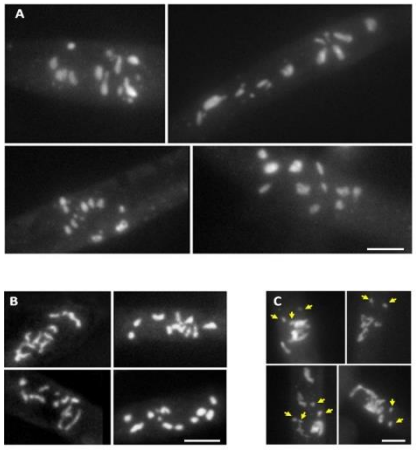

Figure 1. DAPI stained chromosomes of *Colletotrichum kahawae.*

Within each metaphase spread, the relative size of the chromosomes ranged from small to large. In metaphase spreads of Ang29, small dot-shaped chromosomes (Fig. 1C) were observed that could potentially correspond to the minichromosomes seen in the electrophoretic pattern.

Chromosome separations using Pulsed-Field Gel Electrophoresis (PFGE) showed a variable number of polymorphic chromosomes (from one to five minichromosomes (less than 2 Mbp) in the Ck isolates tested (Fig. 2). These minichromosomes appear at different patterns among the tested isolates. Furthermore this difference also appeared in other non-CBD isolates. This is consistent with other reports on plant-pathogenic fungi where polymorphisms in the number of chromosomes among isolates were observed in small sized chromosomes, which can be involved in pathogenicity traits of the various isolates. The presence of different minichromosomes might account for individual isolates having different pathogenicity/aggressiveness characteristics. In fact, there is apparently a correlation between the higher number of minichromosomes separated by PFGE and the higher levels of aggressiveness of the respective isolates (Fig. 3). Results prompt further analyses attempting to correlate variations in chromosome size/number with different levels of aggressiveness for Ck isolates.

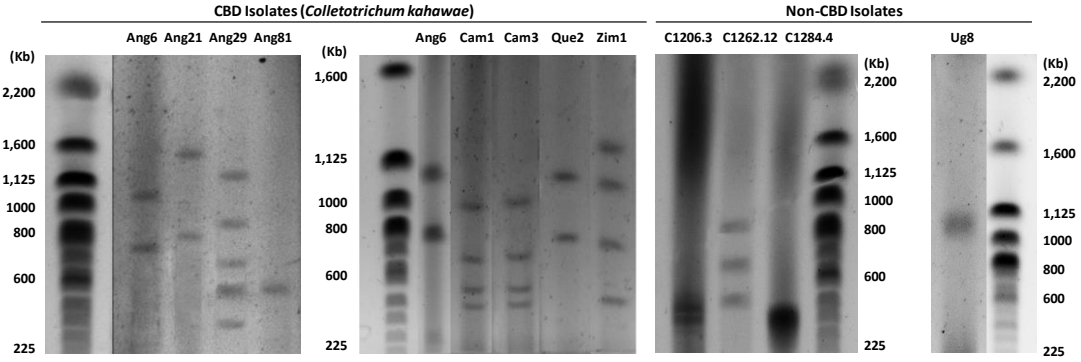

Figure 2. Small-sized chromosomes separated by Pulse Field Gel Electrophoresis.

Chromosomes were separated in a 0.8% Pulsed Field Certified Agarose gel in 0.5x TBE at 12°C. The run time was: A, C, D - 50 hours at 1.5V/cm with a 1000-2500 second switch time ramp at an included angle of 106°, followed by 16 hours at 3.5V/cm with a 100-300 second switch time ramp at an included angle of 106°. B- 32 hours at 3V/cm with a 350-550 second switch time ramp at an included angle of 120°, followed by 13 hours at 4.5V/cm with a 80-120 second switch time ramp at an included angle of 120°. Size marker, 0.225–2.2Mb *Saccharomyces cerevisiae* chromosomal DNA. Non-CBD isolates include *C. gloeosporioides sensu strictu* (Ug8), and isolates clustering in groups defined by as *Colletotrichum* sp. 2 (C1282.4) and *Colletotrichum* sp. 1 (C1206.3 and C1262.12).

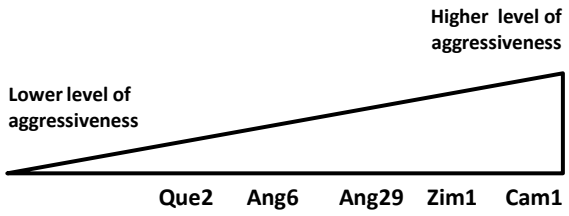

Figure 3. Relative levels of aggressiveness of *Colletotrichum kahawae* isolates.

Inoculations were done in hypocotyls and green berries of *Coffea arabica* 'Caturra' (previous results obtained in CIFC/IICT routine screening).

ACKNOWLEDGMENTS

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Identification and Characterisation of *Hemileia vastatrix* Effectors

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SUMMARY

Hemileia vastatrix is a biotrophic fungus which causes coffee leaf rust, the most important disease affecting Arabica coffee production worldwide, leading up to 40% yield losses if no control measures are applied. Breeding for rust resistance has proved the most economically efficient strategy for crop protection, leading to a sustainable coffee production. The genetics of coffee-rust interaction follows the 'gene-for-gene' theory, enabling the identification of physiological races of the pathogen and of plant resistance factors. Recently, the large-scale identification of fungal gene products that induce or supress a defence response in the host plant (effectors) has led to the development of approaches to identify and characterise plant resistance genes based on the identification and characterisation of fungal effectors.

To better understand the fungal genetic factors that condition coffee–rust interactions, a 454-transcriptomic analysis of three *H. vastatrix* differentiation/infection stages (germinating urediniospores, appressoria and *in planta* haustoria rich sample) was performed. An Expressed Sequence Tags (ESTs) database was generated releasing an exhaustive gene repertoire to be explored. From the 9234 genes reported, 516 were predicted to encode secreted proteins. These constitute candidate effectors, putatively governing the host-pathogen interaction. In this work, these 516 genes are analysed *in silico* for predicted biochemical and functional characteristics. Gene expression profiles for a selection of these genes are also analysed along the time course of key compatible and incompatible interactions, with the purpose of identifying candidate fungal effectors, and under the broader objective of contributing to the identification of the respective resistance gene conterparts.

INTRODUCTION

Coffee leaf rust caused by the fungus *Hemileia vastatrix* Berkeley & Broome is a disease disseminated in nearly all coffee growing countries causing up to 40% of crop losses if no control measures are applied. The studies conducted at CIFC/IICT enabled characterizing 45 physiological races of the pathogen and several resistant coffee genotypes. To better understand molecular factors governing coffee-rust interaction a 454-transcriptomic analysis of three *H. vastatrix* differentiation/infection stages (germinating urediniospores, appressoria and *in planta* haustoria rich sample) was performed. The homology of transcripts to genes known to be involved in pathogenicity in other fungi, namely in appressoria-mediated infection, enabled the identification of an array of putative pathogenicity factors, many of which are expressed as early as during germ-tube elongation. Effectors are usually defined as

"pathogen proteins and small molecules that alter host-cell structure and function". "These alterations either facilitate infection (virulence factors and toxins) or trigger defence responses (avirulence factors and elicitors) or both". The discovery of effector proteins is impaired by the difficulty on its identification. Effectors, and particularly AVR genes, are often very diverse even between species, and rarely present conserved domains with characterized proteins, discouraging the hunt for AVR by sequence similarity to known effectors. Effectors and effector targets are directly linked to how pathogenesis is promoted, and its identification has as ultimate goal breeding plants to pathogen resistance.

In this work we used a bioinformatic approach to detect effector candidates in *H. vastatrix* secretome. First, an identification of secreted proteins which did not show transmembrane domains was conducted. Second, the sequences of putative secreted proteins were analyzed *in silico* for predicted biochemical and functional traits. Finally, a set of genes was chosen for gene expression studies along the time course of compatible and incompatible interactions.

MATERIALS AND METHODS

Bioinformatic analysis of transcripts

A library of 9234 ESTs representing three *H. vastatrix* differentiation/infection stages (germinating urediniospores, appressoria and a *in planta* haustoria rich sample) was screened for putative secreted proteins using a pipeline comprising the SignalP, TargetP and TMHMM programmes. Several annotation tools were employed (Fig. 1).

RT-qPCR gene expression

Biological samples used for RT-qPCR studies comprised *H. vastatrix* isolate 178a resting urediniospores, germinating urediniospores (gU) and appressoria (Ap) and *in planta* samples of *Coffea arabica* leaves inoculated with isolate 178a [genotypes H147/1 (compatible interaction) and H469/16 (incompatible interaction)] collected at 18 h and 1, 2, 3, 7, 14 and 21 days after inoculation (for incompatible interactions samples were also collected at 36h and the experiment ended at 7d). The progression of the infection was monitored by light microscopy. Sample preparation and RT-qPCRs were conducted as before.

CONCLUSION

Secreted proteins

From the 9234 genes reported, 5.6% (516 genes) were predicted to encode putative secreted proteins and analysed *in silico* for predicted biochemical and functional characteristics (Fig.1). The annotation of the 516 transcripts encoding putative secreted proteins and comparison of their relative abundance in each of the three samples showed that a large proportion of transcripts were expressed as early as during germ-tube elongation, with high accumulated expression values (Fig. 2). Ca. 43% of genes present 'No hits' annotation, denoting a high proportion of putative *H. vastatrix* specific genes. Also, different expression profiles were identified considering the RA values obtained. From these 516 transcripts 70% entries present less than 200 amino acids each; 16% are highly enriched in cysteine residues (5-15% of all amino acids); 26%contain a [YFW]xC motif (Fig. 2) and 9% present homology to genes in the "Pathogen Host Interaction" database, among which are the pathogenicity-required genes UBC3 and CLB2 (*Ustilago maydis*), BCP1 (*Botrytis cinerea*), CGB1 (*Cochliobolus heterostrophus*) and ILC1 (*Leptosphaeria maculans*). Despite the higher

number of genes represented in the H library they are less expressed comparatively to the gU and Ap libraries (Fig. 2).

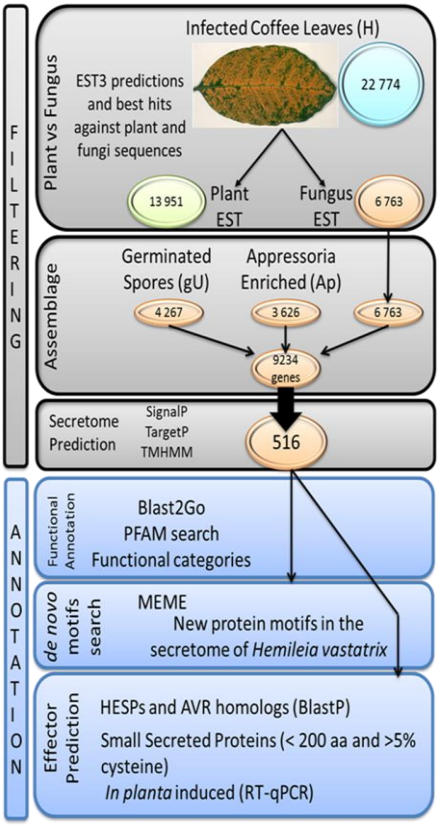

Figure 1. Pipeline for transcriptomic analysis of *Hemileia vastatrix* key differentiation stages, gene assembly, secretome prediction and bioinformatic annotation.

	Number of genes			Relative abundance		
	gU	Ap	H	gU	Ap	H
Secreted proteins	249	242	301	78.22	29.53	25.56
<200aa	79	61	129	31.8625	11.2911	14.0697
>5% Cysteines	27	29	48	8.1476	1.7498	3.7998
[YFW]xC motif containing	54	61	78	6.3661	11.7250	1.5273

Figure 2. Heatmaps of the number of genes and sum of their Relative abundance values (=number of transcripts/transcript length) in the three *Hemileia vastatrix* EST libraries (germinating urediniospores - gU; appressoria - Ap; infected leaves 21 days after inoculation - H) for transcripts putatively encoding secreted proteins. Colour scale: green to red denote lowest to highest values for each gene.

Rt-qPCR Genes expression profiles

The eleven genes selected for RT-qPCR analysis, included three transcripts orthologous of the *Uromyces fabae* Rust Transferred Protein 1 (RTP1) (transcript Hv01043 was detected in Ap and H, and transcripts Hv00303 and Hv00357 were identified in the three EST libraries); two transcripts orthologous of the *Melampsora lini* Haustorially Expressed Secreted Proteins (HESP) (transcript Hv00258 which was identified in the three libraries and transcript 09298 only identified in Ap);one transcript related to a sterol carrier protein (Hv00199) of the Cysteine-rich secretory protein family (pfam00188); three transcripts containing the

DUF3129 domain (pfam11327), to which a pathogenicity-related *Magnaporthe oryzae Gas1* gene belongs, (Hv00125, Hv00409 and Hv00489); one transcript (Hv00231) clustering in the Glycine rich protein family (pfam07172); one transcript (Hv04957) containing the Common in Fungal Extracellular Matrix (CFEM) domain (pfam05730), a fungal specific cysteine rich domain found in proteins with proposed roles in fungal pathogenesis. RT-qPCR expression profiles (Fig. 3) suggested the activation of several genes in germ tubes *in vitro*, as well as in the compatible interaction, namely the early activation of RTP-orthologous Hv00303 and Hv00357 and of the Gas1-orthologous Hv00489, and the late activation of gene Hv00231. On the contrary, the HESP-orthologue gene Hv09298 seems to be specifically activated in the incompatible interaction. The *H. vastatrix* secretome, as those of other rusts, seems to be partially composed by enzymes that may be responsible for basic biological functions of secreted proteins, e.g., plant cell wall degrading enzymes (polysaccharide deacetylase), phospholipases, and detoxification enzymes (thioredoxin, Cu-oxidase_3, Multicopper oxidase). On the other hand, effectors are difficult to identify due to high sequence variation and difficult function prediction, and therefore only a small number of rust effectors has been identified. Two genes from our analysis are good effector candidates, Hv00231 and Hv09298. The first was clearly induced *in planta* (Fig. 3) and has a glycine-rich domain usually associated with protein-protein interaction. In fact, the Pw1 effector group from *Magnaporthe oryzae* comprises small-secreted glycine-rich proteins. The gene Hv09298 was specifically induced during the incompatible interaction, suggesting that it may play a role in the induction of plant immunity towards the rust fungus. The recent availability of genome-wide repertoires of effectors for various pathogens rendered effector-assisted disease resistance breeding successful in various crops.

Figure 3. RT-qPCR expression profiles of 11 *H. vastatrix* candidate effector genes for samples obtained in vitro (germinated urediniospores, gU and appressoria, Ap) and along the time course of a compatible and a incompatible interaction (Fig. 3), expressed as Fold Change (Y axis) by comparison to the expression level in resting urediniospores.

We envisage that the results presented here may contribute to determine molecular factors governing coffee-rust interaction through the identification of candidate effector genes.

ACKNOWLEDGEMENTS

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Characterization of *CcDREB1D* Promoter Region from Contrasting Genotypes of *Coffea canephora* by Homologous Transformation in *Coffea arabica*

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SUMMARY

Sequence variation analysis of promoter and coding regions of *DREB1D* gene from 16 coffee plants (10 from *C. arabica* and 4 from *C. canephora*), displaying different phenotypes (tolerance *vs.* susceptibility) regarding to drought, indicated a low level of diversity at protein level but a high level of diversity in the promoters within coffee plants. Furthermore, we found several indications of association between drought tolerance and the genetic variations on *DREB1D* promoter region, but not with those from the coding region. A comparison of predicted *cis*-acting elements for all the promoter sequences suggest the *DREB1D* is evolving by the rearrangement of *cis*-regulatory elements and, as result the alteration of expression patterns of this gene might play a fundamental role on drought adaptation in coffee. Three haplotypes of *DREB1D* loci likely to be associated to contrasting phenotypes regarding to drought, H_16 and H_15 (tolerant), and H_17 (susceptible) are under characterization by homologous transformation in *C. arabica*.

INTRODUCTION

Climatic variability has always been the main factor responsible for the fluctuation of coffee yields worldwide, and the climate change, as a result of global warming, is expected to present a major challenge to the coffee industry. Despite of the uncertainty surrounds the effect of climate changes on individual producing regions and overall coffee production, significant changes are expect to occur in some regions. Furthermore, the potential impact will not only vary between countries but also within producing areas in individual countries. Climatic changes leads to abiotic stress such as extreme temperatures and drought which are the major environmental stress affecting coffee production in most coffee-growing countries. In several plant species, the *DREB* genes play a key role in responses to abiotic stress. DREBs (Dehydration Responsive Element Binding) are transcription factor proteins that compose abscisic acid (ABA) -dependent and -independent pathways of signal transduction in abiotic stress response, and regulates the expression of several stress-related genes. Natural variability exists for drought tolerance among *Coffea* species, even at the intraspecific level between and within genetic groups in *C. Canephora*. Since the development of molecular markers is one of the major goals for accelerating breeding programs, a study was done to evaluate the sequence variability of the *DREB1D* gene in several *Coffea* genotypes. The promoter and coding regions of *DREB1D* gene were cloned and sequenced from 16 coffee plants (10 from *C. arabica* and 4 from *C. canephora*), most of them characterized by different phenotypes (tolerance *vs.* susceptibility) regarding to drought.

MATERIALS AND METHODS

Plant materials

The plant material used in this study was obtained from a range of genotypes from *Coffea* genus. The genotypes were chosen in order to obtain coverage of genotypic variability in *Coffea* based on criteria such as: genomics resources; diversity of geographical origin; morphological and agronomical difference. A total of 29 genotypes were used in this study (Table 1).

Gene amplification and sequencing analysis

Promoter and coding sequences of *DREB1D* gene were amplified separately by PCR using specific primers pairs. The amplified fragments were cloned and sequenced in both directions with primers M13 forward/reverse using the sequencing reagent kit BigDye Terminator v3.1 (ABI 3129xl Genetic Analyzer, Applied Biosystems). For each genotype, 16 clones were sequenced and a total of 24 sequences were aligned and analyzed for the identification of polymorphisms. The DNA sequences were subdivided into promoter and coding regions including UTR. Sequences were aligned with the SEQMAN 9.1.1 (4). 418 program (DNASTAR Version 2.1.0.97, Copyright 2010-2011, Inc.). Statistics of intraspecific polymorphism within the genotypes were performed using the DnaSP program version 5 (Librado and Rozas 2009).

Generation of transgenic Coffee embryos

To generate binary vectors harboring homologous sequence of *DREB1D* promoter from H_16, H_15 and H_22, the constitutive promoter fragment CaMV35S from pBI121 (Clontech, Palo Alto, CA, USA) was excised so that it could be replaced by each haplotype promoter. Two series of 5'-deletions (–1466/+1 and –762/+1) of the *DREB1D* promoter covering the upstream sequence from the Inr and the 5'-UTR were amplified from *Coffea* genomic DNA of clone 14 and 22 through PCR using specific primers pairs for H_15, H_16 and H_17 haplotypes. The resulting PCR fragments were subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and then sequenced to confirm that no mutations were introduced into the PCR products. The deleted derivatives were cloned into the *Hind*III/*BamH*I sites of pBI121. These recombinant constructs were named pD14-hp15 D, pD14-hp16 D, pD22-hp17 D, pD14-hp15 P, pD14-hp16 P and pD22-hp17 P, according to the nucleotide positions at the 5'-end P (–762/+1) and D (–1466/+1). Each recombinant plasmid was introduced into *Agrobacterium tumefaciens* strain LBA1119 and was used to transform cryopreserved calli of 8 and 12 months from *C. arabica* var. Caturra old via the mediated gene transfer procedure. For the stress treatment, Caturra transgenic embryos were dehydrated on a laminar *flow* cabinet subjected to an air flow of 0.49 m/s for 30 minutes and, then, stained with GUS.

CONCLUSION

DREB1D gene amplification

The *DREB1D* promoter and coding sequences were isolated by PCR on genomic DNAs obtained from the panel of genotypes. The promoter and coding sequences were amplified separately. Specific amplification products were cloned and 16 clones sequenced. 1456 bp

and 738 bp of the promoter and coding region, respectively, were analyzed. Two to three alleles were identified according to the ploidy of each genotype.

Table 1. List of coffee genotypes used to amplify the *DREB1D* promoter and coding region.

Genotype	Ploidy	Haplotype	Genotype	Ploidy	Haplotype	Specie
1.Acaiá 47119	4x	H_02; H_01	10. Mundo novo	4x	H_01; H_32	*C. arabica*
2.Bourbon	4x	H_03; H_04; H_05	11. Obatã	4x	H_01; H_19	*C. arabica*
3.Catuai 25	4x	H_01; H_05	12. Palma 02	4x	H_01; H_33; H_34	*C. arabica*
4.Catuai 144	4x	H_12; H_13; H_14	13. Purpurenses	4x	H_01; H_05	*C. arabica*
5.E238	4x	H_01; H_18; H_19	14. Rubi	4x	H_01; H_37	*C. arabica*
6.Guatemalense Baixo	4x	H_26; H_27; H_28	15. Sabiá	4x	H_05; H_38	*C. arabica*
7.Guatemalense Alto	4x	H_24; H_25	16. San Bernardo	4x	H_01; H_05; H_39	*C. arabica*
8.IAPAR 59	4x	H_01; H_19	17. San Ramon Baixo	4x	H_01; H_40	*C. arabica*
9.Ikatú Colombiano	4x	H_29; H_29; H_31	18. Tupi	4x	H_04; H_19	*C. arabica*
			19. Typica	4x	H_01; H_42	
20. C1007	2x	H_06; H_07	24. Clone 22	2x	H_15; H_17	*C. canephora*
21. C2011	2x	H_08; H_09	25. G2020	2x	H_22; H_23	*C. canephora*
22. C3001	2x	H_10; H_11	26. UW002	2x	H_43; H_44	*C. canephora*
23. Clone 14	2x	H_15; H_16	27. UW099	2x	H_45; H_46	*C. canephora*
28. Psilanthus	2x	H_05; H_35; H_36				*C. benghalens*
29. Eugenioides	2x	H_20; H_21				*C. eugenioides*

Sequence diversity of the *DREB1D* gene

A total of 960 sequences were assembled and only alleles present in at least two different sequences were considered as polymorphisms (these two different sequences could originate either from the same genotype or from different genotypes). Within the 2194 bp of *DREB1D* gene analyzed we identified 145 SNPs. We observed a high conservation of DREB1D proteins among the homologous sequences due to the low level of diversity and the high number of synonymous mutations and neutral changes. Among the 22 SNPs identified in the coding region, 13 are synonymous (do not change the coding amino acid), and 9 are non-synonymous (promote changes on amino acid sequence) of which 8 are neutral substitutions (E41D, V163G, F165S, D168H, G175E, D189E, V191A, G213A) and only one is a non-neutral substitution on the sixth amino acid of the DREB1D protein sequence, isoleucine to threonine (I6T). However, the promoter region displayed a high level of nucleotide diversity, 123 SNPs and 171 INDELs were found in the coffee *DREB1D* promoters. We obtained 21 haplotypes for the coding region and 46 haplotypes for the promoter region among all genotypes analyzed. When we analyzed the promoter haplotype NJ network a combination of population structure and estimated habitat drought tolerance of *C. canephora* accessions was observed suggesting a possible relationship between adaptive variation and haplotype variability (Figure 1). Analysis of genetic diversity in *C. canephora* population showed the occurrence of distinct groups classified according to its geographical origin. One of them, the Congolese group, was subdivided in several groups. Among them two major groups contrasting to drought response, SG1, tolerant, and SG2, susceptible. A comparison of predicted *cis*-acting elements for all the promoter sequences signaled the loss of some regulatory DNA elements. The sequence variation and the loss of some regulatory DNA

elements can be the link between adaptive variation and haplotype variability, and could explain the differences of *DREB1D* gene expression previously observed in leaves of drought tolerant (clone 14) and susceptible (clone 22) clones of *C. Canephora*. In fact, both clones 14 and 22, have one same *CcDREB1D* allelic sequence (hp15), and diverge at a second allele. Thus, the *CcDREB1D* allele in the tolerant 14 (hp16) was considered to be the favorable/tolerant allele and the allele in 22 (hp17) was inferior/sensitive.

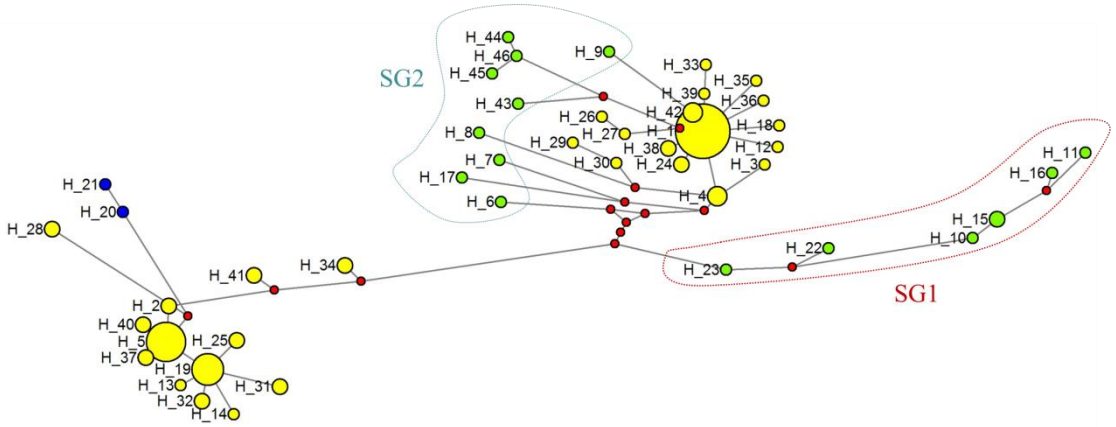

Figure 1. Neighbour-joining analysis of *DREB1D* gene in *Coffea* genus. Separation of *C.arabica* homoeologous haplotypes (yellow) evidence similarity between CaCe subgenome and homologous haplotypes of *C.eugenioides* (blue) as well as similarity between CaCc subgenome and homologous haplotypes of *C.canephora* (green).

Generation of transgenic Coffee embryos

Cryopreserved calli of 8 and 12 months old were successfully transformed by *Agrobacterium tumefaciens* mediated gene transfer procedure. The capacity of *DREB1D* promoter to control the expression of the *uidA* reporter gene was evaluated in a preliminary essay in transgenic embryos of *Coffea arabica* cv. Caturra. After six month of kanamycin selection, torpedo-shaped embryos were dehydrated on a laminar *flow* cabinet under an air flow of 0.49 m/s for 30 minutes and, then, stained with GUS at 37°C for 12 hours. Preliminary results of bioassays checking GUS (*β*-glucuronidase) activities indicate that the observed sequence variations have a direct role in the regulation of *CcDREB1D* expression (Figure 2).

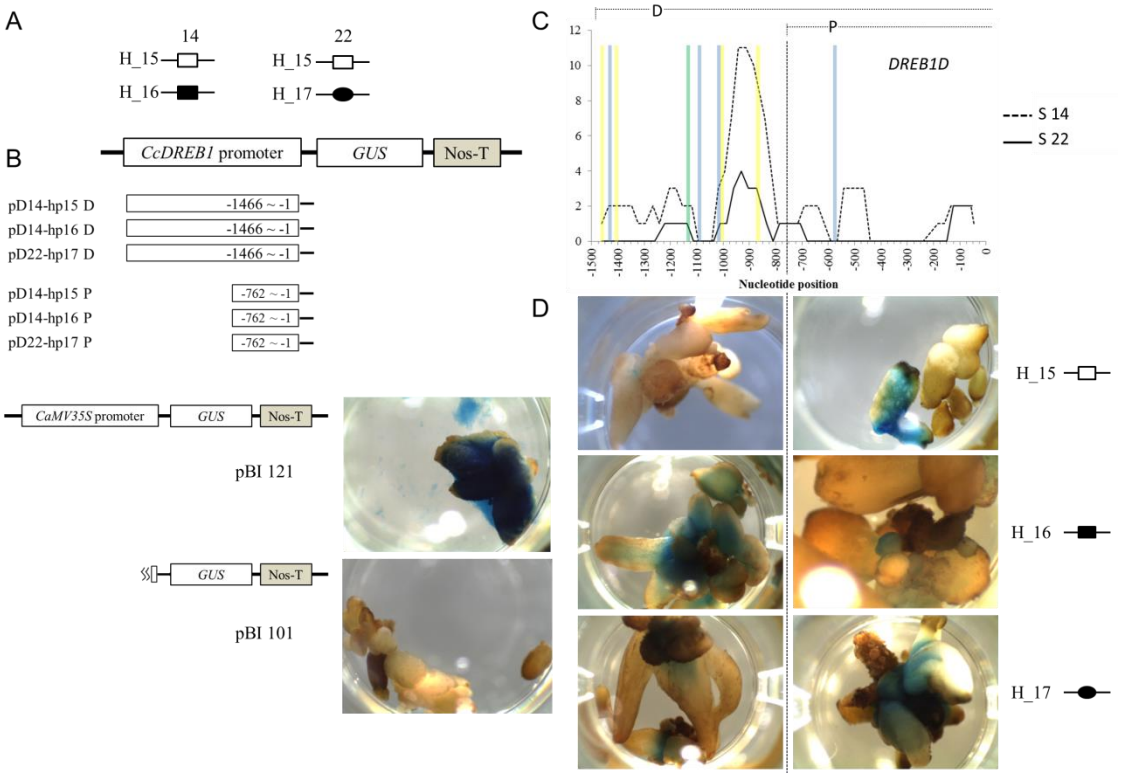

Figure 2. Truncation analysis of the DREB1D promoter region. (A) *DREB1D* haplotypes found in clone 14 (tolerant) and 22 (susceptible). (B) Schematic map of the promoter-*GUS* construct used in this study. (C) Polymorphic sites frequency (S) of the *DREB1D* gene among the genotypes of clone 14 (H_15 and H_16) and clone 22 (H_15 and H_17) in sliding window graphic. *Cis*-acting elements involved in osmotic stress response found in the promoter region of the *DREB1D* gene are indicated in column bars: ABRE-Like (yellow); MYB (blue) and MYC (green). (D) Histochemical localization of GUS activity of the distal promoter (-1466 bp) on the left panel and proximal promoter (-762) on the right panel in *C. arabica* transformed embryos with the pD14 and pD22 vectors.

The proximal promoter of *CcDREB1D* for the three alleles tested (H_15, H_16 and H_17) equally induced the *uidA* gene expression, however, expression of *uidA* under control of the complete *CcDREB1D* promoter was significantly induced in the tolerant allele (H_16) in response to the osmotic stress, whereas, it was not significantly upregulated for the common (H_15) and sensitive alleles (H_17). These results also evidence that the sequence variation present at the first -700 bp of *CcDREB1D* promoter do not interfere the regulation activity of the promoter, probably due to the non-overlapping of SNPs and *cis*-regulatory elements. Though, the higher sequence variation and co-occurrence of SNPs and *cis*-regulatory elements observed between -700 and -1500 bp seems to affect the regulation of *CcDREB1D* promoter in response to drought stress.

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Transcriptome Analysis of Leaves and Fruits of Coffea Eugenioides

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SUMMARY

Coffee is one important agricultural product to several developing countries. It is mainly produced by the allotetraploid *Coffea arabica* and the diploid *C. canephora*. Meanwhile, *Coffea eugenioides* is considered the ancestor of allopolyploid *Coffea arabica*, together with *C.canephora*, but there are few molecular studies in this specie. In this work, we present a large scale gene identification of *C. eugenioides* from RNA-Seq. A *de novo* assembled with all sequences was performed by Trinity and it generated 36,935 contigs which were successfully annotated against non-redundant (NCBI-nr) protein database, GO, Swiss-Prot, InterproScan, PlantCyc and KEGG. In addition, we examined genes differentially expressed in leaves and fruits of *C. eugenioides* using DESeq. A set of genes related to sugar metabolism and fruit ripening were identified in both organs. Finally, 10 highly expressed genes were selected to confirm their expression pattern by qPCR. Our study provides novel information to *C. eugenioides* and it is a valuable source for molecular and genetics studies for *Coffea* and can increase our knowledge about the mechanisms involved in the *C. arabica* homeologs expression. This general repertoire of *C. eugenioides* genes could open new perspectives to develop studies in this specie as well as the genetic improvement of commercial coffee.

INTRODUCTION

Coffea eugenioides is considered the ancestor of *Coffea arabica* allopolyploid, together with *Coffea canephora*. Despite the strategic importance of understanding gene expression of the *C. arabica* coffee ancestors, there are few studies focusing on *C. eugenioides.* Also there is few data in coffee fruit transcriptome.

Advances in new generation sequencing technologies (NGS) such as Illumina / Solexa allowed progresses in the analysis of the transcriptome of various species generating a large volume of data with effective cost. In this work, we used RNA-sequencing (RNA-Seq) data from leaf and fruit from *C. eugenioides* to develop a general characterization with coding genes of this specie.

MATERIALS AND METHODS

Mature fruits and young leaves were harvested of *C. eugenioides* maintained at the Technological Center of Cooperativa Agropecuária e Industrial (COCARI), Mandaguari, PR. After harvesting, samples were immediately frozen in liquid nitrogen and stored at -80 °C

until RNA extraction. Total RNA was isolated and RNA-Seq was done using Illumina HiSeq 200.

The processed reads of both organs were merged and assembled with Trinity assemble to develop the *de novo* assembly. Only contigs >200 bp were used for further annotation and with the dominant isoform. All contigs were compared using BLASTX against the NCBI non-redundant sequence database (nr), with e-value cutoff of 1e-5 and it was done a BLASTN against an EST database developed to *C. arabica*. Also BLASTX was done against sequences from Swiss-Prot. Functional annotation of biological processes and molecular function were performed using BLAST2GO, InterProScan and KEGG database.

Bowtie was used to map the processed reads against the reference *de novo* assembled transcriptome. Mapped sequence counts were processed using the DESeq package to estimate the transcript level. Additionally, BLAST2GO was also used for a GO functional enrichment analysis of exclusive contigs of leaves and fruits, by performing Fisher's exact test. Based on transcriptional activity pattern showed by DESeq results, 10 contigs were selected for expression analysis by qPCR to validate the results.

CONCLUSION

We obtained a total of 8,435,413 Illumina reads: 368,8364 reads from leaves and 4,747,049 reads from fruits. A *de novo* assembled with all sequences was performed by Trinity and they were generated 36,935 contigs which were annotated against non-redundant (NCBI-nr) protein database (63.1% hits), Gene Ontology (48.9% hits; Figure 1), Swiss-Prot (45.8% hits), InterproScan (34.7% hits), PlantCyc (20.8% hits) and KEGG (2.2% hits).

Gene annotations observed similar categories previously reported for *C. arabica* and *C. canephora*. However, top domains like small molecule binding and transferase activity in GO annotations could indicate processes associated with sugar synthesis and transport, terms also found with prevalence in *C. arabica* than *C. canephora*. Furthermore, these terms can indicate proteins related sugar metabolism, especially sucrose, as was seen for pathways in KEGG, where the pathway starch and sucrose metabolism were annotated. Sucrose has an important role in determine the coffee cup quality.

We also examined an overview of differentially expressed genes between leaves and fruits using DESeq. Interestingly, several genes exclusively expressed in fruits did not find similarity in any database. We selected contigs more expressed in each organ to confirm transcriptional profile by qPCR (Figure 2). Annotation using BLASTX against TAIR database of these contigs allowed the identification of the highly expressed genes in leaves: one UDP-Glycosyltransferase superfamily protein (*Ce14433*); a germin-like protein (germin 3, *Ce15205*); a glucose-6-phosphate/phosphate translocator 2 (*Ce2770*); a chitinase A (*Ce14847*); and a BURP domain-containing protein (*Ce10671*). In fruits higher expression levels were also observed from a BURP domain-containing protein (*Ce14834*); a serine carboxypeptidase-like 29 (*Ce13100*); a cytochrome P450, family 79, subfamily B, polypeptide 2 (*Ce13451*); transcription factor-related (*Ce9246*); and an oxidoreductase, zinc binding dehydrogenase family protein (*Ce13525*). These unigenes could be possible candidates to studies focused mainly in fruits and coffee quality.

To our knowledge, this is the first gene catalog for *C. eugenioides* using RNA-seq and the first large report on the analysis of leaf and fruit genes in this specie. A general repertoire of *C. eugenioides* contigs with differential expression in leaves and fruit open new perspectives

for studies in this specie and potential value for breeding assisted by biotechnological tools in coffee.

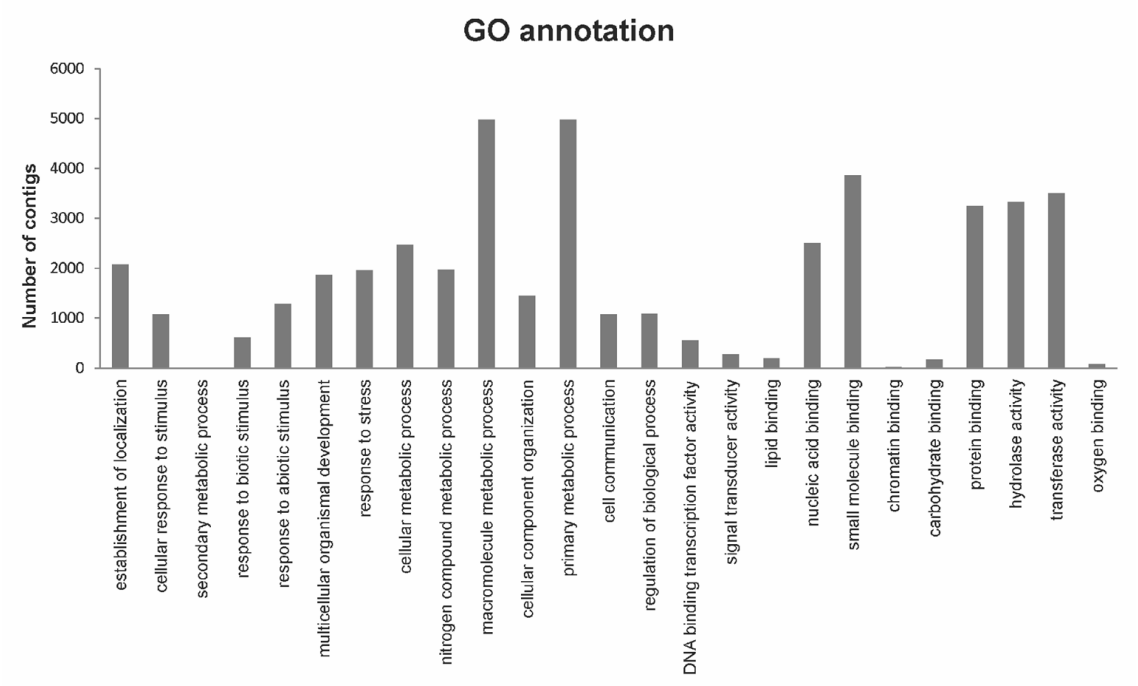
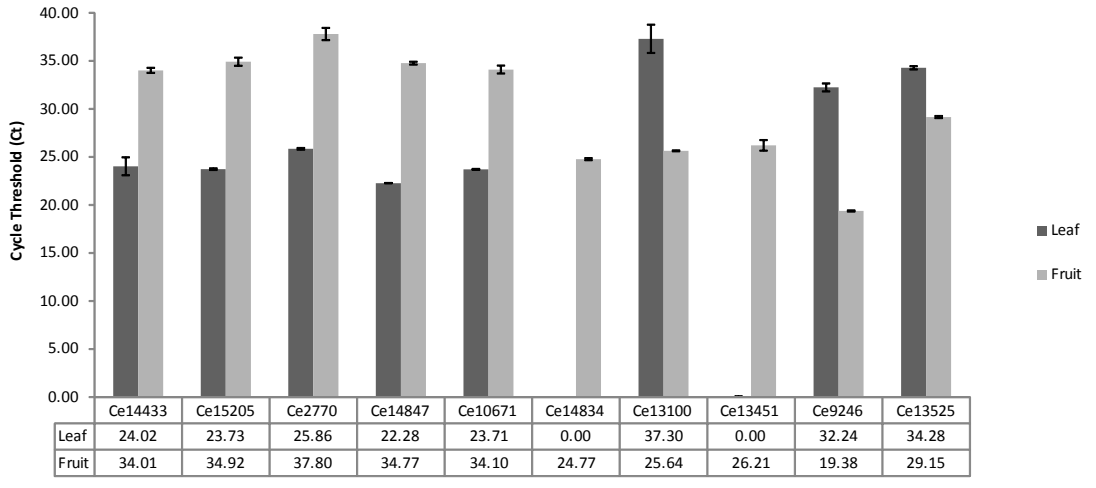


Figure 1. Number of *C. eugenioides* contigs in each functional category based on gene ontology classification. *C. eugenioides* contigs were classified into different functional groups based on a set of GO slims in the Biological Process and Molecular Function category.



	Ce14433	Ce15205	Ce2770	Ce14847	Ce10671	Ce14834	Ce13100	Ce13451	Ce9246	Ce13525
Leaf	24.02	23.73	25.86	22.28	23.71	0.00	37.30	0.00	32.24	34.28
Fruit	34.01	34.92	37.80	34.77	34.10	24.77	25.64	26.21	19.38	29.15

Figure 2. Relative expression values of contigs up-regulated in leaves (green) and fruits (red) organs by qPCR (Ct values scale). Bars represent the standard deviation values.

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Annotation of Transposable Elements in the *Coffea canephora* Genome

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SUMMARY

54.4 million of Roche 454 sequences, 131,412 Sanger BAC-end sequences and 60X Illumina coverage of the 710 Mb genome of a *C. canephora* Double Haploid accession (DH200-94) were generated, assembled and anchored to a genetic map by the COFEE GENOME CONSORTIUM (http://coffee-genome.org). We present the identification and classification of TEs in the *canephora* genome using a combination of *ab initio,* similarity and structure search approaches. We found that about half of the genomic sequences produced are composed of TEs similarly to other sequenced crop species such as banana, papaya, castor bean and soybean. Class I LTR retrotransposons represent the vast majority of identified elements, accounting to 42% of the genome assembly. *Gypsy* elements clearly outnumbering *Copia* elements since *Ty3-Gypsy* family covers 24.1% of the genome. Interestingly non-autonomous LTR retrotransposons elements were detected and classified into a new subgroup of non–autonomous elements. They represent about 11% of the coffee genome. All the annotated transposable elements will be used to study in details non–autonomous elements, the conservation of LTR retrotransposons between coffee and Angiosperm genomes, and the distribution of TEs along pseudochromosomes.

INTRODUCTION

It is now well established that plant genomes are dynamic structures submitted to a wide range of modifications through the activity of Transposable Elements (TEs). TEs are mobile sequences that share several key properties such as the ability to move from one chromosome location to another one, to amplify their copy number within the host genome. TEs contribute to the chromosome structure, organization and evolution and they play a major role in creating structural variation and genetic diversity in plant genomes. TEs were traditionally classified into two main classes according to their lifestyle cycle: Class I or Retrotransposons for TEs moving via an RNA intermediate (following a “copy and paste” mechanism) and Class II or Transposons for TEs moving via a DNA intermediate (having a “Cut and Paste” mechanisms).

MATERIALS AND METHODS

A combination of manual approaches and automatic programs (REPET package V.2.1-RC;) were used to identify, classify and annotate TEs on the *Coffea canephora* genome. The LTR_STRUC algorithm was used to detect LTR retrotransposons, MUST and MITE_hunter to predict MITE and Sine_finder to identify SINE, with the largest contigs or the

pseudochromosomes as input datasets. The REPET TEdenovo package was used to *de novo* identify TEs in the *C. canephora* largest contigs (14,160 contigs). TEs reference sequences were classified according to the REPBASE database (http://www.girinst.org/repbase/) and to our manually annotated TE library. Only non-chimeric repeats predicted by TEdenovo were used to annotate the *C. canephora* pseudomolecules (Figure 1).

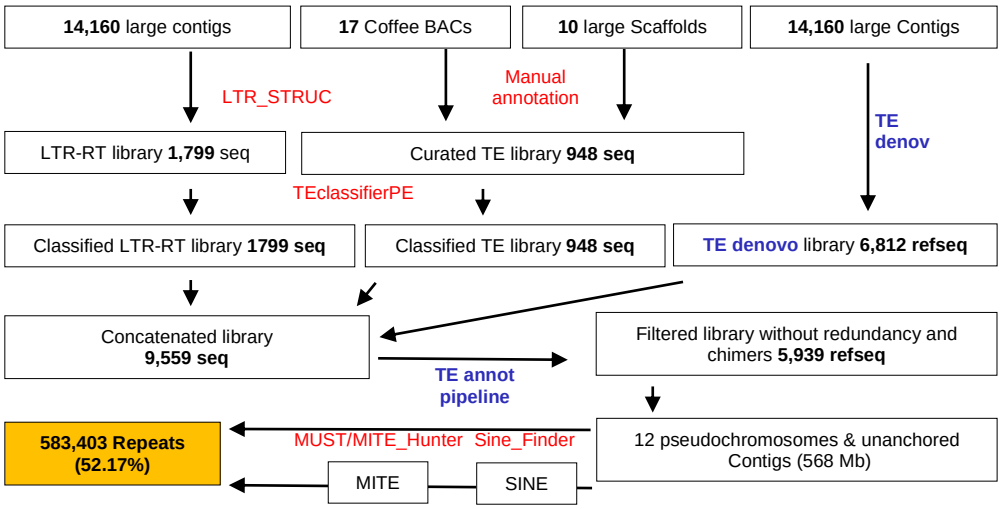

Figure 1. Schematic representation of the annotation process of transposable elements in the *C. canephora* genome.

CONCLUSION

Identification of LTR-retrotransposons

1,799 full-length LTR retrotransposons were *de novo* detected using the LTR_STRUC algorithm. This dataset was classified into *Gypsy* (RLG), *Copia* (RLC) and unknown (RXX) according to their BLASTX matches against the GyDB domain libraries (http://gydb.org). Sequences were clustered into 189 RLC and 388 RLG clusters and confirmed by phylogenetic analyses using their reverse transcriptase RT domains (Figure 2). The insertion times of full-length copies were dated, based on the divergence of the 5' and 3' LTR sequences of each copy. The insertion dates were estimated using an average base substitution rate of 1.3E-8. Our analysis indicates a relatively recent wave of LTR retrotransposon amplification (highest peak at 0.5-1 million years ago) for both RLC and RLG super-families.

De novo TE identification

The REPET TEdenovo package was used to *de novo* identify TEs in *C. canephora* contigs. After clustering, 6,812 reference sequences were kept and classified. On the 6,812 reference sequences, 6,414 fell into Class I elements (or Retrotransposons, 94%). *de novo* detection found a very low number of Class II transposons, few MITEs, and few SINEs, but a huge number of LTR retrotransposons (RLG and RLC) and non-autonomous LTR retrotransposons (RLX, TRIM and LARDS) reference sequences. On the 6,812 reference sequences, 1,393 were classified as "chimeric" (elements with more than one TE classification), and removed from our dataset. These elements are probably "nested" TEs.

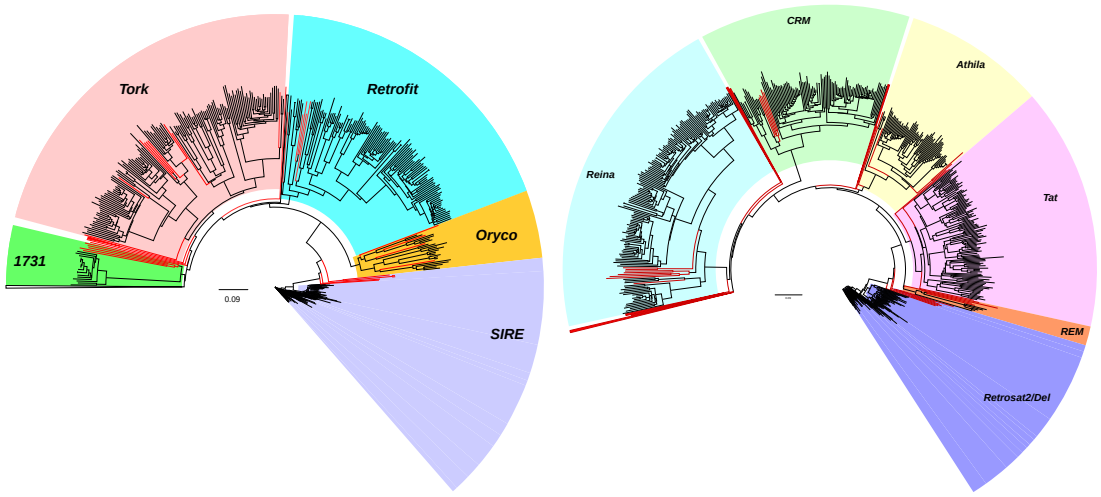

Figure 2. Phylogenetic analyses and classification of *Ty1/Copia* (left) and *Ty3/Gypsy* (Right) LTR retrotransposons.

TE annotation along the C. canephora pseudomolecules

We used the 5,363 (non-chimeric) reference repeats predicted by TEdenovo to annotate the *C. canephora* pseudomolecules. In total we found that TEs account for half of the coffee pseudomolecules (49.2%, Table 1). LTR retrotransposons represent 42% of the sequenced genome. Among them, the *Ty3-Gypsy* family represents the largest part: 24.1% of the genome. The distribution of TEs was plotted and compared to gene density. As observed in other plant genomes, there is an inverse relationship between gene density and repetitive sequences.

Table 1. Percentage of pseudomolecules annotated as transposable elements. TEs are classified according to wicker's rules: RLG: LTR retrotransposons Gypsy, RLC: LTR retrotransposons Copia, RLX: unknown and non-autonomous LTR retrotransposons, RIX: LINE, RSX: SINE, DTX: TIR transposons, DXX: MITEs, DHX: Helitrons, XXX: Unclassified.

Pseudomolecule	RLG	RLC	RLX	RIX	RSX	DTX	DXX	DHX	XXX	Total %
chr1	17.37	6.13	9.58	1.82	0.08	2.70	1.19	0.78	2.06	41.71
chr2	14.16	5.14	8.07	1.35	0.08	3.07	0.95	0.41	2.01	35.24
chr3	18.42	6.44	8.70	1.73	0.06	2.95	1.32	1.12	2.07	42.81
chr4	16.04	6.51	9.07	1.65	0.08	2.83	1.06	0.54	1.96	39.74
chr5	19.03	7.02	9.48	2.10	0.07	2.74	1.23	0.71	1.98	44.36
chr6	15.58	5.72	8.90	1.54	0.08	3.06	0.99	0.58	1.91	38.36
chr7	15.41	5.36	8.67	1.40	0.08	2.91	1.06	0.85	2.21	37.95
chr8	16.26	6.17	10.10	1.93	0.08	3.04	1.17	0.71	2.12	41.58
chr9	20.56	6.53	10.24	2.11	0.08	2.68	1.69	0.44	1.87	46.2
chr10	16.27	6.20	8.74	1.79	0.07	3.14	1.32	0.49	1.93	39.95
chr11	19.08	7.10	10.88	2.02	0.07	2.69	1.02	0.63	1.87	45.36
chrUn	37.38	8.15	14	1.73	0.04	1.57	0.2	0.49	1.09	64.65
Total %	24.14	6.84	10.92	1.73	0.07	2.43	0.80	0.59	1.68	**49.2**

In conclusion, we identified and classified Transposable elements (TEs) using a combination of *ab initio*, similarity and structure-based approaches. TEs account for almost half (49.2%) of the sequenced genome length. The vast majority is class I LTR retrotransposons that were amplified recently (0.5-1.0 million years before present), as suggested by analysis of insertion

times of full-length copies. This analysis indicates that transposable elements are the main and the more dynamic component of the *C. canephora* genome.

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Progress Report on the Sequencing of *Coffea arabica* Genome

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SUMMARY

It is well known that *Coffea arabica* is the result of a cross pollination between two *Coffea* species, very likely *Coffea canephora* and *Coffea eugenioides*. Moreover, *arabica* can set flowers and fruits by self-fertilization and indeed beans can be obtained by a single and isolated plant. Such reproductive behaviour should find some justification in its genome. A genome sequencing project has been initiated to investigate the structure of the allotetraploid genome of Arabica.

High molecular weight genomic DNA was obtained from entire plantlets of *Coffea arabica* var. Bourbon and a BAC library was constructed. 175,872 BAC clones were pooled into 96 pools of 384 clones each and the pools underwent DNA sequencing on next generation sequencing Illumina platform. Whole genome shotgun sequencing was also performed on two Illumina libraries with 500 and 800 bp insert size and on one mate-pair library with inserts of 2 kbp. These libraries were supplemented by the sequencing of cDNA libraries (RNA-seq on Illumina platform) obtained from leaves, root and cherries to use for gene prediction. A preliminary assembly of the genome has been carried out.

The preliminary bioinformatic analysis of the *arabica* genome suggests a high degree of polymorphism between its sub-genomes, in line with the allotetraploid constitution of the *Coffea arabica* genome.

INTRODUCTION

The diploid *Coffea canephora* genome was recently unveiled, but the highly valuable, complex and allotetraploid *Coffea arabica* genome is still obscure. In order to tackle the problem of sequencing a tetraploid genome we followed two independent strategies. At first, we sequenced the genome with the nowadays standard Whole Genome Shotgun (WGS) approach; then we exploited also the hierarchical approach by sequencing and assembling BAC pools. The latter approach gives us the possibility to work on limited portions of the genome where homeologous regions are not present and at the same time drastically reducing the complexity of the genome assembly.

MATERIALS AND METHODS

A BAC library of 175,872 *Coffea arabica* var. Bourbon BAC clones was constructed at Lucigen Corporation (Lucigen Corporation, Middleton, WI). 36,864 BACs were randomly selected, inoculated into 96 384-well plates and grown at 37°C for 22 hours in LB2X medium supplemented with chloramphenicol antibiotic. Once at plateau, the 384 cellular cultures of each plate were joined together and mixed in a single tube. The resulting 96 pools were conserved at -80°C in a solution of LB2X medium and glycerol at 7%.

Bacteriophage Phi29 polymerase was used (IllustraTM TempliPhiTM Large Construct V2 kit, Resnova) to extract DNA from BACs. Briefly, from each pool an alkaline lysis procedure was performed in order to break bacterial cells, then BAC vectors, containing coffee DNA inserts were amplified in vitro with the enzyme Phi29 with isothermic reaction at 20°C for 16 hours. At the end of the reaction, BAC DNA was purified with ethanol and sodium-acetate precipitation and resuspended in distilled water. BAC pools were quantified on a fluorometer (Qubit, Invitrogen) and visualized on agarose gel at 0.8% in TBE 1x buffer.

Samples passing the quality controls were used to construct Illumina libraries with Nextera™ DNA Sample Preparation kit, following the manufacturer's protocol.

Libraries were then purified with magnetic beads AMPure XP (Agencourt) and quantified on Caliper GX (Perkin Elmer). After being balanced in an equimolar way they were loaded with the appropriate index on Illumina HiSeq2000 in order to be sequenced.

Whole-Genome Shotgun library construction was carried out using Illumina TruSeq DNA Sample prep kit Illumina, according to the manufacturer's protocol.

Mate Pair Library v2 Sample Preparation kit was used to construct the 2-3Kbp mate-pair library following the Illumina protocol with no agarose gel size selection. The libraries were validated at Bioanalyzer 2100 (Agilent), quantified by Qubit (Invitrogen) and then loaded on Illumina HiSeq2000.

Data sequenced with either HiSeq200 or MiSeq were preprocessed as follows: quality trimming with ERNE-FILTER with default parameters, adapters were removed with Cutadapt with default parameters but –O 10 –n 2 –m 50, reads were paired again with an internally developed python script, contaminants were filtered with ERNE-FILTER with default parameters but --min-size 50. Contaminants considered were the cloning vector, E. coli and the arabica chloroplast.

Mate pair reads were similarly cleaned and successively separated in real mate pairs and paired end reads with internally developed software.

k-mer analysis was carried out with KMerCounter (http://sourceforge.net/ projects/kmercounter/) on cleaned data.

Each BAC pool was assembled independently with ABySS v1.3.7 with default parameters but k=71, aligner=map, b=1000000, p=0.95, s=500, n=10.

WGS mate pairs were also used to scaffold each BAC pool assembly with SSPACE requesting 10 links to scaffold different sequences.

WGS paired ends and mate libraries were assembled with AllPaths-LG with default parameters but TARGETS=standard, MAXPAR=4, HAPLOIDIFY=False, CLOSE_UNIPATH_GAPS=False. The 900bp insert size library was not used, as the assembler could not process it.

CONCLUSION

Considering an estimated haploid genome size of 1.3Gb, we obtained nearly a 100x coverage with the WGS approach. All reads were produced with Illumina technologies, either HiSeq200 or MiSeq. Different libraries were produced in order to get robust results (see table 1 for details).

Table 1. WGS sequencing. The "Coverage" column refers to an estimated genome size of 1.3 Gb. Trimmed nucleotides refer to sequence data after cleaning as explained in Materials and Methods.

WGS Library	Insert size (bp)	Read length (bp)	Nucleotides (Gb)	Coverage	Trimmed Nucleotides (Gb)
Miseq overlapped	420	300+300	22.5	17.3x	20.8
Hiseq overlapped	180	100+100	34.4	26.5x	27.6
Hiseq	250	100+100	8.2	6.3x	6.0
Hiseq long	900	100+100	20.2	15.5x	18.0
Mate pairs	2000	100+100	42.7	32.8x	31.3
TOTAL			**128.0**	**98.4x**	**103.7**

96 pools were sequenced with each pool containing 384 BACs, for a total of 36,864 BAC clones. Given an estimated insert size of 100kb per BAC we had in total 3,686.4 Mb of physical coverage corresponding to almost 3x of the genome. The raw data corresponds to 488Gb, giving an average sequencing coverage of 132x. Sequencing coverage is not homogenous as it depends on BAC growth. Details are presented in Table 2.

Table 2. BAC pools sequencing. Coverage corresponds to the physical coverage of BAC clones. Trimmed nucleotides refers to data cleaned as explained in Material and Methods.

BAC	Pool	BAC x Pool	Insert size	Coverage	Nucleotides	Trimmed Nucleotides
36,864	96	384	≈100kb	2.84x	488Gb	313Gb

Data cleaning affected more BAC pools data than WGS data with a decrease of 19% vs 36%, respectively. This is due to the presence of the BAC vector sequence and of contaminating *Escherichia coli* DNA in the BAC pools.

Using k-mer distribution analysis, the genome size was estimated to be 1.2Gb, close to the initial estimate of 1.3Gb. Moreover, k-mer analysis shows that for low values of k, *e.g.* 16, two peaks are present, representing the portion of the genome either specific or shared between the two sub-genomes; while for higher values of k, *e.g.* 71, a single peak is present, representing the fact that the two sub-genomes are very different from each other. The two plots of figure 1 also show that there is no peak representing inter-homeolog SNPs (heterozygosity within a sub-genome).

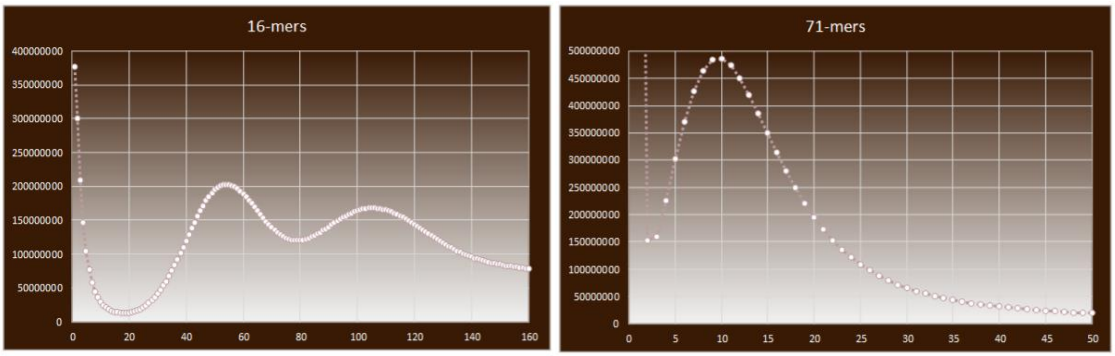

Figure 1. k-mer analysis. The volume of k-mers is plotted against the frequency at which they occur. k=16 and k=71 are used for the two plots.

The WGS assembly reconstructed 931Mb of the genome with L50 over 50kb, while, taking into consideration all BAC pools assemblies, we reconstructed 1.5Gb of sequence. BAC pools assemblies are more fragmented than the WGS assembly with 129,560 sequences and L50 of 23kb. See table 3 for details.

Table 3. WGS and BAC pools assembly. BAC pools are presented as sum of the 96 distinct assemblies.

	WGS	BAC pools
Total length (Mb)	931	1,512
# sequences	36,770	129,560
Minimum Length (bp)	1,000	1,000
Average length (bp)	25,314	11,671
Maximum length (bp)	379,195	182,942
L50 (bp)	51,803	22,732

Going forward, we will proceed with the merging of the two sources. The initial idea was to merge them with GAM-NGS as per the Norway spruce assembly but now with the *Coffea canephora* genome available we might use that information as anchoring point for the BAC pool sequences.

ACKNOWLEDGEMENTS

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Supercritical Fluid Extraction of Spent Coffee Grounds: Optimization of Oil and Diterpenes Extraction Using Experimental Design and Response Surface Methodology

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SUMMARY

Box–Behnken design of experiments and response surface methodology were applied to optimize the supercritical fluid extraction (SFE) of spent coffee grounds (SCG) oil with diterpenes enrichment (kahweol, cafestol and 16-O-methylcafestol) using pure or modified CO_2. The effects of pressure (140–190 bar), temperature (40–70ºC), and ethanol as cosolvent (0–5 wt.%) were evaluated.

The maximum of 11.97% ($g_{oil}/100_{gSCG}$) was obtained for oil extraction using 190 bar/55ºC/5wt.% ethanol. However, for the enrichment in diterpenic compounds the best operating conditions were 140 bar/40ºC/ no ethanol used as co-solvent. The measurement of extraction curves near optimized conditions confirmed the trends of the statistical analysis and revealed that SFE enhances diterpenes concentration by 75-129%, to a maximum of 102.90 $mg_{diterpenes}\ g^{-1}_{oil}$, at the expenses of reducing the extraction yield between 20% and 69% in comparison to *n*-hexane extraction.

INTRODUCTION

Around 6 million tons of spent coffee grounds (SCG) are produced every year. The large variability of SCG composition in terms of carbohydrates, proteins and phenolic compounds makes this residue a potential raw material for industrial processes. Recent studies have shown SCG potential as a source of green energy like biofuel, and oil or isolated molecules for the pharmaceutical, cosmetic and food industries. In fact, SCG contain several human health related compounds, diterpenes in particular, have been related to anticarcinogenic properties.

Roasted coffee yields between 7 and 17% (wt.) of oil, which is mainly composed of fatty acids esterified with glycerol (triacylglycerols, around 78 wt.%) and diterpenes (around 15 wt.%). The main diterpenes found in SCG are cafestol, kahweol and 16-*O*-methylcafestol, which esterify the fatty acids. The total amount of these compounds in SCG depends on coffee species and on the brewing method, e.g. when dealing with *espresso* coffee the kahweol levels range between 1.2–8 $mg\ L^{-1}_{oil}$, while cafestol levels are between 4–16 $mg\ L^{-1}_{oil}$. Among the methodologies used, the supercritical fluid extraction (SFE) (mostly using carbon dioxide as the extraction solvent) has been proved an efficient, environmental attractive, and selective method for oil, and more specifically for diterpenes extraction. Nevertheless, the latter is only achievable upon an optimized combination of the SFE operating conditions with the intrinsic physical characteristics and chemical composition of the vegetable matrices under study. For this purpose, both design of experiments (DoE) and response surface

methodology (RSM) were applied to study the influence of pressure, temperature and cosolvent (ethanol) in the extraction yield of SCG oil, as well as to identify the operating conditions that better enhance diterpenes concentration (C_{Dit}) in extracts.

MATERIALS AND METHODS

Soxhlet extraction

Espresso spent coffee grounds (SCG) were obtained from a commercial batch of Delta Cafés Platina (Portugal). The SCG samples were dried according to the ISO/DIS 11294-1993, following the method of oven drying at 105°C for 8 h. The oil reference sample was obtained from 45 g of SCG by Soxhlet extraction with *n*-hexane for 4 h at 80ºC.

Supercritical fluid extractions (SFE)

SFE were performed in an apparatus developed at Department of Chemistry of University of Aveiro. The scheme of process can be visualized on the publications of Passos et al., together with the corresponding full description of the set up. In each run 60 g of SCG were introduced in the extraction vessel and a constant CO_2 mass flow rate of 12 g.min^{-1} was applied. The results were expressed in weight percentage of dry biomass.

Diterpenes profile

The total diterpenes content in the extracted oil was determined by HPLC-UV using a reverse-phase column after saponification with KOH/ethanol, using diethyl ether for the solvent extraction of a 40 mg sample as described by Barbosa et al. using a procedure adopted from Rafael et al. The mobile phase was a mixture of methanol/water (85:15, v/v) using a flow rate of 0.7 mL min^{-1} with detection at 220 nm. The identity of individual diterpenes was ensured by comparing their retention times with authentic standards. The quantification was made using external calibration curves of concentration *vs.* peak areas, whose coefficients of correlation were higher than 0.99.

DoE and RSM

Response surface methodology was applied to evaluate the relevance of 3 experimental factors: pressure (*P*), temperature (*T*), and ethanol concentration in the supercritical solvent (ethanol wt.%) using three different levels: 140-165-190 bar, 40-50-70ºC and 0-2.5-5.0 (wt. %), respectively. A Box–Behnken design comprising 15 experiments was chosen to study total extraction yield (η_{Total}) and total diterpenes concentration (C_{Dit}) in extracts. Experimental results submitted to RSM analysis were described by a second order polynomial function:

$$Y = \beta_0 + \sum_{\mathrm{i}=1}^{\mathrm{k}} \beta_{\mathrm{i}} X_{\mathrm{i}} + \sum_{\mathrm{i}=1}^{\mathrm{k}} \beta_{\mathrm{ii}} X_{\mathrm{i}}^2 + \sum_{\mathrm{i}<j}^{k} \beta_{\mathrm{ij}} X_{\mathrm{i}} X_{\mathrm{j}} \qquad (1)$$

where Y is the studied response (whether η_{Total} or C_{Dit}), β_0 is a constant, β_{i} are model coefficients linked to linear effects, β_{ii} are coefficients related to quadratic effects, and β_{ij} $\beta_{\mathrm{ij}}$are coefficients for interaction effects. STATISTICA software (version 5.1, StatSoft Inc., Tulsa, USA) was used for statistical treatment of the results.

CONCLUSION

In the Soxhlet experiment the oil yield achieved 15 wt.%. From the experimental Box-Behnken design assays (Table 1) under supercritical conditions the maximum oil yield of 12 wt.% was obtained in Run 8 (190 bar, 55ºC, 5 wt.% EtOH). However, the maximum oil yield was obtained at different conditions from those of the maximum diterpenic concentration of 107 $mg\,g_{oil}^{-1}$ which was obtained in Run 5 (140 bar, 55ºC, 0 wt.% EtOH). Such results emphasize the pertinence of optimization through design of experiments methods.

Table 1. Results for the total extraction yield (η_{Total}, wt.%) of supercritical fluid extraction of spent coffee grounds samples used in the optimization work. Soxhlet results are also shown for comparison.

Run	P (bar)	T (ºC)	EtOH (wt.%)	η_{Total} (wt.%)	C_{Dit} (mg g_{oil}^{-1})		Run	P (bar)	T (ºC)	EtOH (wt.%)	η_{Total} (wt.%)	C_{Dit} (mg g_{oil}^{-1})
1	140	40	2.5	10.21	57.45		9	165	40	0	8.98	87.59
2	140	70	2.5	1.99	42.47		10	165	40	5	11.60	55.84
3	190	40	2.5	11.66	53.26		11	165	70	0	1.93	94.39
4	190	70	2.5	8.74	62.94		12	165	70	5	8.94	66.76
5	140	55	0	4.61	107.44		13	165	55	2.5	10.62	62.42
6	140	55	5	8.79	56.52		14	165	55	2.5	10.52	60.07
7	190	55	0	9.16	74.57		15	165	55	2.5	9.97	64.88
8	190	55	5	11.97	53.81		Soxhlet				15.03	52.69

Concerning the individual profiles of the diterpenes, their effective affinity to the SC-CO_2 seems to follow the same trend (Figure 1A), kahweol being the most abundant in accordance with its concentration in the matrix (respectively 48, 32, and 28 $mg\,g_{oil}^{-1}$ for kahweol, cafestol, and 16-*O*-methylcafestol in Run 5).

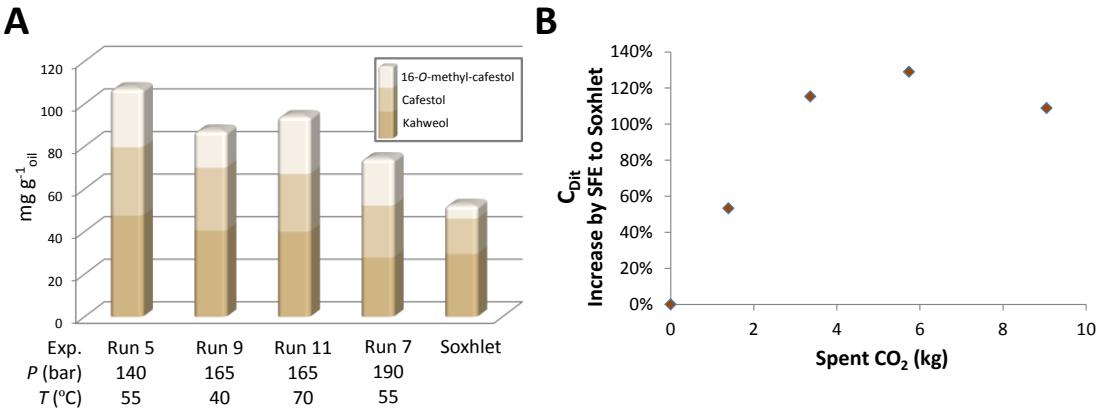

Figure 1. A) Total diterpenes concentration (C_{Dit}) in the SFE of SCG with individual diterpenes profile. Data are graphed for 0 wt.% cosolvent. B) Percentage of total diterpenes concentration (C_{Dit}) increment to Soxhlet extract (53 $mg\,g_{oil}^{-1}$) in the supercritical cumulative extracts obtained along time (expressed in terms of mass of spent CO_2). SFE operating conditions: 140 bar/55ºC/0 wt.% EtOH.

From the 15 runs, and despite Soxhlet extraction, when the oil yield was the highest (15.03 wt.%), all SFE runs (exception for run 2) led to extracts richer in diterpenes. Figure 1A exemplifies the increased percentage in total diterpenes concentration in the oil obtained in

Run 5 (Table 1) when compared to the 53 $mg\,g_{oil}^{-1}$ of total diterpenes concentration in oil obtained by Soxhlet extraction (30, 17 and 6 $mg\,g_{oil}^{-1}$ of kahweol, cafestol, and 16-O-methylcafestol, respectively). Such results show a more selective character of SFE to obtain SCG oils richer in diterpenic compounds. In addition, the extraction curve measured at 140 bar/55ºC/0 wt.% EtOH (Figure 1B) exhibits a parabolic shape that reaches a maximum value of 121 $mg\,g_{oil}^{-1}$ at 5.7 kg_{CO2}, At this point, the C_{Dit} value obtained by SFE is 2.3 times greater than the reference from Soxhlet extraction.

In the whole, extraction curves using optimized operating conditions (particularly in the case of 190 bar, 55ºC and 0 wt.% EtOH) demonstrated the great selectivity advantages of the SC-CO_2 to produce SCG oil enriched in diterpenes. Such enhancement is obtained at expenses of a lower total extraction yield (in relation to Soxhlet results). However, this trade-off seems favourable to SFE by the C_{Dit} increment, within 53-129%, while η_{Total} losses stay within 20-69%.

ACKNOWLEDGEMENTS

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Research and Development in the Field of Roast Exhaust Gas Treatment during Coffee Production with the Main Focus on Energy Saving and NOX Reduction

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SUMMARY

A new procedure is at present investigated in the PROBAT Technical Center. It concerns a catalyzer with which considerably lower NOx values are achieved and which is more energy efficient than the current systems. The miscellaneous exhaust air treatment systems do also considerably differ with regard to NOx and N2O. Looking at the NOx transformation in the exhaust air treatment systems of the coffee industry special emphasis was also placed on the transformation process from NOx to N2O, i.e. on the determination and development of N2O during the exhaust air cleaning.

INTRODUCTION

With growing environmental consciousness the demands in the field of the exhaust gas treatment are rising as well. Various approaches exist to clean roast exhaust gases released during the coffee production. This involves the complete catalyzer technology from standard to low-temperature catalyzers, thermal cleaning as well as the Proforte introduced onto the coffee market by PROBAT, and zeolite filters for smaller roasting units etc.

CONCLUSION

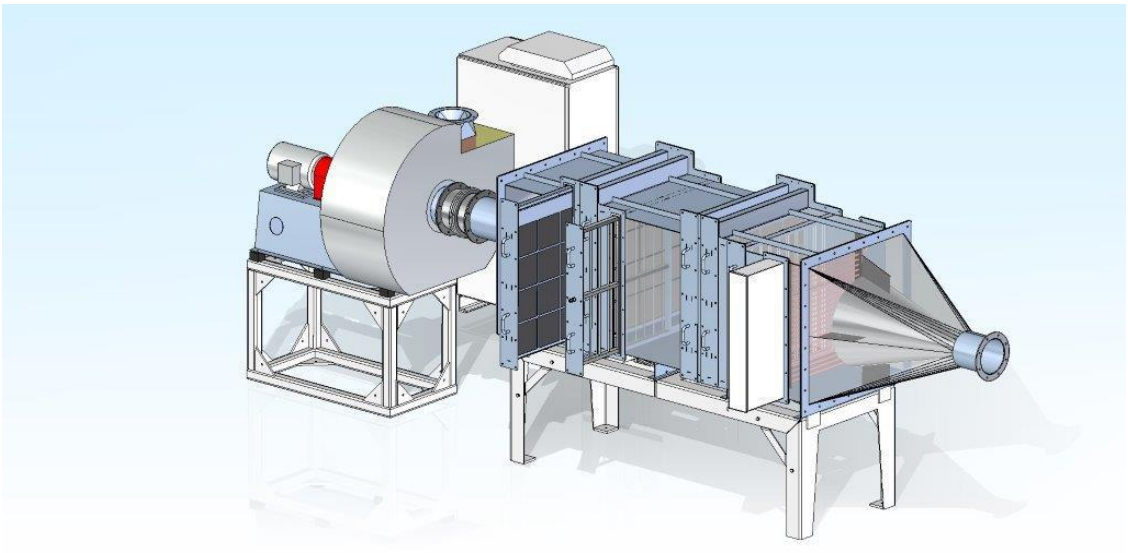
The new catalyzer is operating according to a method where emissions are converted through catalytic oxidation by means of ozone (O_3). The temperature of reaction on the catalyzer is already reached with very low temperatures. This means a considerable energy saving compared with the current systems.

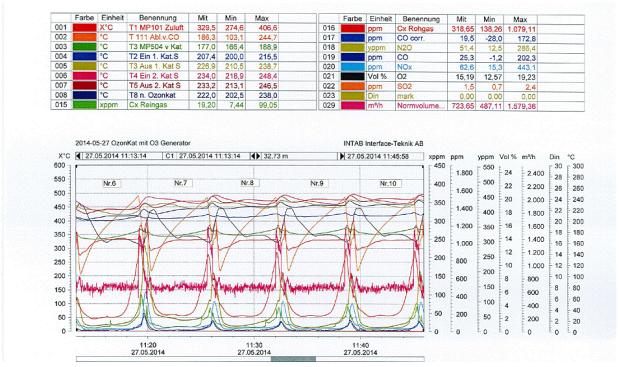
A series of bench scale tests shows that the roasting process emissions can be degraded more effectively and lower emission values occur. For this purpose a modular catalyzer has been developed and applied on an industrial roaster under real roasting conditions.

Table 1.

Example for medium roasted Arabica Coffee		O_3 catalyzer	standard catalyzer
C_x	[mg/m²n]	32	39
CO	[mg/m²n]	24	54
NO_x	[mg/m²n]	127	421
working temperature	[°C]	170	450
heating energy	[%]	0	100
total energy	[%]	40	100

With this technology the demanded values of the German regulations VDI 3892 and TA-Luft are complied with resp. significantly dropped below.





FOLLOW UP

Due to the fact that very low NOx values were achieved a new question arose:
What happens to the other nitrogenous compounds during NOx reduction?

Detailed measurements at the PROBAT Technical Center showed a significant increase of N_2O values during the NOx reduction process.

The whole array of questions concerning NOx reduction needs further research and tests to understand this conversion process in its complexity.

Solubilization of Coffee Bean Cell-Wall Using Enzyme of Microorganisms Degrading Coffee Arabinogalactan Protein and Food-Processing Cellulases

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SUMMARY

We succeeded to isolate coffee bean AGP degrading bacteria. The enzyme of the culture broth and cellulase could completely solubilize sliced coffee beans. We carried out to extract the AGP, and prepared the crude-AGP (>1,500 kDa). Many microbial enrichment cultures and screenings using the AGP were carried out to find the AGP degrading enzyme, and three bacteria producing the enzyme were isolated in the result. The isolated bacteria were identified by analysis of 16s rDNA, and named as *Chitinopaga* sp. KSM45, *Ensifer* sp. KSM81, and *Burkholderia stabilis* KSM97, respectively. The enzyme of the culture broth could degrade the AGP to the lower ones. Combination of the AGP degrading enzyme and some food processing cellulases were very effective to digest the cell wall, and showed completely solubilization of the slices of the coffee beans.

INTRODUCTION

Are coffee beans soluble? Coffee beans cell wall is very hard and insoluble, this nature prevent new approach of coffee production and its uses. For example, about a half of roasted coffee beans are remained in the brewing, and the lees are co-produced and discarded. The coffee brew-lees are hardly digested or fermented. Although the enzymatic digestion-trials have been reported, there are a little reports of the high or efficient digestion. Generally, mannanase would be described as an efficient enzyme to digest the cell wall of coffee beans, but the ideal reports are hardly to find. Mannanase are really effective for roasted-fragmented soluble galacto-mannans such as mannan of coffee breu, but is not effective for the one of the coffee bean cell wall itself. We have been studying to clear the factors to digest and enzymatically solubilize the cell wall of coffee beans. If AGP of coffee bean were easily to degrade, then the coffee beans will be turned to be easy digestible. Then new and more better or effective coffee production will be possible, and new food materials also could be made.

We previously showed a selected cellulase collapsing the cell wall, and predicted the arabinogalactan protein of the coffee bean (C-AGP) would be an important factor to inhibit the cellulase digestion with covering and gluing galactomannans and celluloses. Coffee bean AGP is reported as covalently linked to protein, and the efficient extraction is difficult even by boiling with strong alkali. Some other plants AGP were enzymatically digested, and the part structures were reported. AGP of radish was enzymatically digested and reported in 1990 , and recently one of AGP of wheat flour was reported. However, complex branched galactan chains of AG or AGP itself were also known to be resistant to enzymatic digestion, the yield is generally below only 10-20%. Further more, high molecular type of AGP such as one of

coffee beans or gum Arabic was practically very difficult to digest. It would be due to the highly branched structures.

We noted that C-AGP from coffee bean was found in cellulase digestion, and it was high molecular one (> 1,500 kDa). We thought the C-AGP was obtained, then the C-AGP degradator would be isolated to use for the efficient enzymatic digestion.

In this report, we would like to show the preparation of the C-AGP was carried out from the selected cellulase digestion, subsequently, screenings for the microorganisms to produce C-AGP degrading enzymes were carried out from soils or fermented foods, and then some microorganisms were successfully isolated from the enrichment cultures at last. Their enzymes produced in the broth reacted on the C-AGP, and gave low molecular ones. Furthermore, some combinations of the broth-enzymes and food processing cellulases were very efficient to soluble sliced coffee beans.

MATERIALS AND METHODS

Coffee beans

Green coffee beans (*Coffee arabica*), the unroasted coffee beans, and their cracked and milled powders were gifts from UCC Ueshima Coffee Co., Ltd., Kobe, Japan.

Enzymes

Cellulase (for food-processing cellulase from *Trichoderma reesei*, 1000 units/mL) was a gift from Godo Shusei Co., Ltd. (Tokyo, Japan).

Preparation of crude coffee bean AGP

Cellulase digestion of coffee beans were principally same as previously reported method. Green coffee beans were milled to the powder (507 g) and defatted with 1 L of hexane for over night. The defatted beans were filtered, washed with hexane and dried off with air. The defatted-milled green coffee beans (468 g) were autoclaved with 2500 mL of 0.1 M NaOH at 121 ºC for 20 min. The treated beans (364 g) were washed with water and 0.1 M acetate buffer (pH 5.0) and placed in 5 L beaker with 0.05 M acetate buffer (pH 5.0), a 5%v/v enzyme solution (4800 mL, 0.05 M acetate buffer, pH 5.0) was mixed with stirring at 40 ºC for overnight, and the reaction was stopped by autoclaving (121 ºC for 20 min). Formed precipitates from the cellulase were centrifuged to remove (8,000 rpm, 10 min). The supernatant was dialyzed in cellulose tubes in deionized water of 20 L, 3 times for overnight, and then the supernatant was filtered and condensed with 50 kDa membrane system kit (sartorious stedim biotech, VIVAFLOW 200, 50,000 MWCO PES). The C-AGP solution (16 g estimated as sugar) was obtained and stored at -20 ºC. For microbial screening, the crude C-AGP solution was used directly. For enzyme degrading analysis, the C-AGP sample was freeze-dried, and the 1% solution was applied to purify on a Sephacryl S-200 column, the first protein peak (>1,500 kDa) was collected and used for the enzyme assay.

Screening of microorganisms

The isolation sources were used as various cheeses, Japanese miso, fermentative vegetables, and soil samples. Enrichment cultures were carried out with 276 sample sources and medium of C-AGP (0.1%) and ammonium sulfate or polypeptone as the first stage. The each enrichment culture was inoculated on the plate of C-AGP (~0.1%) and polypeptone (0.001%)

containing pH indicator of brom thimol blue (80 mg/L), and then grown yellowish or yellowish green colonies were isolated as the secondary stage. The each strain was cultivated in the same medium, and the supernatant and the C-AGP (0.1%, 0.1M phosphate buffer, pH 7.0) were mixed and reacted for 3-24 hr. The C-AGP degrading ratio was estimated using HPLC system.

C-AGP degrading analysis

One mL of the partially purified C-AGP (0.1M phosphate buffer, pH 7.0) and 0.125 mL of diluted broth of AGP degrading bacterium were incubated at 37 ºC for 3-15 hr, and the mixture was analyzed using HPLC size exclusive column of TOSO G3000SWXL system. The decrease of the C-AGP peak was estimated as the enzyme activity.

HPLC system

HPLC system (DP8020, Toso, Tokyo, Japan) consisting of a molecular sizing column (TSK-Gel G3000SWXL, 7.8 × 300 mm, Toso), a difference refraction indicator RI8020 (Toso), and an optical photometer UV8020 (Toso) for detection. HPLC analysis conditions: elute, 0.1M phosphate buffer pH 7. 0.1M Na2SO4; flow rate, 1 mL/min; Column, TSK-gel G-3000 SWXL (I.D. 7.8 mm x 300 mm); sample size; 60 μ L.

Digestion of the cell walls of sliced green coffee beans

The green coffee beans were autoclaved with water at 121 ºC for 10 min. The cooled beans were sliced, and the sliced sections of the coffee beans were incubated in a 96 micro-holes plate with 1% the selected cellulase from *Trichoderma reesei* and broth of the C-AGP degrading bacterium *Chitinophaga* sp. KSM45 at 37 ºC for 24-72 hr.

Light microscopic observation

The microscopic observations and photos were done using an Olympus BH-21 (Olympus Optical Co., Ltd., Tokyo, Japan) light microscope and a digital DP-II microscope photographic device.

CONCLUSION

Crude C-AGP preparation

The C-AGP was obtained from the selected cellulase digestion. The cellulase was *Tricoderma reesei* of GODO TCF reported previously in efficient digestion of coffee cell-wall. Using the cellulase, most of arabinose of C-AGP, >95%, were removed, and the high molecule C-AGP (>1,500 kDa) was partly obtained in the digestion. Most of proteins of the cells were removed by autoclaving treatment with 0.1M NaOH as the first step, and the C-AGP was obtained from the cellulase digestion as high molecular protein fraction. The C-AGP was mainly composed of galactans (95%) and protein (5%), and this one was used as major carbon source to assimilate in the enrichment culture described below. Most of the C-AGP arabinoses (>95%) were removing, and it was convenient to exclude some arabinose assimilating microorganisms.

Screening and isolation of C-AGP degrading microorganisms

Various microorganism sources were tried on the enrichment culture as the first screening, the C-AGP was not easily assimilated in the synthetic medium. Additive polypeptone helped to assimilate the C-AGP, clear yellowish colonies, which were formed acids from the C-AGP, were also hardly to find, however, some yellowish or yellowish green colonies were found in the results. Most of the grown colonies were also isolated, and the microorganisms were cultured in the medium of C-AGP-polypeptone. Isolated microorganisms (155 strains) were checked the degradation activity for the C-AGP using HPLC analysis, and three strains were finally isolated. Three bacteria producing C-AGP degrade enzyme were analyzed their 16s rDNA in TechnoSuruga Laboratory Co., Ltd. (Shizuoka pref, Shimizu city, Japan), and the strains were identified and named as *Chitinopaga* sp. KSM45, *Ensifer* sp. KSM81, and *Burkholderia stabilis* KSM97, respectively (Figure 1).

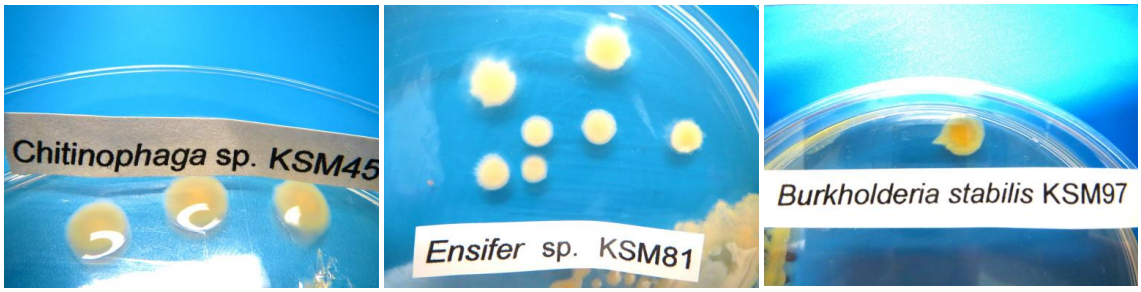

Figure 1. The isolated AGP degrading bacteria of *Chitinophaga* sp. KSM45. Three strains of AGP degrade-enzyme producing bacteria were isolated and identified by 16S rDNA analysis as *Chitinophaga* sp. KSM45, *Ensifer* sp. KSM81, and *Burkholderia stabilis* KSM97, respectively.

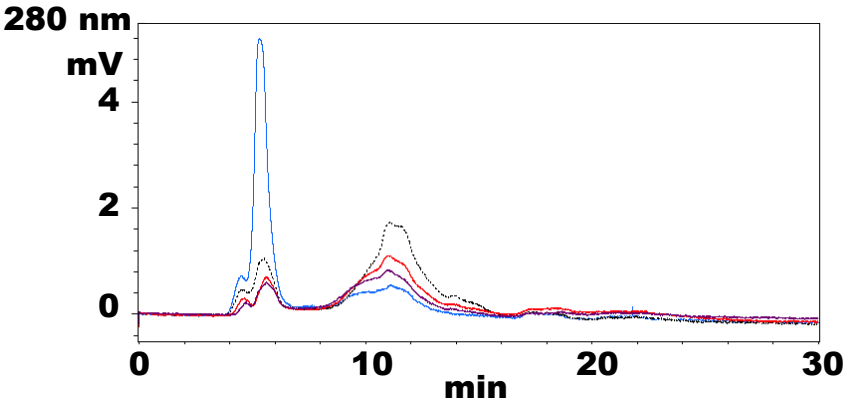

Figure 2: Degradation reaction of C-AGP. High molecular C-AGP (4-6 min) was gradually degraded, and the digested one was monitored as lower one (9-12 min). Reaction conditions: One, two, and four-fold diluted culture broth of *Chitinophaga* sp. KSM45 (0.125 mL) and AGP (0.1%, 1.0 mL, 0.1 M phosphate buffer pH 7.0) were mixed and reacted for 15 hr. The C-AGP was gradually degraded, and gave the lower one.

Degrading reaction of C-AGP

Three bacteria produced the C-AGP degrading enzyme in the culture broth. The C-AGP was gradually digested and decreased, and the lowered molecular ones were detected (Figure 2). In the cultivating with C-AGP, the lower ones were also gradually degraded and decreased,

and then the C-AGP was well assimilated in the final. C-AGP itself is known as hard to digest, C-AGP degrading enzymes of these bacteria are to note.

Solubilization of sliced coffee beans

The C-AGP degrading enzyme gave strongly assist cellulase in digesting and solubilizing coffee bean cell wall. In this test, complete solubilization was found in the enzyme mixture shown in Figure 3. In the microscopic figure, the sliced coffee bean cell wall was digested and fragmented, and hardly found in the reaction mixture. We certainly reported efficient digestion of coffee bean cell wall, and collapsing and partly solubilization of the cell wall was occurred, but we could not achieve complete solubilization of the cell wall. This solubilization was observed on some combinations of food processing cellulases. The C-AGP was digested and removed, and then the coffee bean cell wall could be easily digested and solubilized by the cellulases. The solubilization of the coffee bean would be first report.

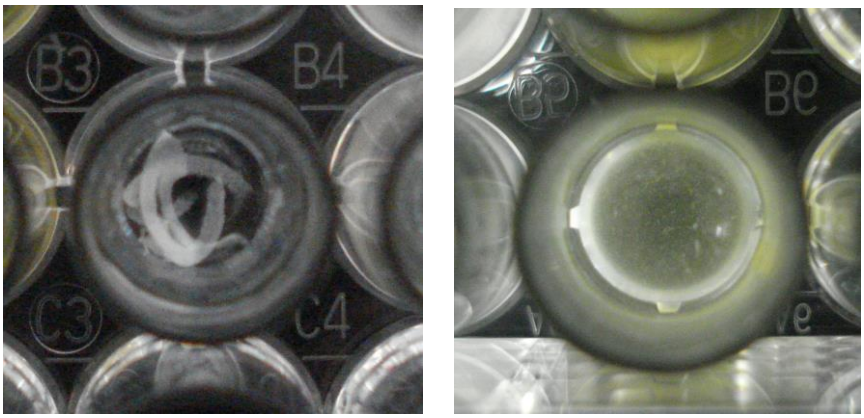

Figure 3. Solubilization of slices of coffee beans by AGP degrading enzyme and cellulase. Some slices of coffee beans were incubated in the mixture of culture broth and 1% cellulase of *Tricoderma reesei* (GODO TCF). Left is the blank test and the right is the positive test. The slices were digested and solubilized, and some muddiness was due to pieces of the residual digested cell wall (right).

We predicted that the C-AGP was a key to digest for coffee bean cell wall in the previously report. Coffee bean cell wall is principally made of galacto-mannan, cellulose and AGP. C-AGP itself is hardly to digest, although the enzyme digestion is possible in the reported models. Indeed, AGP was hard to assimilate, and the microorganisms screening was hard to isolate. A novel and specific enzyme was obtained in the result.

Our results will make possible to digest or to liquefy coffee beans, and more better and new coffee production or new food functional materials from coffee beans also will be possible. Furthermore, it will be useful and helpful for the enzymatic or microbial treatment of the coffee spent ground.

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Yields of the Total Metabolite Extracts from Grains of *Coffea arabica L.* Collected in Two Consecutive Years

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SUMMARY

Most chemical compounds present in *Coffea arabica* grains are polar, or have multiple groups with this feature in their structures (proteins, carbohydrates, chlorogenic acids and caffeine). The aim of this work was to determine the yields of extracts obtained from mature beans of *Coffea arabica* L., harvested in two consecutive years (2010 and 2011), applying four solvents (ethanol, acetone, dichloromethane and hexane) and their binary, ternary and quaternary mixtures. Coffee plants were grown in two plant densities (6,000 and 10,000 plants ha^{-1}) and planting patterns (square – Q or rectangular - R). In the two harvests, the highest yield of crude extract was obtained with the ethanol-acetone mixture followed by pure ethanol, both polar solvents. In 2011 the binary interaction of ethanol: acetone was more relevant for Q_{10}, Q_6 and R_{10} than in 2010. The Q_6 treatment showed the higher coefficients for the ethanol: acetone interaction in both years. Since this plant, arrangement has more light availability and the production of higher amounts of polar metabolites, such as flavonoids and tannins, highest yields with polar solvent mixtures could be expected.

INTRODUCTION

The chemical composition of raw arabica coffee depends on the coffee species and variety, soil type, crop management, climatic conditions, ecophysiological constraints, harvest dates and roasting procedures. Thus, coffee beans originating from different production conditions result in varying quality characteristics and sensorial attributes.

The use of different solvents in the metabolite extraction process of coffee beans allows obtaining extracts of diverse compositions, according to the polarity of the extracting solvent. Most of the compounds present in the grains of *Coffea arabica* are polar, or have multiple groups with this feature in their structure (proteins, carbohydrates, chlorogenic acids and caffeine). One might expect that the use of polar solvents in the extraction process would produce the highest yields. Statistical mixture designs are useful tools for this purpose. Their associations with fingerprint techniques, such as spectroscopic and chromatographic ones, permit the identification and approximate quantification of the extracted metabolites. The aim of this work was to calculate the yield of extracts obtained from mature beans of *Coffea arabica* L., provided by harvests in two consecutive years, applying four solvents (ethanol, acetone, dichloromethane and hexane) and their binary, ternary and quaternary mixtures.

MATERIALS AND METHODS

Plants of *Coffea arabica*, cv. IAPAR 59, were cultivated in two high densities (10,000 plants ha^{-1} and 6,000 plants ha^{-1}), in two planting patterns (rectangular – R and square - Q) in

experimental fields of the Agronomic Institute of Paraná (IAPAR), Londrina (23°18'S and 51°17'W), Brazil. The four treatments were identified as Q_{10}, R_{10}, Q_6 and R_6. The low cut was performed in 2008. Biennial production of coffee was considered and visually mature berries were collected June 7, 2010 (1st year) and May 11, 2011 (2nd year). The samples of green beans were frozen with liquid nitrogen, crushed and sieved.

The choice of the best experimental condition for obtaining the extracts was performed using a Simplex Centroid Design for four components, using ethanol (Exodo), acetone (Fmaia), dichloromethane (Anidrol) and hexane (Anidrol). Four pure solvents, six binary, four ternary and one quaternary mixtures were investigated. Each extract was prepared with 2.50 g of milled bean and 60 mL of extracting solvent. The mixture was placed in ultrasonic bath (Ultracleaner 1400 Unique). The temperature was maintained at 15 ± 2 ° C with ice (Figure 1a). After the first three days of extraction, the samples in contact with the extracting solvent were stored in the refrigerator for 48 hours and then, an extraction cycle was completed (Figure 1b). The extraction was considered exhaustive when a complete overlap occurred between the UV-Vis spectra of two consecutive extractions. The spectrophotometer used was a Thermo Scientific Evolution 60.

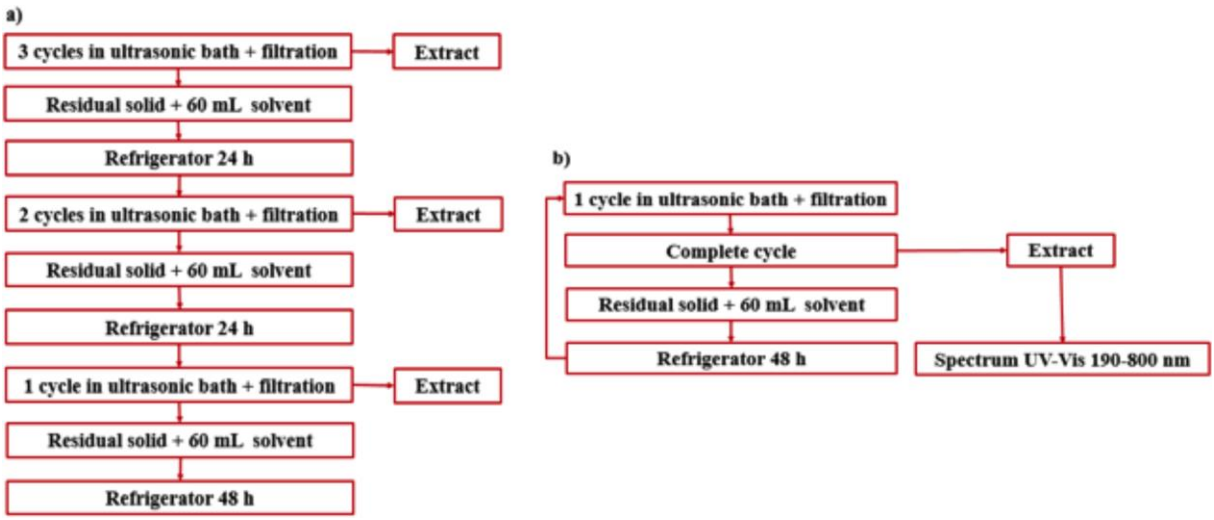

Figure 1. (a) Scheme of extraction during the first three days, and (b) extraction after the first three days.

The extracts were evaporated in a rotary evaporator and lyophilized in a Virtis freeze-SP Scientific until constant weight, and the extraction yield was calculated based on dry basis moisture content. The statistical models and the mixture response surfaces were obtained with the Statistica 7.0 software.

CONCLUSION

The extraction yields of metabolites, expressed in percentages, are shown in Figures 2 (a) and (b). For the two harvests, the highest yield of crude extract was obtained with the ethanol-acetone mixture followed by pure ethanol, both polar solvents.

An increase in the yield of four to five times was obtained from extractions with hexane comparing the 1st to the 2nd harvest. The composition of these extracts has being analyzed to understand the difference in their nonpolar metabolite constituents. To evaluate the effect of extracting solvent on the yields obtained, mixture models were tested. The linear model showed lack of fit for all treatments. Then, the quadratic and the special cubic models were

tested. The quadratic model reproduced the experimental data for the 1st harvest (Table 1). Both quadratic and special cubic models were used to reproduce the experimental data of yields for the 2nd harvest. The R_{10} yield data adjusted properly with the quadratic model and the Q_{10}, Q_6 and R_6 data were adjusted precisely with the special cubic model (Table 2). The model response prediction was represented by ŷ. The independent variables were represented by e = ethanol, a = acetone, d = dichloromethane and h = hexane. The standard errors of the parameters are shown in parentheses.

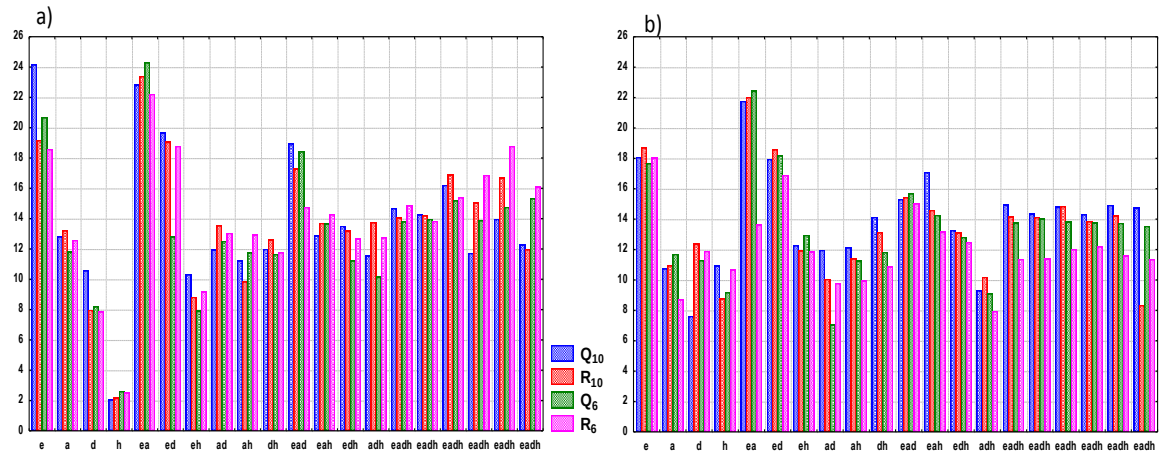

Figure 2. Yields from the extracts of beans of *Coffea arabica* IAPAR 59: (a) 1st (2010) and (b) 2nd harvest (2011).

Table 1. Quadratic model equations for the four treatments – 1st harvest.

Treatment	Quadratic model equations
Q_{10}	ŷ = + **24.56e** + 13.23a + 10,88d – **17.63eh** + **17.96dh** (±1.46) (±1.46) (±1.46) (±6.14) (±6.14)
R_{10}	ŷ = +**19.47e** + 14.88a + 8.93d + **22.15ea** + 15.19ed + **27.84dh** (±1.60) (±1.33) (±1.47) (±6.79) (±6.77) (±6.78)
Q_6	ŷ = +**20.58e** + 11.99a + 8.22d + 2.84h + **32.07ea** -15.90eh + 12.11ah + **22.15dh** (±1.11) (±1.11) (±1.11) (±1.11) (±4.96) (±4.95) (±4.95) (±4.96)
R_6	ŷ = +18.16e + 13.40a + 8.70d + **20.47ea** + 16.03ed + **20.36ah** + **25.00dh** (±1.59) (±1.59) (±1.59) (±7.31) (±7.31) (±7.31) (±7.31)

Table 2. Quadratic and special cubic model equations for the four treatments – 2nd harvest.

Treatment	Quadratic and cubic special model equation
Q_{10}	ŷ =**+18.09e** + 10.77a + 7.63d + 10.94h **+ 28.98ea** + 19.98ed – 9.32eh + 10.71ad + (±0.39) (±0.39) (±0.39) (±0.39) (±1.89) (±1.89) (±1.89) (±1.89) + 4.75ah + 19.01dh – 59.33ead **+ 38.49eah** – 83.74adh (±1.89) (±1.89) (±11.80) (±11.80) (±11.80)
R_{10}	ŷ = **+19.62e** + 11.44a **+ 13.79d** + 9.70h **+ 23.11ea** (±1.61) (±1.61) (±1.46) (±1.46) (±7.40)
Q_6	ŷ = **+17.67e + 11.69a + 11.31d** + 9.25h **+ 31.42ea** + 14.78ed – 5.05eh – 17.75ad + (±0.26) (±0.26) (±0.26) (±0.26) (±1.21) (±1.11) (±1.21) (±1.11) + 5.20dh – 40.35eah (±1.11) (±7.88)
R_6	ŷ = **+18. 02e** + 8.70a + 11.89d + 10.67h + 8.01ad – 9.69eh **+ 29.85ead** + (±0.39) (±0.39) (±0.39) (±0.39) (±1.92) (±1.92) (±12.00) **+ 34.13eah** – 67.12adh (±12.00) (±12.00)

Pure ethanol, acetone and dichloromethane showed significant effects for extraction of coffee bean components obtained in Q_{10}, R_{10} and Q_6 of the 1st harvest. Hexane and dichloromethane interaction was significant in all models, at the 95% confidence level. The four solvents were significant for Q_6 of the 1st harvest. The highest coefficient was obtained for a binary interaction of ethanol: acetone in R_{10}, Q_6 and R_6 at the 95% confidence level.

The four pure solvents (ethanol, acetone, dichloromethane and hexane) were significant for the treatments of the 2nd harvest. The binary interaction of ethanol: acetone became more relevant for Q_{10}, Q_6 and R_{10}. The highest yield for R_6 was extracted with the ternary mixture ethanol: acetone: hexane (Table 2).

The Q_6 treatment showed higher coefficients for the ethanol: acetone interaction in the two harvests (Tables 1 and 2). The plants in the square pattern with lower density had more light availability and the production of higher amounts of polar metabolites (such as flavonoids and tannins) under these conditions has been previously observed. Hexane (nonpolar) had the lowest yields for all plantings.

The contour plots for yields predicted by the equations in Tables 1 and 2 are given in Figures 3 (1st harvest) and 4 (2nd harvest). Higher yields (in red) were observed for polar solvents or their mixtures.

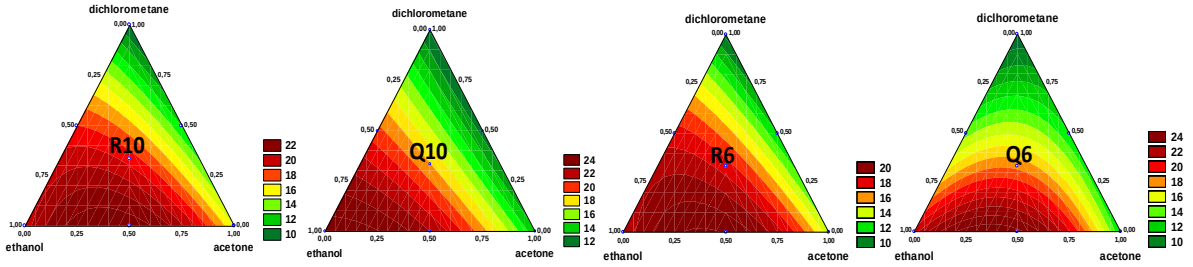

Figure 3. Mixture response surfaces obtained for the ethanol, dichloromethane, acetone proportions, for *Coffea arabica* cv. IAPAR 59 1st harvest (2010).

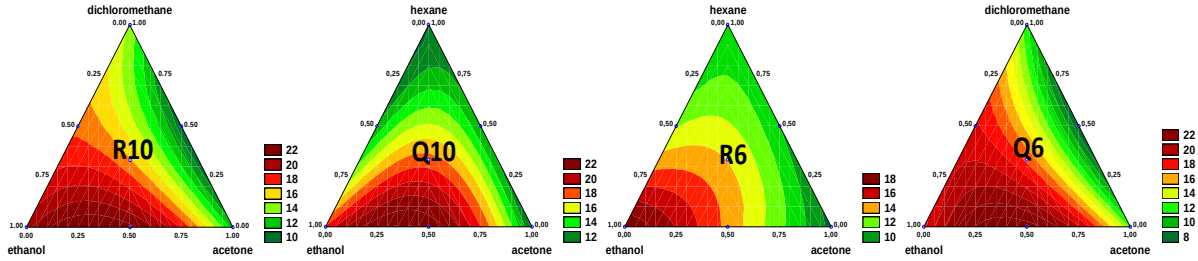

Figure 4. Mixture response surfaces obtained for the ethanol, dichloromethane, acetone and hexane proportions, for *Coffea arabica* cv. IAPAR 59 2nd harvest (2011).

The extraction process was laborious and slow, but it did show the best solvents for the highest yield under these production conditions. The polar solvents and their mixtures had the highest yields in this process as expected. Spectroscopic and chromatographic analyses are necessary to define the *biennial production* and the planting arrangement impacts on synthesis of specific metabolites in coffee beans.

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Content and Solvent Extraction in Coffee Leaves from Brazil

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SUMMARY

The aim of this work is the quantitation of phenolics compounds in the methanolic extracts in the *Coffea arabica* leaves in Brazil – Ceará (Guaramiranga), Minas Gerais (Bom Sucesso and Manhumirim) – and a available commercial souce. Our preliminary studies show that the methanol extract of *Coffee* leaves commercial tea analysed by HPLC-MS contain mangiferin, isomangiferin, epicatechin, procyanidin B1 chlorogenic acid (4 isomers), isorhamnetin glucoside, rutin glucoside, quercetin glucoside, and a rutin isomer. Infusions studies with powder leaves of a commercial Brazilians source show that release of phenolics is temperature dependent and that release at 100 °C is virtually instantaneous and equivalent to that of prolonged methanol extraction.

INTRODUCTION

Tea and coffee the most popular beverages in the world, have been consumed for thousands of years. Tea is produced by brewing the dried leaves and buds of *Camellia sinensis*. Coffee berries are dried once ripe, roasted at various temperatures and then ground and brewed. Many studies have shown a relation between the consumption of tea and coffee and their potential disease prevention properties, which may be due to their polyphenol contents.Mangiferin, as one of the many examples of polyphenolic compounds, appears to have considerable promise as a natural disease chemopreventant. Mangiferin has also been reported to have strong scavenging effects against reactive oxygen species which attack molecules including DNA, proteins, and lipids, contributing to the development of several chronic diseases including cancer. Recently has been reported the phenolic composition in Coffea leaves showing the presence of mangiferin, and because of that the potential cancer chemopreventive effects of coffee-leaf tea and beverages have to be considered. Chemoprevention is a very promising means to control cancer and there is a growing interest in the use of natural products, as consumer awareness of their possible beneficial health effects increases.

The aim of this work was the quantitation of phenolic compounds in the methanolic extracts of *Coffea arabica* leaves from Brazil – Ceará (Guaramiranga), Minas Gerais (Bom Sucesso and Manhumirim) – and a available commercial source.

MATERIALS AND METHODS

10 g of dried material from coffee leaves were extracted for 3 hours with hexane in a Soxhlet apparatus to remove lipid. After drying, the material was further extracted for 3 hours with methanol, three times, the solutions were pooled and evaporated to dryness under reduced pressure. One milliliter of each extract was dissolved in methanol and analyzed by analytical HPLC and HPLC-ESI-MS. The major polyphenols were quantitated using analytical HPLC.

In order to assess efficiency of phenolic compound extraction by infusion, varying amounts (100-1000 mg) of coffee leaf powder from the commercial source were transferred to 15 mL Falcon tubes in duplicate and 10.0 mL of double distilled boiling water were added and maintained in a water bath at 100°C for 20 minutes. The solutions were filtered and 1.0 mL was set aside for HPLC-ESI-MS. To assess the rate of extraction, duplicate 100 mg samples of coffee leaf powder were transferred to 15 mL Falcon tubes and 10.0 mL of double distilled boiling water were added and maintained in a water bath at 100°C for 1 to 30 minutes. The solutions were filtered and 1.0 mL was set aside for HPLC-ESI-MS. Finally, to assess the effect of temperature on efficiency of extraction, duplicate 100 mg samples of coffee leaf powder were transferred to 15 mL Falcon tubes and 5.0 mL of double distilled water at temperatures from 35-100°C were added and maintained in a heating block for 5 minutes. The solutions were filtered and 1.0 mL was set aside for HPLC-ESI-MS.

CONCLUSION

Our preliminary studies show that the methanol extract of commercial *Coffee* leaf coffee tea analyzed by HPLC-ESI-MS contain the compounds shown in Figure 1.

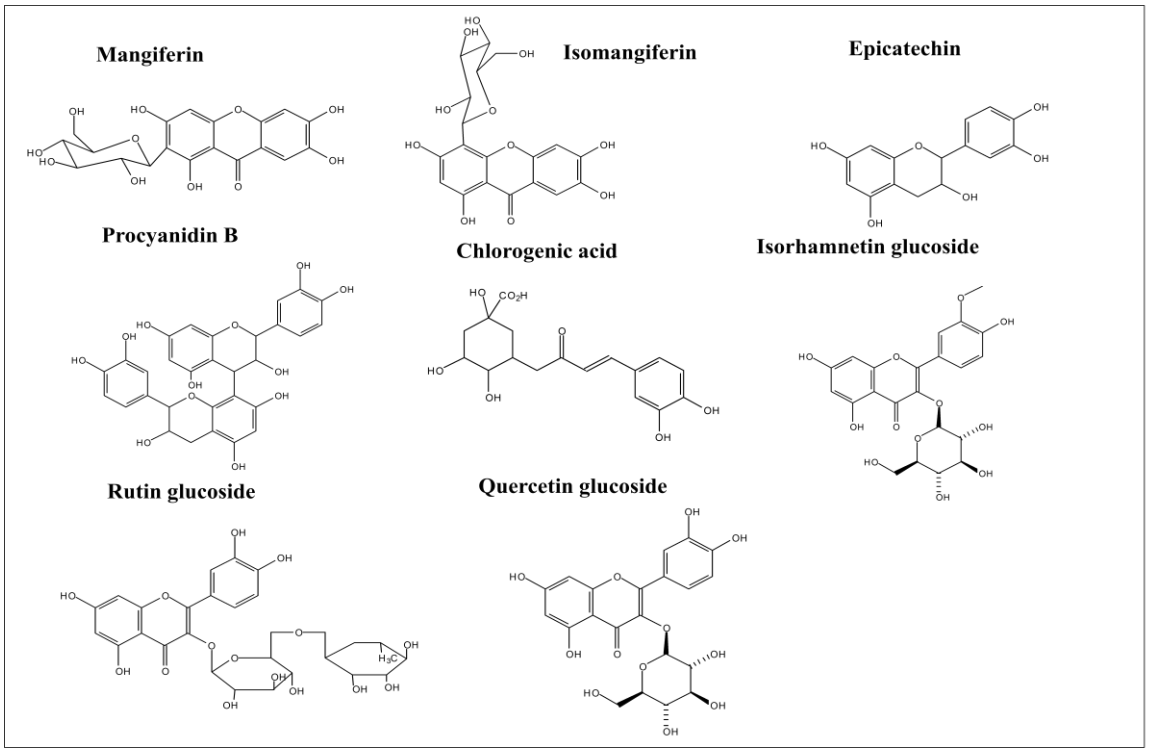

Figure 1. Compounds identified in methanol extract of coffee leaves commercial tea.

Overall, the concentration (Table 1) of mangiferin detected in the methanol extracts was in the range 0.48-4.17 g/kg whereas isomangiferin was in the range 0.19-0.80 g/kg.

Table 1. Content of Mangiferin and Isomangiferin in Coffee Leaves from Brazil

Species	Mangiferin (g/kg; % of total)	Isomangiferin (g/kg; % of total)	Total mangiferins (g/kg ± SE)
Minas Gerais:- Bom Sucesso-Conventional-old leaves	1.21 (81)	0.28 (19)	1.49 ± 0.19
Minas Gerais:- Bom Sucesso-Conventional-young leaves	4.17 (84)	0.80 (16)	4.97 ± 0.11
Manhumirim - Organic	0.48 (71)	0.19 (29)	0.67 ± 0.03
Ceara:- Guaramiranga	3.24 (89)	0.40 (11)	3.64 ± 0.01
Commercial variety	2.56 (84)	0.49 (16)	3.05 ± 0.16

Infusion of varying amounts (100-1000 mg) of Coffee leaf powder (Commercial variety) with boiling distilled water and maintenance at this temperature for twenty minutes revealed (Figure 2) that release of mangiferins showed a linear dose response (r = 1.00; *P* = 0.0001). The amount released from 1g was only 47 % lower than that after prolonged extraction with methanol.

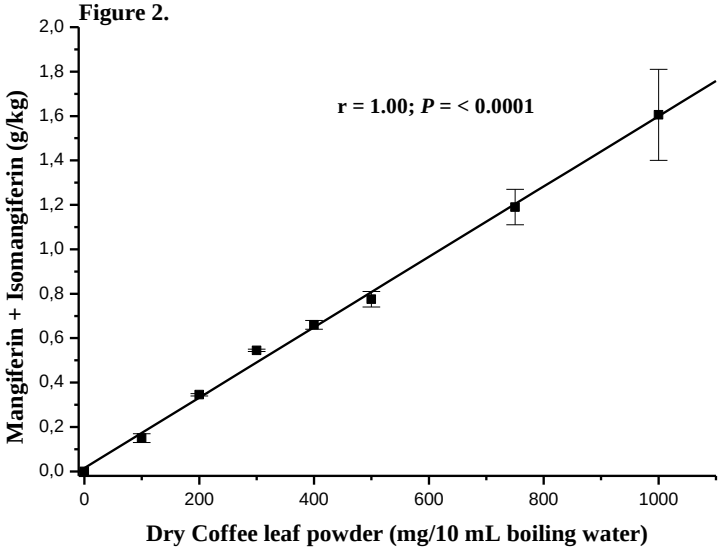

Figure 2.

The release of mangiferins by infusion with boiling water was instantaneous with 53 % extraction after one minute only, with no increase after 30 minutes (data not shown). By infusion, release of mangiferins from Coffee leaf powder was strongly temperature dependent (Figure 3). At 35°C, release of mangiferins was 59% efficient rising to 88% at 85°C and to 100% at 100°C. Therefore, infusion of Coffee tea leaves in the usual manner, releases greater than 50 % of the available mangiferins present as compared to methanol extraction.

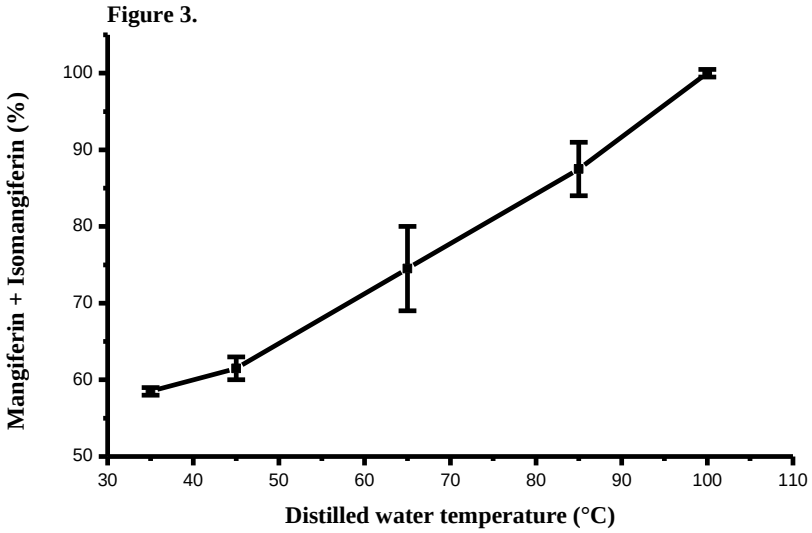

Figure 3.

Our data showing total mangiferins at concentrations in the range 0.67-4.97 g/kg, (which are 53 % soluble by infusion), indicate that the leaves of commercially cultivated *Coffea* species are an useful natural source of mangiferins, and consumption of Coffee leaf tea brews, may contribute significantly to elevated intake of these potentially health-promoting phenolic compounds, the exact relevance of which needs to be determined by future bioavailability and epidemiologic studies. Infusions studies with powder leaves of a commercial Brazilian source show that release of phenolic compounds is temperature and amount dependent and that release at 100 °C is virtually instantaneous and solubilises over 50% compared to prolonged methanol extraction.

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Stable Radical Content and Antiradical Activity of Roasted Arabica Coffee

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SUMMARY

The roasting of coffee beans generates stable radicals within melanoidins produced by non-enzymatic browning. The thermal treatment has further been suggested to increase the antioxidant capacity of coffee brews. Herein, we examined radical content and AO capacity of brews prepared from roasted Arabica beans sourced directly from an industrial plant. Using electron paramagnetic resonance spectroscopy, we observed signals arising from Fe^{3+}, Mn^{2+} and at least three distinct stable radicals (species I, II, III) as a function of roasting time. Species I was ascribed to the endogenous radicals present in green beans, species II was observed only within beans roasted for intermediate times, while species III corresponded to roasting-induced radicals that predominated at longer roasting times. In coffee brewed from the dark-roasted beans, the roasting-induced radical was harboured within the high molecular weight (>3 kD) melanoidin-containing fraction at a concentration of ~10 nM, whilst the low molecular weight (<3 kD) fraction exhibited the highest AO capacity using DPPH as an oxidant. This activity was not dependent on Mn- or Fe-based complexes within the brew. While other non-AO activities of the roasting-induced radical and metal complexes may be possible *in vivo*, we conclude that the antiradical activity of brewed coffee is dominated by low molecular weight phenolic compounds.

INTRODUCTION

Although free radical content of roasted coffee has been studied under laboratory conditions that emulate industrial roasting processes a controlled, time-dependent study of the free radical content from beans sourced directly from an industrial roaster is yet to be investigated. We examined *Coffea arabica* (Arabica) beans from an industrial roasting plant in order to measure the stable radical content of in-tact and ground beans as a function of roasting time and the relationship between the radical content and AO activity in coffee brewed from these beans. Using electron paramagnetic resonance (EPR), we identified as many as three different stable radical species as a function of roasting times ranging from 2–12 min. The intensity of these signals increased upon grinding, and also following ageing of the grounds, which may result from the exposure of a greater surface area and/or gradual dehydration, both of which will result in an increased microwave penetration depth. In brewed coffee solutions, we conclude that the radical is localized within phenolic-containing melanoidins and has no antiradical activity.

MATERIALS AND METHODS

Coffea arabica beans were sourced from Brazil and roasted at temperature up to 220°C under industrial conditions. During the roasting process, coffee beans were sampled at 2, 4, 6, 8, 10

and 12 minutes. To prepare coffee brews, 3.5 g of 12 min-roasted beans was coarsely ground in a marble mortar and pestle. Within 15 min of grinding, 20 mL 'MilliQ' grade water (Millipore) was added and brewed at 92 ± 1 °C in a beaker on a heat block with constant stirring. After 5 min, the solutions were rapidly cooled on ice and clarified using a low binding Millex® 0.45 μm PVDF syringe filter (Millipore).

High molecular weight (HMW) and low molecular weight (LMW) fractions were collected by passing the brew through an Amicon Ultra 3kD centrifugal ultrafiltration device (Millipore). The resulting solutions were either used immediately or stored at –80°C prior to further use.

Continuous-wave EPR spectra were acquired at 25°C using an E500 X-band spectrometer (Bruker) fitted with a Bruker super-high-Q probehead (ER 4122SHQE).

Whole beans were inserted directly into the probehead by attaching them to the end of a 3 mm ID quartz EPR tube using paraffin film (Bemis Company, Inc). Six beans from each roasting condition were first weighed on a microbalance, then inserted into the centre of the microwave probehead with the "face" of the bean being approximately parallel to the direction of the static magnetic field. Solution measurements of coffee brews were made at room temperature using a quartz flat cell (Wilmad, WG-808-Q).

Antiradical assays were performed in 1:1 MeOH/water solutions in the presence of 50 μM 2,2-diphenyl-1-picrylhydrazyl (DPPH; Sigma-Aldrich) and variable concentrations of brew. A dose response curve was computed by plotting the DPPH• concentration (absorbance maximum at 517 nm) as a function of brew concentration after 60 min incubation at 25°C. The EC50 was computed by non-linear least squares fitting of the dose response curve.

CONCLUSION

In-tact coffee beans exhibit EPR spectra comprising broad, anisotropic signals characteristic of high spin Fe^{3+}, Mn^{2+} (not shown) and narrow signal(s) corresponding to a stable radical(s). Figure 1A shows the EPR spectra of the stable radical in whole coffee beans and in freshly-ground beans that were roasted for variable times ranging from 2–12 min. At maximum roasting (12 min), the spectrum was characterized by an effective spectroscopic splitting factor of $g_{\text{eff}} \approx 2.004$ and a linewidth of 7 G, consistent with the free radical species previously observed in roasted coffee and instant coffee, in addition to several other beverages and foodstuffs including caramelized glucose. Quantification of the free radical intensity (determined by double integration of the first derivative signal) showed a monotonic increase as a function of roasting time except for a local maximum after 6 min roasting, which was associated with the presence of a broad component underlying the more narrow signals present at short and long roasting times (Figure 1B). Through spectral algebra and simulations, we were able to isolate and characterize three separate radical species (I–III) as a function of roasting time. The distribution curves for in-tact and ground beans are shown in Figure 1B. Relative to the total radical intensity measured at the longest roasting time within each series, we observed that the EPR signal of species II was enhanced upon grinding the beans (Figure 1B v–vi).

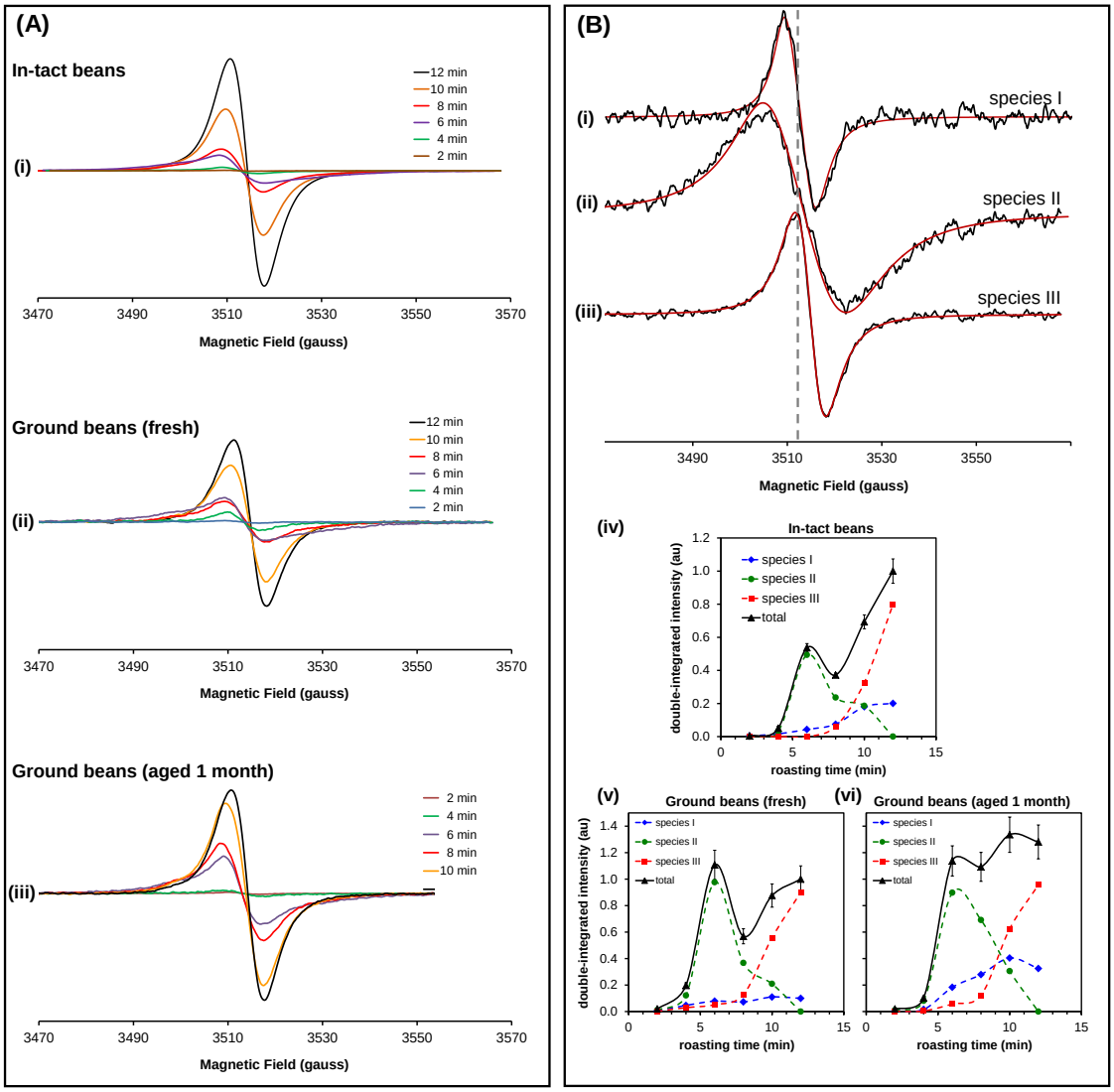

Figure 1. (A) Dependence of the radical EPR spectrum on roasting time for (i) in-tact beans, (ii) freshly-ground beans and (iii) the same grounds aged 1 month. (B) Experimental (black lines) and simulated (red lines) EPR spectra of (i–iii) three putative radical species present in whole and ground coffee beans. The experimental spectra from (iv) in-tact beans, (v) freshly ground and (vi) same grounds aged 1 month, were decomposed into contributions from species I–III. The intensity in *v-vi* is comparable, but the intensity in *iv* is not directly comparable with *v-vi*.

The room-temperature EPR spectrum of coffee brewed from dark (12 min) roasted beans consisted of a sextet of lines of width 25–30 G and spaced by 90–100 G arising from the orientationally-averaged interaction of the electron spin with the $^{55}Mn^{2+}$ nucleus (I = 5/2), together with a single-line radical species of width ~5 G (Figure 2A). The isotropic $\langle g \rangle$ = 2.0039 of the radical extracted by hot water was consistent with that of the predominant species (III) in the ground 12 min-roasted beans. We determined whether Mn^{2+} was likely bound to melanoidins or existed as LMW species by recording the EPR spectra of LMW (<3 kD) and HMW (>3 kD) fractions of brewed coffee solution. From the intensity of the Mn^{2+} spectra in Figure 2A, we estimated that ≈75% of the Mn^{2+} is contained within the LMW fraction. On the other hand, we estimated that >95% of the stable radical is contained within the HMW fraction, indicating that the radical resides within the melanoidin structure.

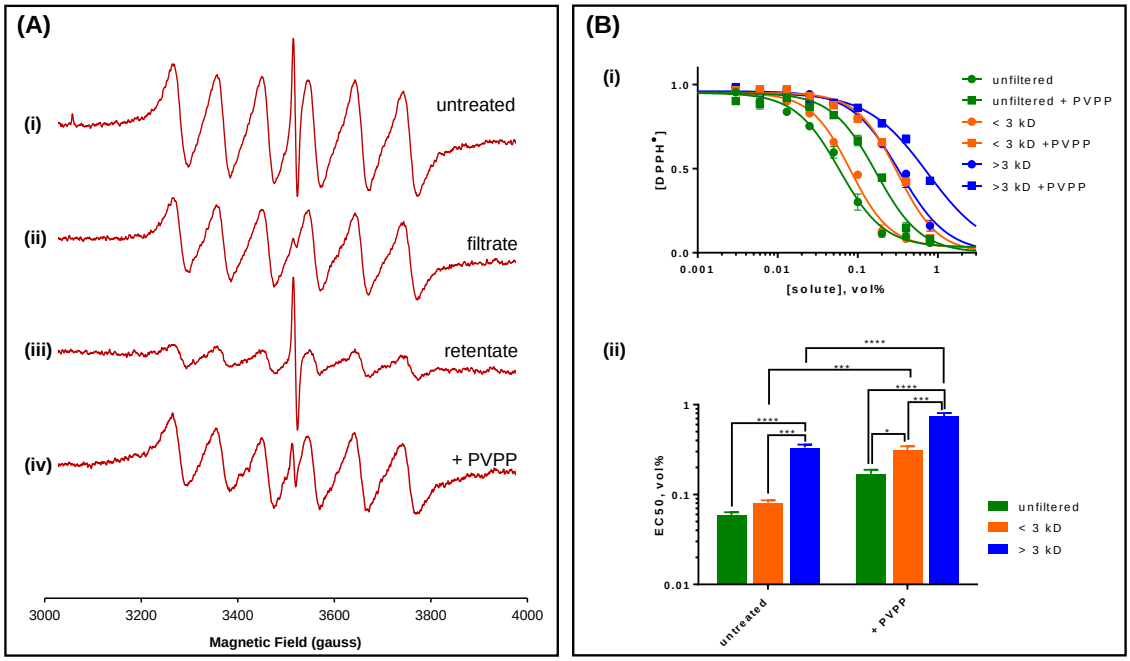

Figure 2. (A) Room temperature EPR spectra of coffee brew following various treatments. (i) untreated brew; (ii) species < 3kD; (iii) species > 3kD; (iv) unfiltered brew treated with PVPP. (B) Reduction of DPPH• to its hydrazine form DPPH-H in the presence of brewed coffee. (i) Dose response curves; (ii) EC50 values calculated from the curves. The brew was optionally pre-treated with PVPP, followed by filtration through a 3 kD MWCO membrane. Data represent the mean and SEM obtained from three brews prepared and assayed independently. For comparative purposes, the EC50 of Trolox under identical conditions was 11.3 ± 1.6 µM. Significant differences were calculated by two-way ANOVA using Holm-Šídák's multiple comparison test: *******P* **< 0.0001, ******P*** **< 0.001, *****P*** **< 0.01, ****P*** **< 0.05. (B) shows the dose response curves for the antiradical activity of brewed coffee in 1:1 MeOH/water and of coffee solutions subjected to various treatments. The EC50 of the retentate was more than 5-fold higher than the unfiltered brew, demonstrating that the highest antiradical activity is contained within the LMW fraction (< 3kD). Extraction of phenolics by PVPP treatment increased the EC50 of the filtrate four-fold, suggesting a predominant role of polyphenols (non-bound to HMW melanoidins). This is consistent with the linear correlation between phenolic (gallic acid) content and AO activity observed by Brezová et al for ground and soluble coffee and the finding of Bekedam et al. that phenolic AOs dominate the overall AO activity of coffee brews despite melanoidins possessing measurable AO activity.**

Treatment with DTPA, a high affinity chelator of metal ions, caused no significant changes to the retentate EC50, indicating that metal binding to coffee melanoidins or flavonoids, particularly Mn^{2+}, does not contribute to the antiradical activity of brewed coffee. The radical species present in the brew maintains a constant intensity in the presence of DPPH (Figure 3). Moreover, no evidence for formation of new stable radical species was observed, indicating the EPR-detectable stable radical in the brewed coffee possesses no significant AO activity as measured by the DPPH assay.

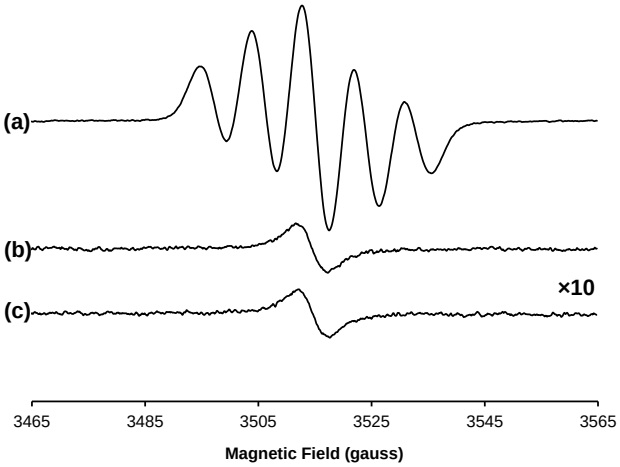

Figure 3. Antiradical activity of brewed coffee does not consume or generate stable coffee radicals. Room-temperature CW-EPR spectra of 1:1 MeOH/water solutions containing (a) 50 µM DPPH• alone, (b) brewed coffee added to an equal volume of 100 µM DPPH• in MeOH, and (c) brewed coffee added to an equal volume of MeOH. Data represent results of assay performed on a single brew. For clarity, the vertical scale is an order of magnitude higher spectra in *b* and *c* as compared with *a*.

This study applied EPR spectroscopy and *in vitro* antiradical assays to study the free radical content and antiradical capacity of *Coffea arabica* sourced from an industrial roasting plant. The present research demonstrated that a number of stable radical species are formed during roasting and their intensity profile varies with roasting time and upon subsequent grinding and ageing. The stable radical(s) present within dark-roasted beans are unrelated to the antiradical activity of coffee brewed from those beans; however, this does not preclude a functional role for these radical species in non-antioxidant mechanisms *in vivo* following coffee consumption, or in variations to flavor profile during storage and ageing.

LN Research activity has been carried out in the framework of POR-FESR 2007-2013 NUTRIHEART project.

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Chemical Characterization of Ethanolic Extracts from Spent Coffee Grounds

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SUMMARY

This work has been obtained and characterized, as far as its functionality is concerned, the spent coffee grounds ethanolic extract from espresso coffee in capsules. The antioxidant activity (DPPH), the sun protection factor and the volatiles profile have been evaluated. The coffee oil content in all extracts were superior to 98%(w/w). The volatiles profile came about to be typical of roasted coffee oil. By means of principal component analysis from chromatographic areas, extracts were discriminated or grouped, respectively for different samples. This results showing the repeatability of the extraction process employed. Regarding the coffee oils ethanol-based, the antioxidant activity ranged from 18.4 to 23.6 (mg extract mg $DPPH^{-1}$) and sun protection factor from 2.27 to 2.76. From the characterization performed in this study, it can be inferred that ethanol extracts from capsules of espresso spent coffee grounds may exhibit chemical properties of industrial interest. The feasibility of their application should be evaluated in cosmetic and food industries.

INTRODUCTION

The consumption of espresso coffee in capsules has grown exponentially in the past decade, generating large amounts of spent coffee grounds. Many studies have been developed aiming at the reutilization of agro-industrial co-products which may contain substances of high value for the industrial sector. The use of a renewable solvent in this extraction process is a technological alternative that provides a gradual reduction of the petroleum by products, reducing consequently environmental risks in the edible oil usines. Ethanol for hexane replacement presents good prospects, since this solvent can be produced from different renewable sources at competitive prices.

Roasted coffee has about 20 % of oil which, after extraction, is commonly used in food industry once it has high value as natural flavoring, due to its pronounced and pleasant aroma. In soluble coffee industry, the roasted coffee oil is incorporated as flavour r. In the other hand, in some cosmetic formulations, the coffee oil is used in the natural products formulations for block the harmful UV radiation to the skin, and to moisturize, lubricate and improve skin texture, and promote regeneration of the hydrolipidic film of the same.

This study aimed to characterize the oil extracted with ethanol using the spent coffee grounds from espresso capsules. To this end the profile of volatiles compounds by HS-SPME-GC-MS, the antioxidant activity and the sun protection factor (SPF) in the oil was evaluated

MATERIALS AND METHODS

The lyophilized samples were incubated at 60 °C with anhydrous ethanol in a ratio of 4: 1 (ETOH: dry spent coffee grounds, w/w) in a thermostated shaker under constant orbital agitation of 2000 rpm for 30 min. The solution was then vacuum filtered to separate the liquid extract of the bran and finally, ethanol was distilled off, also under vacuum on a rotary evaporator to obtain the crude ethanolic extract.

To evaluate the profile of volatile compounds, 0.20 g of the ethanol extract were heated at 60 °C in 5.0 ml sealed vial for 20 min. Then Polydimethylsiloxane/Divinylbenzene/Carboxen (50/30 mM) fiber was exposed to the headspace of the vial for substances adsorption for 10 min. The desorption of the compounds was made by the fiber introduction for 5 min, in the chromatograph injector to 240 °C on splitless mode (Agillent 6890). Chromatographic column DBwax, J & W Corp., 25 mx 0.2 mm internal diameter x 0.25 mm thick film was employed; temperature program: 45 °C (5 min)//4 °C min^{-1}/230 ºC, maintaining the final temperature for 10 min. The mass spectra were obtained with a mass selective detector Agilent Model 5973 operating in the electron ionization mode (70 eV), transfer line temperature of 280 °C and the ion source to 220 °C. In order to differentiate the samples as their volatile composition, we used the multivariate principal component analysis (PCA) of the chromatographic areas obtained by HS-SPME-GC-MS using the Unscramble Software version 10.1 (CAMO CORP).

Determination of antioxidant activity was carried out by DPPH• method, which is measured the ability of the sample to stabilize the free radical DPPH•. This method is based on absorbance reduction of DPPH• (1,1-diphenyl-2-picryl-hydrazyl) to DPPH (1,1-diphenyl-1-picryl hydrazine) at 515 nm by the action of reducing compounds. The antioxidant activity of standard 5-caffeoylquinic acid (5-CQA), which is the main substance responsible for the coffee antioxidant activity, was also measured.

To evaluate the FPS, we used the methodology proposed by Bendova et al. (2007), In this case ethanolic extract was prepared in absolute ethanol at a concentration 0.20 $mg.mL^{-1}$, for which the spectrophotometric scan was performed on the region between the wavelength (λ) of 290 and 320 nm with measurements at 2 nm intervals. The SPF was calculated using the expression proposed by Mansur (1986).

CONCLUSION

Yield values of shaker ethanolic extraction ranged from 15.1 to 17.4% by mass. Due to its polar feature, ethanol extracted substances responsible for the flavor, the antioxidant activity and the sun protection factor.

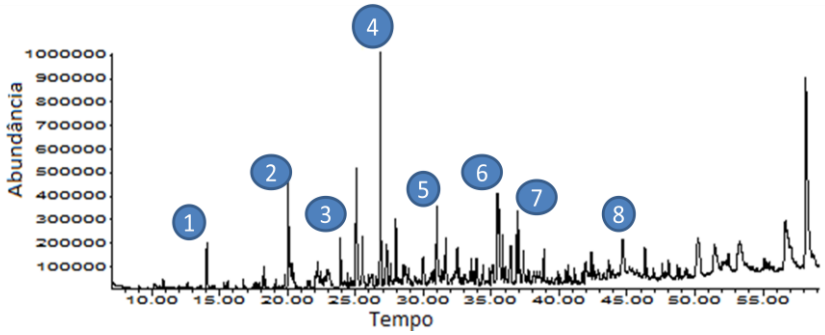

Figure 1. Chromatogram of spent coffee grounds ethanolic extract. *Coffee flavour substances, identified by GC-MS and IRL: 1) 2,5-dimethyl pyrazine, 2) Furfural, 3) 1 methyl 2-acetyl pyrrole, 4) 2-furan methanol, 5) 2-methoxy phenol, 6) 2-acetyl pyrrole, 7) phenol, 8) 3-ethyl phenol.

About 180 chromatographic compounds were detected in all ethanolic extract samples studied. In a first analysis, the similarities between the mass spectra obtained by the detector and the chromatograph spectra software library were observed. It were considered identified the substances that the similarity of the spectra showed that greater than or equal to 90% mass. From the statistical analysis performed with six injections of the same sample, we evaluated the coefficient of variation of the peak areas of each substance (less than or equal to 10%). In the end, the HS-SPME-GC-MS technique was shown to be reproducible for 70 compounds.

Were detected and identified flavour coffee substances classes as pyrazines, furans, pyrroles, alcohols, acids, phenolics, ketones, pyridines, terpenes, lactones, aldehydes, thiophenes, tiazolas, ethers, pyrans and benzoxazolas. Besides the comparison of mass spectra, the linear retention indices (LRI's) are in accordance with LRI's reported in the literature for the type of column chromatography used. Thus, the qualitative determination of substances is given by two tools, a comparison of mass spectra and LRI.

Figure 1 shows a ethanolic extract of espresso grounds chromatogram obtained by HS-SPME-GC-MS technique in the present study. It is possible to observe the complexity of the volatile profile by the amount of chromatographic peaks and indicating the signs of some of the principal substances.

As the volatile profile is similar to that reported in the literature for the oil roasted coffee, the product proves to be potentially applicable in the food industry (candies, chocolates, ice cream, instant coffee) as a flavor enhancer, given their pronounced flavor.

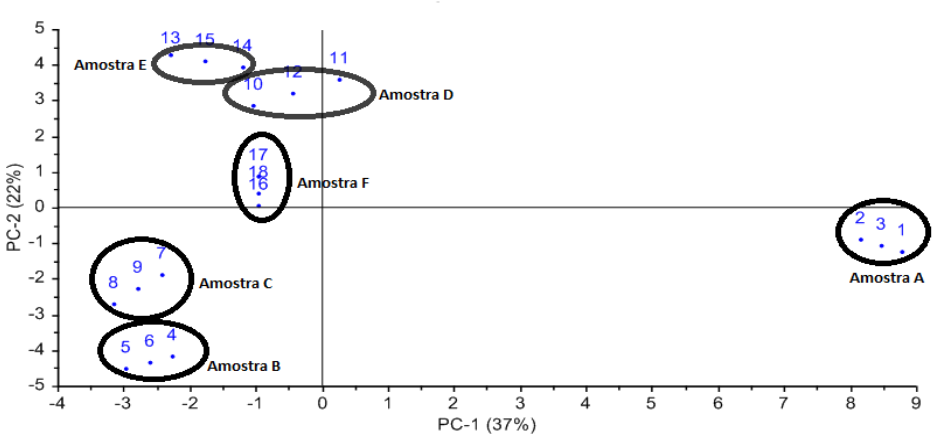

Figure 2. Multivariate Principal Component Analysis (PCA) of the coffee ethanol extract volatiles profile.

Figure 2 shows that the differentiation of samples of different origins or blends, by means of multivariate analysis of chromatographic data, confirms the applicability of this statistical technique into arrays with large numbers of chemical compounds and classes, and is consistent with other studies that have discriminated differents coffee samples by applying PCA to the data volatiles profile. Additionally, this result indicates that the thermodynamic properties of the solvent defines the main compounds removed to extract and for this reason, the technique also allowed to discriminate the solvent used in the process because it was group replicates of the same sample.

Table 1. Antioxidant activity of sold in capsules spent coffee grounds ethanolic extract.

Sample	Antioxidant Activity ($mg_{extrato}/mg_{DPPH\bullet}$)
A	$23,6 \pm 1,44^{b}$
B	$19,4 \pm 1,07^{a}$
C	$19,1 \pm 1,15^{a}$
D	$19,0 \pm 2,52^{a}$
E	$18,4 \pm 2,11^{a}$
F	$19,2 \pm 1,98^{a}$
5-CQA (Standard)	0,38 ±0,023

**Results are expressed as mean ± standard deviation of three replicates.*
**Values followed by different letters are significantly different (by Fisher's LSD test p <0.05).*

The average antioxidant activity of ethanolic extracts of spent coffee grounds, provides the mass needed to reduce 50% of DPPH• radicals. The results are in the range 18.4 to 23.6 mgextrato / mgDPPH• (Table 1), values 18-62 times higher than the value presented by standard chlorogenic acid 5-CQA. Dias et al. (2005) reported a 5-CQA content of 0.515 $g.100g^{-1}$ coffee (0.5%) in middle degree of roasting.

Table 2. Sun Protection Factor (SPF) of coffee grounds ethanolic extract.

Sample	Sun Protection Factor (SPF)
A	$2,44 \pm 0,0386^{a}$
B	$2,76 \pm 0,0651^{b}$
C	$2,51 \pm 0,0664^{c}$
D	$2,69 \pm 0,0553^{d}$
E	$2,27 \pm 0,0579^{e}$
F	$2,29 \pm 0,0212^{e}$

**Results are expressed as mean ± standard deviation of three replicates.*
**Values followed by different letters are significantly different (by Fisher's LSD test p <0.05).*

The sun protection factor (SPF) ranged from 2.27 to 2.76 (Table 2). Samples with different compositions of arabica and robusta coffee types showed no significant difference in the power minimizing the effects of UV rays.

The extraction with ethanol generated a product with high oil content and, because of their polar character, also water soluble substances were abtained. Those kind of substances couldn´t remove by hexane, a solvent commonly used in oils industry. These compounds are associated with features that add value to the product as flavour, antioxidant activity and sun protection factor. From the characterization performed in this study, it can be inferred that ethanol extracts from capsules of espresso spent coffee grounds may exhibit chemical properties of industrial interest. The feasibility of their application should be evaluated in cosmetic and food industries.

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Chemical Compounds and Spectral Signature, Instrument of Origin in Three Colombian Coffee Zones

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SUMMARY

In order to develop tools to identify origin in Colombian Coffee zones, 3877 coffee samples were taken for three consecutive years on farms, to represent the higher variability of coffee production system in terms of management, harvest season, varieties and origin of the coffee areas of the departments of Cauca (1006 samples) , Nariño (925) and Huila (1946). The samples were evaluated by Near Infrared Spectroscopy - NIRS (SSig) and by determination of 15 chemical compounds (ChC) by NIRS prediction equations; discriminant functions (DF) for each region on the SSig and ChC were developed. Differences in the coffee SSig from each region based Mahalanobis distance were determined. While the DF for the three regions evaluated was 87 % in the overall percentage of correct classification using ChC, the SSig analysis rated 100%; among the findings, Cauca coffee samples shared characteristics with Nariño coffee by 11 %. These results were used by the Colombian Coffee Growers Federation for supporting the claims for protection of the Denomination of Origin "Café de Cauca", "Café de Nariño" and "Café del Huila" in the Industry and Commerce Superintendency in Colombia.

INTRODUCTION

The Colombian coffee is recognized in the international market for high quality product, but the high variability in the coffee area in terms of climate conditions, soil type and production systems (shade, semi – shade, sunlight) with only arabica varieties planting, enable differentiation and segmentation of coffee production at the regional level.

The Colombian Coffee Growers Federation began a strategy which seeks to acknowledge the quality of different coffees zones, under the concept of Protected Geographical Indication (GI) and Protected Designation of Origin (DO), these are a recognition of the quality of product, which is derived solely and primarily the geographical environment in which it is produced, and which are associated natural factors such as climate and soil, and humans, thus, currently recognize the GI "Café de Colombia“ (SIC, 2005; OJEU, 2007).

The coffee regions of Cauca, Nariño and Huila in recent years have held important positions in high quality competitions such as the Cup of Excellence and are required by different actors in the coffee marketing chain Value (Figure 1).

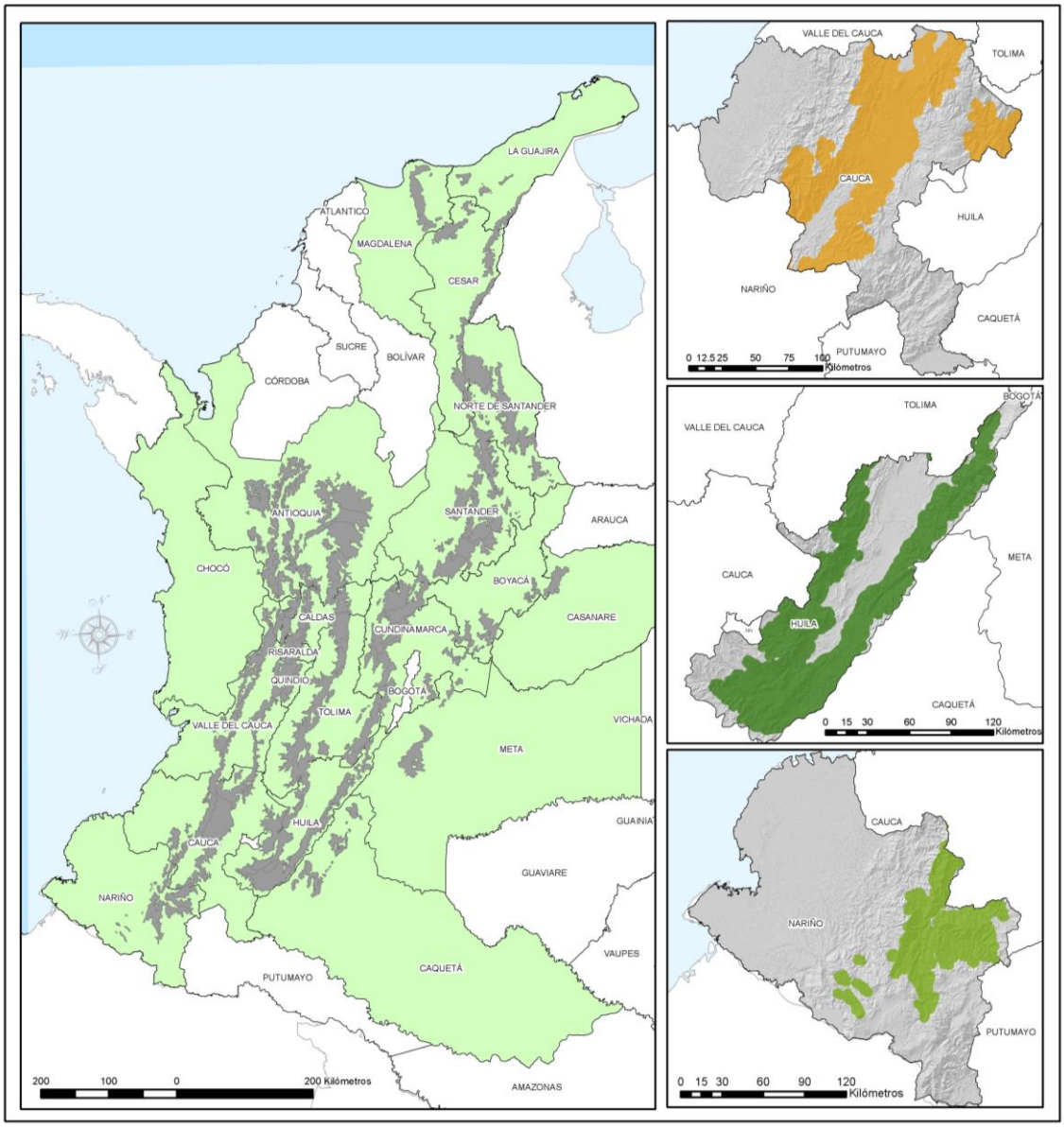

Figure 1. Coffee regions of Cauca, Nariño and Huila with sites sampled.

MATERIALS AND METHODS

In order to develop tools to identify origin in three Colombian Coffee zones (Cauca, Nariño and Huila), 3877 coffee samples were taken in several years on farms, to represent the higher variability of coffee production system in terms of management, harvest season, varieties and origin of the coffee areas of the departments of Cauca (1006 samples) , Nariño (925) and Huila (1946) using the methodology described by Oberthür (2011). The samples were evaluated by Near Infrared Spectroscopy - NIRS (SSig) using the monochromator spectrophotometer FOSS, model 6500 (Perstrop Analytical Inc., 1201 Tech Road, Silver Spring, MD 20904, USA); the quantification of 15 chemical compounds (ChC) was performed using prediction models developed in Cenicafé (Cenicafé, 2011), the discriminant functions (DF) for each region on the SSig and ChC were developed.

RESULTS AND DISCUSSION

The table 1, showed the descriptive Statistics for fifteen compounds associated with coffee quality for the three coffee regions, the average behavior of the Arabica species for the three areas.

Table 1. Chemical composition by origin.

CAUCA					*NARIÑO*				*HUILA*			
Compound	**Min**	**Means**	**Max**	**Est_dev**	**Min**	**Means**	**Max**	**Est_dev**	**Min**	**Means**	**Max**	**Est_dev**
Caffeine	1.04	1.32	1.61	0.09	1.02	1.32	1.57	0.09	1.09	1.32	1.55	0.08
Trigonelline	0.68	0.93	1.20	0.07	0.68	0.94	1.22	0.07	0.86	1.08	1.28	0.07
CQA3	0.11	0.33	0.49	0.08	0.10	0.29	0.39	0.04	0.21	0.39	0.56	0.06
CQA4	-0.15	0.47	1.05	0.24	-0.08	0.26	0.65	0.12	0.42	0.61	0.79	0.06
CQA5	0.88	2.90	4.03	0.44	1.09	3.10	3.98	0.52	2.48	3.19	3.98	0.25
CQAT	3.52	5.05	6.74	0.63	3.46	4.70	5.54	0.28	5.46	6.22	7.16	0.26
Saccharose	3.50	5.09	6.45	0.43	3.96	5.03	7.86	0.41	4.33	5.43	6.72	0.38
Lipids	11.41	15.00	17.72	0.99	12.19	14.74	16.67	0.71	13.31	15.58	18.11	0.78
Arachidic	1.47	2.67	4.01	0.40	1.49	2.82	4.09	0.38	1.74	3.08	4.27	0.36
Behenic	0.36	0.75	1.14	0.15	0.53	0.80	1.10	0.09	0.77	0.96	1.19	0.08
Oleic	2.97	8.63	15.45	2.09	5.61	9.60	14.32	1.42	5.77	9.55	13.37	1.18
Linoleic	32.15	41.17	48.41	2.16	33.71	40.61	50.78	1.93	30.87	39.75	45.57	2.31
Linolenic	1.03	1.41	1.84	0.12	1.13	1.45	1.83	0.11	1.09	1.33	1.60	0.09
Palmitic	24.78	34.89	47.55	3.08	25.34	34.14	43.25	2.69	27.00	33.73	47.52	3.08
Stearic	3.73	7.04	10.12	0.99	4.40	7.10	10.18	0.99	5.84	7.97	10.50	0.69

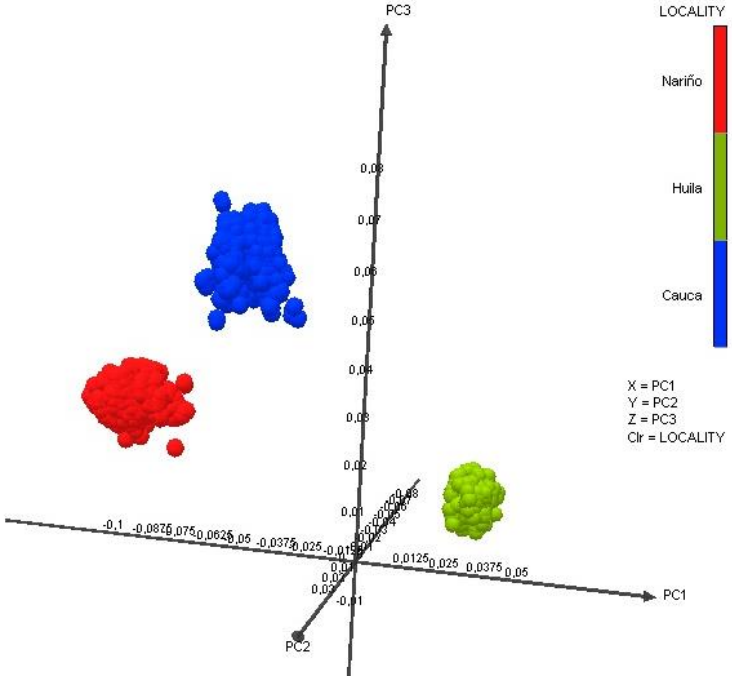

Figure 2. Spectral signature by locality (Biplot of the first 2 Principal Components).

The DF for the three regions evaluated was 87 % in the overall percentage of correct classification using ChC and using SSig analysis was 100%; Cauca coffee samples shared characteristics with Nariño coffee by 11 %, especially from areas of Mercaderes, Florencia and Bolivar.

CONCLUSION

These results were used by the Colombian Coffee Growers Federation for supporting the claims for protection of Origin "Café de Cauca", "Café de Nariño" and "Café del Huila" in the Industry and Commerce Superintendence in Colombia, are being used to further authenticate these three coffee regions.

ACKNOWLEDGEMENTS

Ministry of Agriculture and Rural Development of Colombia, for financing the Genome project.

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Model Development Using Nirs and 16-Omc Content for Detecting Possible Adulterations of the "100% Colombian Coffee" (pc422)

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SUMMARY

In order to implement a better quality control for the "100% Colombian Coffee" product, samples of arabica coffee from Colombia and its admixtures in proportions from 5 to 50 percent with Robusta coffee from Ecuador, Vietnam and Indonesia with three levels of roasting were evaluated. Samples were analyzed by Near-Infrared Spectroscopy (NIRS) and multivariate calibration methods. Raw data were pretreated before the calibration process in order to remove any non-relevant spectral information that could be associated with roasting level, grinding differences and any other transformation process for coffee. A variable normal standardization (SVN), detrending the signal and its first derivative were the best pretreatments for the sample data. From there a Modified Partial Least Squares regression model (MPLS) was built. Furthermore, samples were divided into two groups, for calibration and external validation; Correlation coefficients for calibration and cross-validation were 0,998 and 0,997 respectively, with a calibration error of 0,94. As a further step, a quantification of 16-O-methylcafestol diterpene (16-OMC), a chemical compound indicator of Robusta coffee presence, was made. These results suggest that the NIRS technique can be used as a quality control tool for identifying adulterations in roasted Colombian coffee and the quantification of 16-OMC can be used for confirmation. This work will be used on NIRS network implemented by the National Federation of Coffee Growers to detect adulterations of Colombian coffee at all shipping points.

INTRODUCTION

In the two coffee species commercially important, Coffea arabica and Coffea canephora, which differ not only in morphology and genetics but also in chemical composition and organoleptic qualities, is possible to detect coffee admixtures between Robusta and Arabica in levels up from 5% (Puerta, 1999). Detection of these admixture percentages is more difficult in roasted coffee and this makes relevant to have tools that can check authenticity of coffee either with direct or indirect methods. In this situation, chemical compounds can act as differential agents between these two coffee varieties. The lipid fraction of the coffee has compounds that allow to differentiate between arabica and robusta coffees. The 16-O-metylcafestol (16-OMC) is only present in C. canephora while the Cafestol, though is present in both species, its concentration in C. arabica is greater and Kahweol is almost zero in C. canephora.

In order to develop rapid and useful methods of check authenticity of 100% Colombian Coffee, Near Infrared Spectroscopy -NIRS- technology and High-performance liquid

chromatography –HPLC- were evaluated for admixture percentage estimation and also dilucidate the possible origin to C. canephora sample using 16-O-metylcafestol.

MATERIALS AND METHODS

Samples roasted of arabica coffee from different origins in Colombia was mixed with three international origins of Robusta (Ecuador, Vietnam and Indonesia) in pure stage and different proportions of blends were evaluated 5, 10, 15, 20, 25, 30, 35, 40, 45, 50%, at two levels to roasted (high and low).

The samples were analyzed by Near Infrared Spectroscopy NIRS, the calibration process using different pre-treatment from the original data in order to remove irrelevant information that spectrum, such as the degree of roasting, grinding and other aspects associated with transformation process is performed. The quantification to 16-OMC was by high efficiency liquid chromatography HPLC-UV.

CONCLUSION

NIRS analysis for the samples were divided into groups and external validation calibration regression modified by partial least squares (MPLS). It was found that a normal standardization variable (SNV), a correction of the signal by the algorithm Detrend and first derivative, were the best pre-treatment to remove such information, the determination coefficients were 0.998 and 0.997 for calibration and cross-validation, respectively, with a calibration error of 0.94 (Fig. 1).

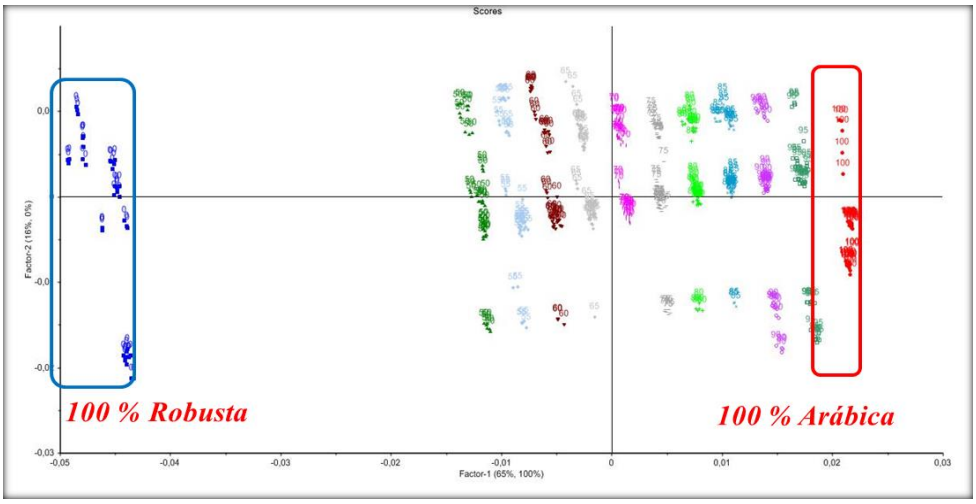

Figure 1. Representation of the first two principal components of samples of pure roasted coffee (100 robusta - 100 Arabica from Colombia) and pooled from 5 to 50% based Arabica – Robusta.

The number of tests performed to quantify the 16-OMC was 144, for Indonesia 72, and 36 for Ecuador and Vietnam respectively. The coefficients of variation for three replicates of each sample of roasted coffee were between 0 and 8.22%. The figure 2 showed the chromatographic profile for three percentages of Robusta.

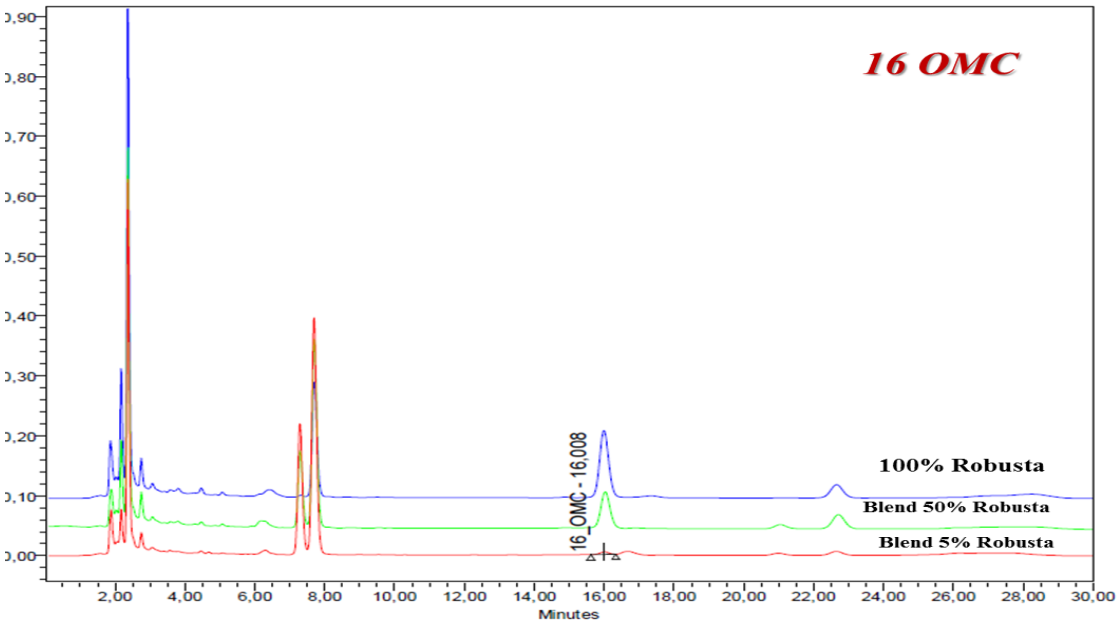

Figure 2. Chromatographic representation Robusta Coffee blends (100%, 50%, 5%).

The ranged values for 16-OMC were between 83.0 ppm (5%) and 1485.3 ppm (100% robusta) in low level in roasted and between 96.9 (5%) and 1844.4 ppm (100%) high roasting for Indonesia. For Ecuador values ranged from 101.8 (5%) and 2117.9 ppm (100%) in low roasting and between 145.5 (5%) and 2725.1 ppm (100%) for high roasting. For Vietnam the values were between 53.9 (5%) and 1082.6 ppm (100%) for a low roasting and 68.7 (5%) and 1457 ppm (100%) to a high roasting. (Fig. 3).

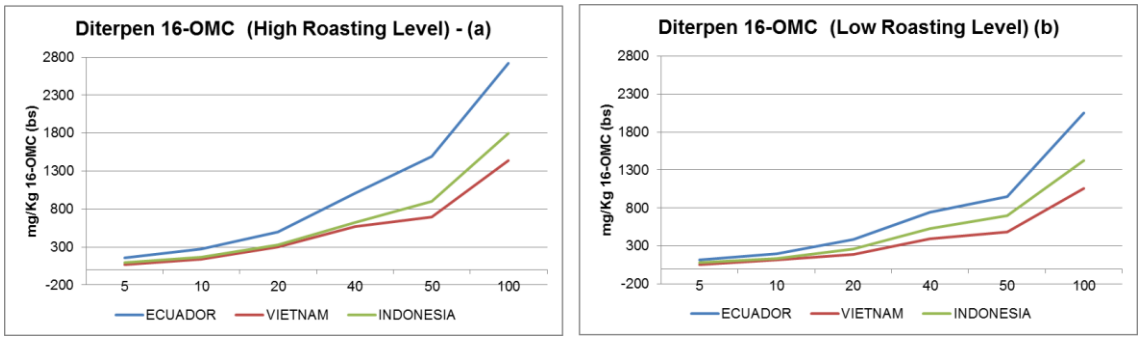

Figure 3. Average composition of diterpen 16-OMC blends roasted Arabica coffee from Colombian coffee with Indonesian, Ecuador and Vietnam Robusta coffee a) Roasting High b) Roasting Low levels.

A directly proportional relationship between the content of 16-OMC and the degree of roasting roasted coffee samples was found. Ecuador had the highest values of 16-OMC, with variations in content depending on how the sample roasting. Vietnam had the lowest values. (Fig. 4).

The results were validated with commercial coffee samples from supermarkets in Colombia, which identified the presence of Robusta and estimate the percentage of blends (Fig. 5).

Table 1.

% Robusta	Minimun	Average	Maximun
5	53.9	98.0	166.6
10	107.4	172.3	279.1
15	198.4	223.3	246.7
20	192.4	324.5	514.6
25	310.6	378.2	432.9
30	389.1	457.4	523.0
35	461.4	524.2	593.3
40	389.5	643.6	1014.0
45	585.0	674.5	767.3
50	483.6	869.8	1508.1
100	1016.8	1768.7	2725.1

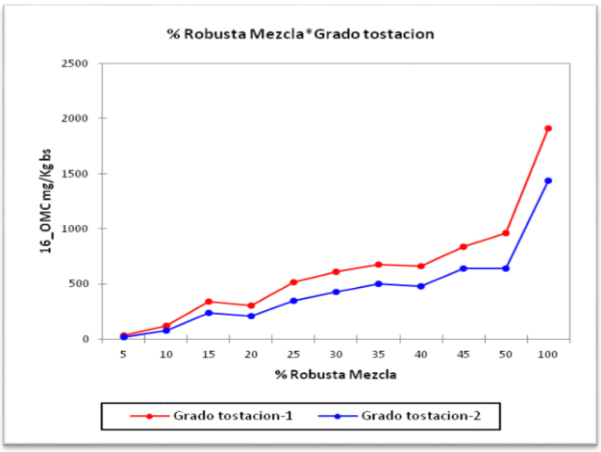

Figure 4. Quantification of 16-OMC in blends of different origins according to the degree of roast.

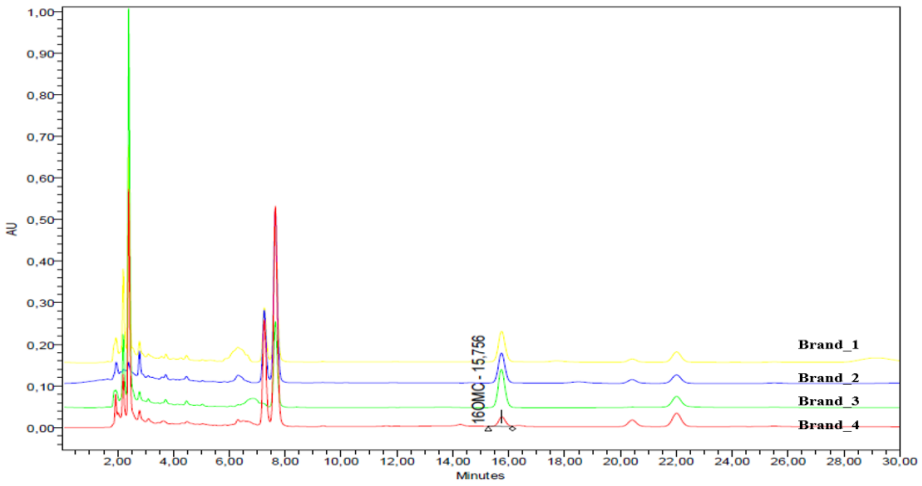

Figure 5. Quantification of 16-OMC in different brands in Colombia.

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Ministry of Agriculture and Rural Development of Colombia, for financing the Genome project.

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Metabolite Profile of Parchment Coffee Beans Harvested under Different Brazilian Environmental Conditions

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SUMMARY

Coffee is a product valued for its beverage quality, especially in international trade. The different environmental conditions provided by altitude and slope exposure of fields are popularly known for affecting final coffee quality. Understanding these effects on coffee quality is highly important for the specialty coffee production chain. Therefore, the aim of this study was to understand the influence of different environmental conditions on the metabolite profile and on the final quality of different coffee varieties grown in the Serra da Mantiqueira (Brazil) and processed by the wet method. The experimental samples were harvested over three crop seasons (2009/10, 2010/11, and 2011/12), with the experimental design consisting of 3 altitude ranges (<1000 m, 1000-1200 m, >1200 m) and two genotypes (Acaiá and Yellow Bourbon). Metabolite profiling was performed using the Gas Chromatography-Quadrupole/Mass Spectrometry - GC-Q/MS technique, and sensory evaluation was performed according to the methodology proposed by the Specialty Coffee Association of America - SCAA. The dataset containing the metabolite profile and the total sensory score was pretreated by generalized least squares weighting - GLSW (a=0.001), and analyzed by Principal Component Analysis - PCA. The altitude ranges clearly influenced the differentiation of samples from both varieties, Acaiá and Yellow Bourbon. However, higher variance could be observed for the Yellow Bourbon variety on Principal Component 1, which separated the group of samples harvested above 1200 m from the ones harvested below that altitude. The metabolites found in higher levels in the Yellow Bourbon demucilaged beans harvested above 1200 m, which were responsible for such separation, were L-serine, L-aspartic acid, L-asparagine, glucose, and fructose. In addition, this coffee variety also showed higher scores in sensory analysis.

INTRODUCTION

Coffee is one of the most important commercial commodities traded in the world. Currently, the demand for specialty coffees in the market grows at a greater rate compared to regular coffees. Specialty coffees are coffee beans that shows high beverage quality and great flavor, have characteristics linked to their origin and do not have any defect. The term "coffee quality" has a particular meaning for each class in the coffee production chain, and it can be significantly different for the farmer and for the consumer. Although Brazil is the largest coffee producer, its coffees are still known worldwide as being average, cheap, flat, and with prominent astringency. Coffees from other origins, such as Central America, Africa, and Asia, are very well known for their high quality and notable sensory attributes. Different origins may affect metabolic responses to environmental conditions. Moreover, the species *Coffea arabica* L. and *Coffea canephora* show great differences in their chemical composition.

The metabolite profile of coffee seeds can significantly change due to several factors, such as genetics, environment, and post-harvest methods. Thus, the cup quality or sensory profile may be directly related to the metabolite profile.

Differences in the metabolite profile involve a network of phenomena that are not easy to explain and that still require much more study to be understood. Therefore, it is necessary to explore changes in the metabolite and the differences that are responsible for defining final coffee quality so as to provide techniques to the specialty coffee production chain. In addition, these studies could help improve the strategies for coffee breeding programs.

MATERIALS AND METHODS

Experiments were carried out in the municipality of Carmo de Minas (-22.12º, -45.13º), which is located in the Serra da Mantiqueira, Minas Gerias, Brazil. The experimental design consisted of samples of two coffee genotyes (Acaiá and Yellow Bourbon) harvested at three different levels of altitude (under 1000 m, from 1000 m to 1200 m, and above 1200 m) over three coffee crop seasons (2009/10, 2010/11, 2011/12). The Acaiá genotype was chosen for the experimental design mainly because it has red fruit skin and high average yield, and because it is one of the most cultivated varieties in Carmo de Minas.

The metabolite profiling was performed according to and the sensory analysis was performed according to the Specialty Coffee Association of America (SCAA) methodology.

The dataset was pretreated by generalized least square weighting (GLSW) and subjected to Principal Component Analysis (PCA). Both procedures were performed using MATLAB 7.9.0 (The MathWorks^{TM}, MA, United States) and PLS Tool-box 5.2.2 (Eigenvector Research, Inc., WA, United States).

CONCLUSION

PCA of demucilaged x Acaiá x altitude

Figure 1 shows the PCA score scatter plot of principal component 1 (47.37%) and principal component 2 (29.67%) of the metabolite profile of the demucilaged Acaiá coffee seeds harvested in the three altitude ranges (<1000 m, 1000-1200 m, and >1200 m). The demucilaged Acaiá samples harvested in these three ranges of altitude showed a clear differentiation. However, separation by PC1 was observed for the samples harvested in the 1000-1200 m altitude range from the other two ranges, <1000 m and >1200 m. The samples harvested in the two altitude ranges, <1000 m and >1200 m, were separated by PC2. Although these results showed differentiation between the samples harvested in different ranges of altitude, a pattern in the metabolite profile in accordance with increasing altitude could not be observed. It could be due to the processing method for demucilaging, which may change the final metabolite profile regardless of differences in altitude.

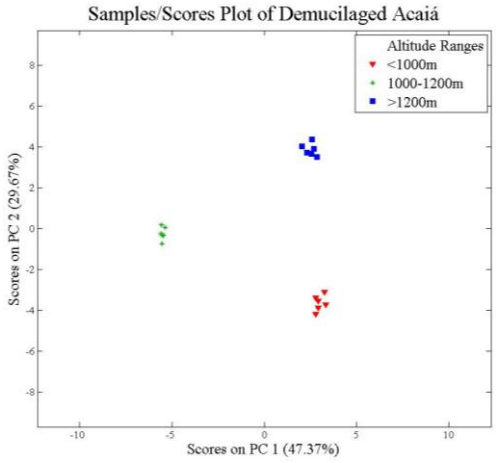

Figure 1. PCA score scatter plot of principal component 1 (47.37% of total variability) and principal component 2 (29.67% of total variability) of the metabolite profile differentiating demucilaged Acaiá coffee seeds harvested in three different ranges of altitude (▼) <1000 m, (✳) 1000-1200 m, and (■) >1200 m. Each class of altitude ranges in the dataset was pretreated by WGLS, alpha = 0.001.

The features that had the greatest influence on the separation of the samples harvested in the 1000-1200 m altitude range were oxalic acid, L-aspartic acid, phenylalanine, fructose, and galactinol. As previously observed, the samples harvested below 1000 m and above 1200 m were not separated by PC1, but by PC2 (Figure 2A).

The metabolites present in higher levels in the demucilaged Acaiá coffee samples harvested below 1000 m were L-valine, glycerol 1-phosphate, and 5-CQA. The coffee samples harvested above 1200 m included L-isoleucine, L-proline, L-serine, trigonelline, putrescine, gluconic acid, myo-inositol, and galacturonic acid.

Gluconic acid and galacturonic were present in higher levels in demucilaged Acaiá coffees harvested above 1200 m compared to the samples harvested below 1000 m. Lactic acid and gluconic acid were also present in the demucilaged Acaiá coffees. These sugar acids may increase the acidity and the sweetness of the coffees harvested above 1200 m of altitude. In addition, acidity and sweetness are valued attributes when evaluating coffee quality. Coffees with high intensity of these parameters will probably be highly scored.

In contrast, the demucilaged Acaiá samples harvested below 1000 m of altitude showed higher levels of L-valine, glycerol 1-phosphate, and 5-CQA, which contributes to bitterness. The presence of these compounds in coffee seeds harvested below 1000 m may explain their lower scores when compared to the samples harvested >1200 m.

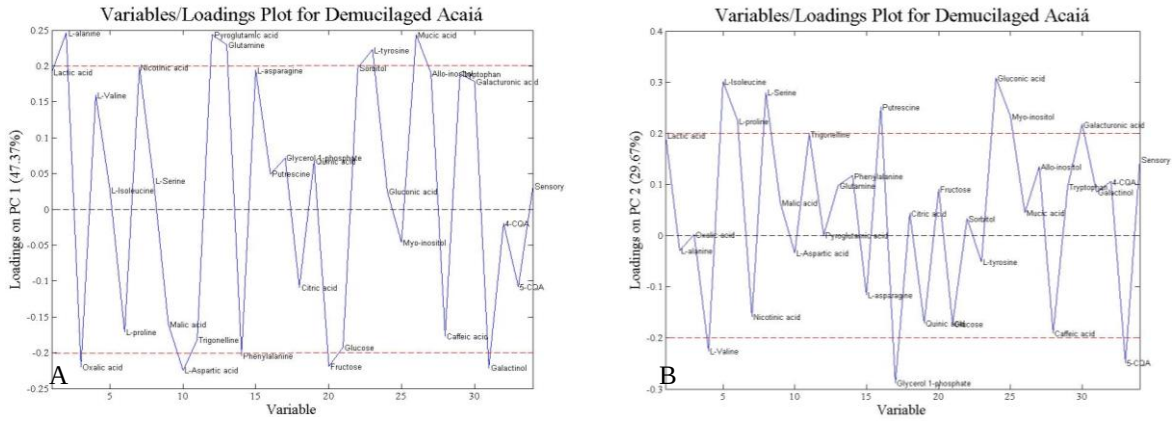

Figure 2. A) PC1 metabolite loadings of the PCA of the demucilaged Acaiá coffee seeds. B) PC 2 metabolite loadings of the PCA of the Natural Acaiá coffee seeds.

PCA demucilaged x Yellow Bourbon x altitude

PCA score scatter plot of PC1 (55.44%) and PC2 (20.04%) of the metabolite profiles of demucilaged Yellow Bourbon coffee seeds harvested in three ranges of altitude (<1000 m, 1000-1200 m, and >1200 m) (Figure 3). The samples harvested above 1200 m were different from the ones harvested below that altitude by PC1. Conversely, the samples harvested below 1000 m and between 1000-1200 m were separated in two different groups; however, they showed much lower variance in PC2 as compared to PC1.

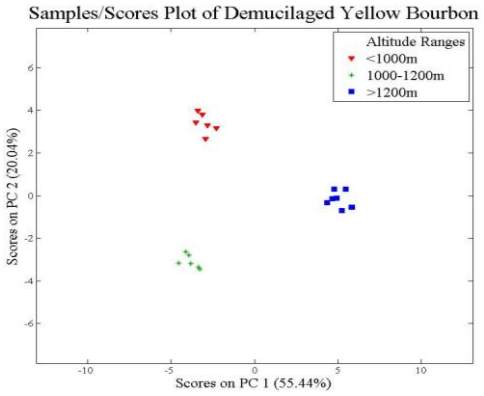

Figure 3. PCA score scatter plot of principal component 1 (40.23% of total variability) and principal component 2 (29.6% of total variability) of the metabolite profile differentiating demucilaged Yellow Bourbon coffee seeds harvested in three different ranges of altitude (▼) <1000 m, (*) 1000-1200 m, and (■) >1200 m. Each class of altitude ranges in the dataset were pretreated by WGLS, alpha = 0.001.

Figure 4A shows the metabolite loadings of PC1, differentiating the demucilaged Yellow Bourbon coffee genotype harvested in three ranges of altitude <1000 m, 1000-1200 m, and >1200 m. The features that had the greatest influence on the separation of the samples harvested above 1200 m were L-serine, L-aspartic acid, L-asparagine, fructose, glucose, and mucic acid.

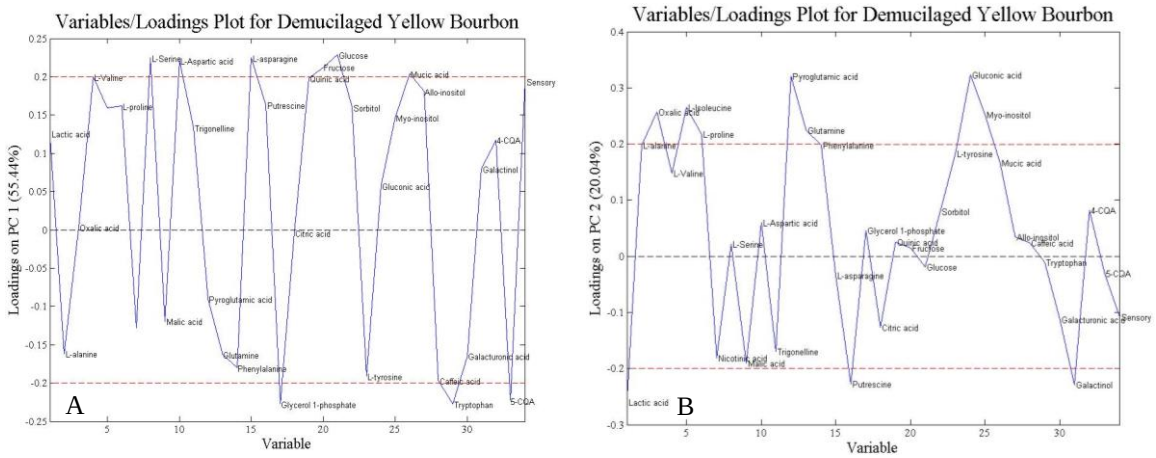

Figure 4. A) PC1 metabolite loadings of PCA of the demucilaged Yellow Bourbon coffee seeds. B) PC1 metabolite loadings of PCA of the demucilaged Yellow Bourbon coffee seeds.

The sensory score did not reach the 0.2 threshold for separation of the samples; however, it indicated that samples harvested above 1200 m showed higher scores than the other ones. This could indicate a higher correlation between the compounds found in those samples and the sensory score.

Among amino acids found in the Yellow Bourbon coffee samples, L-serine provides a bitter taste, and L-aspartic acid and L-asparagine provide an acidy taste. L-aspartic acid and L-asparagine are the main amino acids found in coffee seeds. In addition to contributing to increased acidity, the asparagine detection threshold is very low, which means that a very small amount can be detected by humans. In the same direction, L-aspartic acid would help to increase an acidic taste, but at much lower rates than asparagine, considering that it has lower threshold detection for human detection. The presence of these compounds in high rates in coffee seeds would indicate higher sensory quality. The features that had the greatest influence on the discrimination of the samples into two groups, those harvested below 1200 m (<1000 m and 1000-1200 m) and the ones harvested above 1200 m, included glycerol 1-phosphate, tryptophan, and 5-CQA. The samples harvested below 1000 m showed more compounds that may increase bitterness in coffee. The compounds 5-CQA and tryptophan increase the bitter taste. In contrast, glycerol 1-phosphate increases sweetness as a sugar alcohol.

Even though this analysis covered about 33 metabolites, we are aware that there are many more metabolites present in coffee seeds, comprising, volatile and non-volatile compounds.

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Chromatographic Profiling of Instant Coffee Carbohydrates Using Hpaec-Pad

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SUMMARY

Typical commercial instant coffee production involves the hydrolysis of complex poly-saccharides found in roasted coffee to soluble mono, di and oligo-saccharides which are found in the final product.

Instant coffee sugars profiling can be used for both process control evaluation and for the analysis of commercial products authenticity. Common high-performance-anion-exchange-chromatography with pulsed-amperometric-detection (HPAEC-PAD) analytical methods of instant coffee profiling are based on a two-step analysis; first, free sugars profile is determined, next, total sugars are analysed following a complete acidic hydrolysis of the coffee sample.

In this work we developed a chromatographic profiling method which utilizes a moderate sodium-acetate gradient elution that allows a total sugar profiling of instant coffee samples in a single step. This method was employed to evaluate the carbohydrates found in different production yields of the same coffee product, and for the detection of coffee adulteration with high M.W. oligo-saccharides.

INTRODUCTION

Green coffee consists of approximately 40% carbohydrates, of which the larger part are poly-saccharides with different degree of polymerization (DP). During coffee roasting, the composition of free sugars as well as high molecular weight poly-saccharides changes significantly. Roasted beans extraction during commercial instant coffee production is performed at elevated temperatures and pressure, resulting with the hydrolysis of complex poly-saccharides to soluble mono, di and oligo-saccharides which are found in the final product.

Instant coffee sugars profiling is essential for process control evaluation, and also for the analysis of commercial products authenticity. Common high-performance-anion-exchange-chromatography with pulsed-amperometric-detection (HPAEC-PAD) analytical methods of instant coffee profiling are based on a two-step analysis; first, free sugars profile is determined, next, total sugars are analysed following a complete acidic hydrolysis of the coffee sample (1).

In this work we present a HPAEC-PAD method for the profiling of instant coffee free sugars and oligo-saccharides (with DP of 3 and above) in a single run. This profiling can be used to detect adulteration of instant coffee with high M.W. sugars, and also to evaluate the dependence of resulting sugar profile depending on instant coffee process parameters.

MATERIALS AND METHODS

Coffee samples. Instant coffee samples were produced by Strauss-Coffee BV.

Sample preparation. Each coffee sample (0.5 gr) was re-suspended in 150 ml distilled water, filtered and sugar profile was analyzed using Dionex HPAEC equipped with pulsed amperometric detector.

HPAEC conditions. Dionex separation was performed with isocratic elution 1mM NaOH (0-40 min) followed by gradient elution with 500 mM Na-Ac (40 – 120 min, 0 to 50%)

CONCLUSION

Instant coffee (0.5 gr) was re-suspended in distilled water, filtered, and injected to Dionex HPAEC-PAD. First 40 minutes resulted with the separation of free mono and disaccharides. Following 40 minutes, the gradient elution resulted with distinctive sugar peaks representing oligo-saccharides found in instant coffee with increasing degree of polymerization (Figure 1).

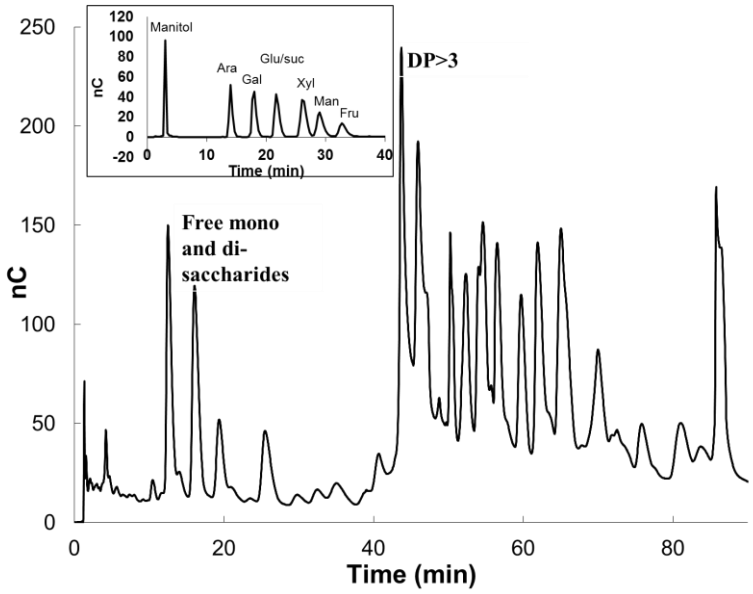

Figure 1. Typical HPAEC-PAD profile of instant coffee. Up to 35 minutes, free sugars elute, while oligosaccharides are visualized between retention time of 40-90 minutes. The inset shows a typical separation of standard sugars found in instant coffee.

This visualization of the total carbohydrate profile found in instant coffee was used to 1) compare carbohydrate profile of instant coffee produced with different yields, 2) Detect coffee adulteration with high M.W. oligosaccharides.

Compression of instant coffee sugar profile depending on production yield

The sugar profile of the same instant coffee, produced with yield of either 2.3 or 2.7 kg green coffee/instant coffee was evaluated (Figure 2).

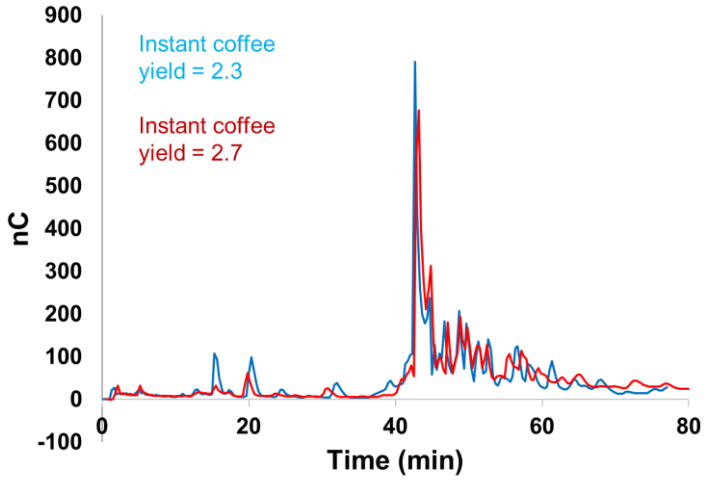

Figure 2. Sugar profile comparison between the same instant coffee produced with different yields. Blue line represents the sugar profile of instant coffee which yielded 2.3kg green coffee/instant coffee while red line represents coffee which yielded 2.7 kg green coffee/instant coffee.

As can be seen, the main differences observed are the higher levels of free sugars (mainly mono-saccharides), as well as DP=3 sugars found in coffee sample which was produced in higher yield, while the levels of sugars with DP=4 and up, are comparable between the two samples. This could be explained by the improved hydrolysis of complex sugars taking place in this type of process.

Detection of coffee adulteration in instant coffee

Five percent of either inulin or malto-dextrin were added to an instant coffee sample. A single run of these coffee samples using the displayed method revealed distinctive peaks of high M.W. sugars found only in the adulterated sample (Figure 3).

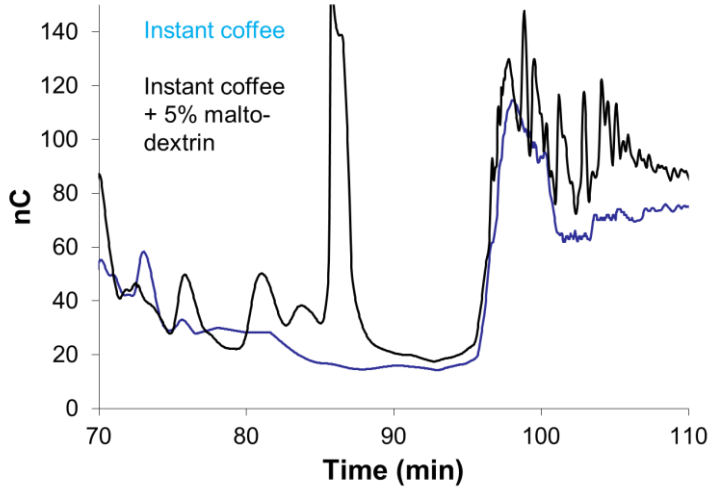

Figure 3. Sugar profile comparison of instant coffee and instant coffee adulterated with 5% malto-dextrin. Blue line represents regular instant coffee, while black line represents the same coffee which was supplemented with 5% malto-dextrin. Plot is focused on elution time of more than 70 minutes where distinctive peaks are observed only in the sugar supplemented sample.

This result indicates the potential of using such a method to detect instant coffee adulteration

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Application of Taste Sensor to Coffee Industry

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SUMMARY

TS-5000Z is a taste-sensing system with artificial lipid-based membrane sensors developed in Japan. It is a kind of electronic tongue, which is very popular tool for developing food products. In this study, it was applied to coffee industry.

The taste-sensing system was useful especially in blending coffee. A data base containing the taste data and the price of each sample was constructed to support product development. If the taste data of the target, allowance for each taste, and limiting condition for the blend were set, optimization of the price within the taste allowance could be performed with a simple PC program.

The cost for the products of soluble coffee and roasted and ground coffee could be reduced without spoiling the quality.

INTRODUCTION

S. ISHIMITSU & CO., LTD. is one of the biggest green coffee importer in Japan, which has big roasting factories in the group. Research and development office supports product developments of our customers by scientific approaches.

TS-5000Z (http://www.insent.co.jp/en/products/ts5000z_index.html) is a taste-sensing system with artificial lipid-based membrane sensors developed by Prof. K. Toko and Intelligent Sensor Technology, Inc. in Japan. It is a kind of electronic tongue, which shows high correlation with sensory evaluation and high reproducibility of measured results. The system is getting very popular tool for developing food products but coffee. We tired to apply it to coffee industry, because the system has possibility to numerize the taste of coffee without keeping a lot of well-trained cuppers. Some applications to soluble coffee and roasted and ground coffee were shown in this study.

MATERIALS AND METHODS

Roasted and ground coffee (R&G coffee)

Products from all over the world were collected for maketing. Both arabica and canephora from main coffee producing countries were roasted in several degrees of roast with some profiles as parts of blend. Each sample was ground and sieved. 15.0 g of 710 – 850 um sized ground coffee was extracted in hot water (90 ^{o}C) for 3 minutes and filtrated for the measurement. More than 5000 samples were processed so far.

Soluble coffee

About 100 kinds of soluble coffee were collected from all over the world for blending. 2 g of each sample was dissolved with 140 g of boiled water for measurement.

Taste Sensor Syste

TS-5000Z (Intelligent Sensor Technology, Inc.) was applied to quantification of the taste of coffees. There was a clear correlation between the outputs by the system and the sensory evaluations of our blender. The output of acidity and bitterness were mainly used for calculations. Zero point for each taste is that of Nescafe Gold Blend.

Blender

An exquisite blender is target for comparison, who is a "Q grader" licensed by coffee quality institute (https://www.facebook.com/rdishimitsu), and also a "J.C.Q.A. Certified Master in Coffee" licensed by Japan Coffee Qualification Authority (http://kentei.jcqa.org/).

CONCLUSION

Data base construction

At the beginning of the trial, we started to collect many kinds of soluble coffee and R&G coffee from to construct a data base. It was easy to visualize the distribution of tastes, which was useful for marketing. An example of the taste map is shown in Figure 1. It could be easily understood that the taste of the third wave coffees all over the world were almost same all over the world.

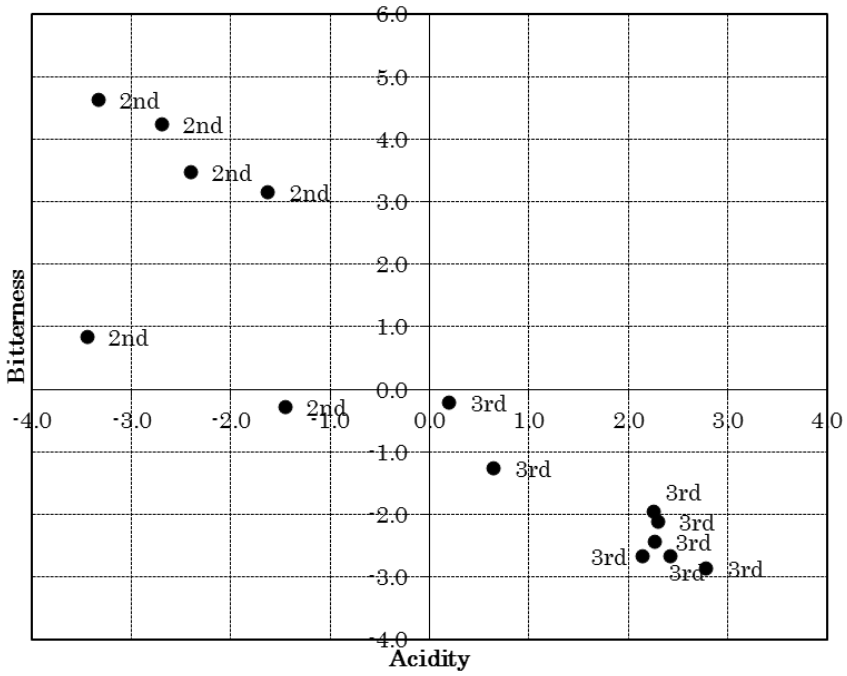

Figure 1. Example of taste map. Changes in trend of taste between the 2nd wave coffee and the 3rd wave coffee could be understood easily.

Optimization of blend

We tried to use the data base as the reference for blending in the next stage. Colombian arabica and Vietnamese canephora were blended in varying proportions for the measurements. The observed values and the predicted values are listed in Table 1. The predicted values were calculated according to the average weighted by the proportions. The differences in both values were less than 1.0, which meant within the allowable range. We tried to expand to multicomponent system, and construct a support system for blending.

The price data for each sample was added to the data base. In addition, taste data of the target, allowance for each taste, and limiting condition for the blend were also necessary for calculation. This was so called “linear programming problem”, and optimization of the price within the taste allowance could be performed with a simple PC program.

This method changed our way of blending as shown in Figure 2. The whole process of product development was used to be dependent on the skill of the blender. But now, only a slight adjustment after the calculation is the job of the blender for us.

Table 1. Differences between the observed values and the predicted values.

Colombia	Vietnam	observed		estimated		difference	
%	%	acidity	bitterness	acidity	bitterness	acidity	bitterness
100	0	-3.46	0.03	-	-	-	-
80	20	-4.61	0.27	-4.92	0.17	0.31	0.10
60	40	-5.79	0.46	-6.37	0.31	0.58	0.15
40	60	-7.14	0.56	-7.82	0.45	0.68	0.11
20	80	-8.79	0.67	-9.27	0.59	0.49	0.08
0	100	-10.73	0.74	-	-	-	-

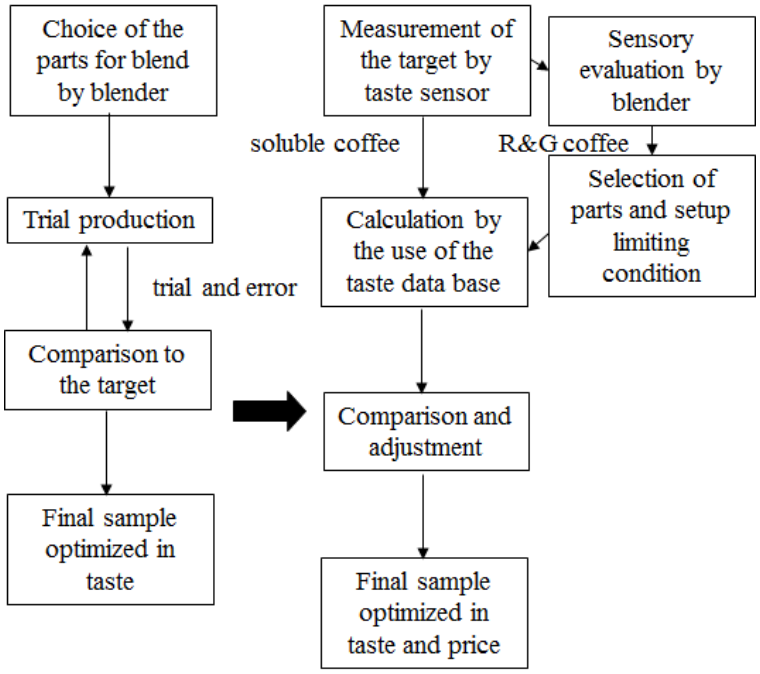

Figure 2. Changes in the procedure of coffee blending. The procedure in the left side is standard. The speed and the similarity to the target is dependent on the skill of the blender. Taste sensor can reduce dependence on the blender. Not only the taste but also the price can be optimized by the use of taste sensor.

Application to soluble coffee

Dozens of requests have been processed, and significant results were achieved in soluble coffee products with this method. It took half a day to make a sample, which was faster than the quick job by the well-trained blender. The prices of samples were roughly over 10 % cheaper than the estimated prices on average. It was not too much to say that the data base system supported by taste sensor exceeds a well-trained blender in ability to meet market needs.

Application to R & G coffee

There was a problem to apply the method to R&G coffee products, because contribution of the flavor to the sensory evaluation was much higher than the case of soluble coffee products. Blender played an important role during the processing. It was necessary to select parts and set some limiting conditions for calculation based on sensory evaluation by a well-trained blender. An example of the application is shown in Table 2. After the optimization, the cost of the product decreased and the manufacturing efficiency got better.

Table 2. Example of the effects of optimization of R&G product.

	parts 1	parts 2	parts 3	parts 4	parts 5	parts 6	parts 7	parts 8
before optimization	27%	20%	3%		13%	20%	3%	14%
after optimization	40%			40%				20%
	Acidity	Bitterness	Yen/kg					
before optimization	-	-	768.7					
after optimization	0.7	-0.2	714.0					

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Primary and Secondary Metabolites from Green Beans Collected from Vertical Canopy Layers Related with the Sensorial Attributes of Coffee Beverage

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SUMMARY

The isomers of chlorogenic acids (CQAI), and other chemical compounds in green coffee beans, contribute on formation of complex sensorial attributes of coffee beverage. The aims of this study were to estimate the chemical composition, including CQAI, in green coffee beans collected from canopy vertical layers of *Coffea arabica* L. (cv. IAPAR 59) and to associate this composition to the sensorial attributes of coffee beverage.The plants were grown in two densities (6,000 and 10,000 plants ha^{-1}), and twoplanting patterns (PP),square (Q) and rectangular (R), resulting in four combinations (Q_6, Q_{10}, R_6 and R_{10}).Two 40cm-thick layers in vertical profile of coffee canopy were considered (upper layer - $L_u$and inferior layer - L_i).The concentration of fats, proteins, sucrose, reducing sugars, phenolic compounds (PC), chlorogenic acids and caffeine in green coffee beans were performed by NIRS. The mass/volume ratio was considered as the green beans density. The following isomers: 3-*caffeolquinic acid* (3-CQA), 4-*caffeolquinic acid*(4-CQA), 5-*caffeolquinic acid*(5-CQA), 5-feruloylquinic acid (5-FQA), *3,5-dicaffeoylquinic* acid (3,5-diCQA), 4,5-*dicaffeoylquinic* (4,5-diCQA) and 3,4-*dicaffeoylquinic*acid (3,4-diCQA) were determined by ultra fast liquid chromatography. Principal component analysis (PCA) was applied to associate the coffee beans physicochemical components to the plant density, planting patternsand canopy layers. The PCA was efficient to discriminate between two PPs, showing that beans originated from rectangular ones contented chemical composition that was associated to better beverage quality. The free-choice profile technique (FCP) was employed to estimate the sensorial attributes of coffee beverage. FCPindicated that the beverage from $L_i$expressedsweetness, bitterness, browncolorandlow astringency. In PCA those samples were related to high concentrations of 3,4-diCQA, sucrose, caffeine and proteins.The PCA and FCP analysis showed the influence of berry position on plant architecture. The berries maturated in the self-shaded layer (L_i) had the chemical composition that resulted in more appropriated beverage attributes than those ones formed and maturated in sun-exposed (L_u) layer. In the second production year, the rectangular PPs originated berries with better chemical and sensory attributes.

INTRODUCTION

Numerousaromatics compounds (volatiles and non volatiles),responsible for aroma and taste of coffee beverage,are formed in reactions between chemical compounds of green coffee beans during roasting.The participation of these aromatic compounds on the sensorial quality of roasted coffee is difficult to evaluate,therefore one approach the characterizationof their precursors in the green beans.

The main precursors of aromatic compounds are lipids, proteins, sugars, caffeine and chlorogenic acids. When coffee beans are heated, proteinsreact with sugars forming various

compounds responsible for the aroma and color of the beverage.The lipids are related to the retention of aromas, while caffeine contributes to the bitter taste. The phenolic compounds, such as chlorogenic acid isomers (CQAI), are related with bitter, astringency and acidity of the beverage.

External factors such as species, cultivar, maturity stage and post-harvest processes strongly influence the concentration of chlorogenic acids in coffee beans. In the Arabica coffee species, the concentration of CQA is about 8% dry mature fruit. The major isomer is the5-CQA, which corresponds to 80% of total CQA. Other common isomers found in coffee beans are 3-*caffeolquinic acid* (3-CQA), 4-*caffeolquinic acid*(4-CQA), 5-feruloylquinic acid (5-FQA), *3,5-dicaffeoylquinic* acid (3,5-diCQA), 4,5-*dicaffeoylquinic* (4,5-diCQA) and 3,4-*dicaffeoylquinic* (3,4-diCQA). The presence of the isomers 3-4-CQA shows significant and positive correlation with temperature of coffee growth, while the 5-CQA content is not affected by this factor. The relationship between the concentration of CQA and diCQA gives a good indication o fbean maturity and coffee quality. The CQA content are related to sensory characteristics of coffee beverage, especially astringency, acidity and bitter taste. The 3,4-isomers and 3,5-diCQA are associated with lower quality of the coffee.The aims of this study were to estimate the chemical composition, including CQAI, in green coffee beans collected from canopy vertical layers of *Coffea arabica* L. (cv. IAPAR 59) and to associate this composition to the sensorial attributes of coffee beverage.

MATERIALS AND METHODS

The IAPAR 59 cultivar was planted in 1995 in the experimental field of IAPAR, Londrina - PR. The plants were grown in two plant densities (6,000 and 10,000 plants ha^{-1}), and in square (Q_6 and Q_{10}) and rectangular (R_6 and R_{10}) planting patterns (PP). Coffee plants were pruned in 2008, and the harvest of the second production year (2011) was evaluated. All treatments had three replicates (plots). Two 40cm-thick layers in vertical profile of coffee canopy were considered (upper-L_u and inferior-L_i).The collected berries were dried in sun at concrete yard until 12.5% of water content. After drying, the beans were benefited (removal of bark and parchment) and all defects have been removed.

The beans were frozen with liquid N_2 and ground to a particle size of 0.5 mm (mill Perten 3600).Concentration of fat, proteins, sucrose, phenolic compounds (PC), chlorogenic acids (CQA), and caffeine in green coffee beans were performed by NIRS technique. The beans mass/volume ratio was considered green beans density (BD). The following isomers: 3-CQA, 4-CQA, 5-CQA, 5-FQA, 3, 5-diCQA, 4, 5-diCQA and 3, 4-diCQA were determined by ultra fast liquid chromatography.

Coffee beans (100g) were roasted at 210-220 °C for 8 to 10 minutes, and weight loss ranged from 13-14%. Coffee beverage was prepared with 70g of roasted and ground coffee in 1000 ml of purified water heated to 96-98 °C. The mixture was filtered through filter paper (Melitta 102) and the beverage was tested until 30 minutes after preparation.Panelists evaluated four coffee samples in each session and beverages were presented in disposable cups (50 mL), served at between 60 and 65 °C without the sugar addition.

All sensory evaluations were carrying out in individual booths under a daily light. Among the candidates for panelists were selected those that recognized correctly over 80% of the basic tastes and flavors in a test of panelist selection. The free-choice profile technique (FCP) was employed to estimate the sensorial attributes of coffee beverages. Repertory grid method was used to generate an individual set of terms for each 12 selected panelists. The individual score cards were prepared by the descriptors indicated by each panelist, and the intensity of individual

attributes was tagged in an unstructured scale (10 cm), with extreme quantitative expressions, located on the left and right end.

The XLSTAT statistical software program was used for analyzes of sensory and physicochemical data. Generalized Procrustes analysis (GAP) was used to analyze the data obtained in the FCP. Principal component analysis (PCA) was applied to associate the coffee bean physicochemical components to plant density, planting pattern and canopy layer.

CONCLUSION

Berries collected in the L_i and $L_u$in vertical profile of coffee canopy showed variability in concentration of the principal chemical compounds (Table 1). The variability in the green beans density indicated differences in fruit development, and the sucrose and PC contentssuggest differences in the maturation levels between studied PP, plant densities and canopy layers.

Table 1. Minimum, maximum and mean values and of principal compounds of the coffee samples from different PP, plant densities and two layers on vertical profile of coffee canopy.

	Minimum	Maximum	Means
Proteins (Pro)	13.48	15.76	14.52
Caffeine (Caf)	1.40	1.65	1.54
Sucrose (Suc)	6.63	7.94	7.32
Reducingsugars (RS)	0,23	0,49	0,33
Fat	13.32	14.82	14.26
Phenoliccompounds (PC)	5.56	6.51	5.74
Chlorogenicacids (CQA)	6,88	8,18	7,51
Beans density (BD)	0.56	0.61	0.58
5-CQA (c5q)	4.84	5.64	5.22
4-CQA (c4q)	0.70	0.82	0.74
3-CQA (c3q)	0.48	0.57	0.52
5-FQA (f5q)	0.36	0.44	0.40
3,4-diCQA +3,5-diCQA(dicq3.4+3.5)	0.78	0.93	0.85
4,5-diCQA (dicq 4.5)	0.40	0.48	0.44

The PCA approach was used to discriminatethe treatments (density PP and layers) considering the chemical composition of coffee beans(Figure 1A).Berries collected fromL_u in Q_{10} and R_{10} were associated to high BD, fat, CQAand 5-CQA. Beans collected fromQ_6L_u and Q_6L_ishowed high phenolic compounds content, in this study measured as CQAI, PC, CQA.All the berries collected in rectangular PP, with exception of $R_{10}L_u$, were associated to high content of proteins, caffeine, sucrose and 3,4-diCQA +3,5-diCQA isomers.The PCA was efficient to discriminate between two PPs, showing that generally, beans originated from rectangular ones contented chemical composition that could have the positive impact on beverage quality.

The beverages originated from beans collected in $L_i$of R_{10}, R_6 and Q_6 were characterized with strong coffee body, acidity, sweetness and bitterness than beverages originated from beans collected in L_u of Q_{10}, Q_6 and R_{10} (separation under the D1 on Figure 1B). Under the D2 of the Figure 1B were separated the beverages originated on rectangular PP (with exception of $R_{10}L_i$)

from those ones collected on square PP. The rectangular PP was characterized with strong aroma of roasted coffee and astringency (under D2).

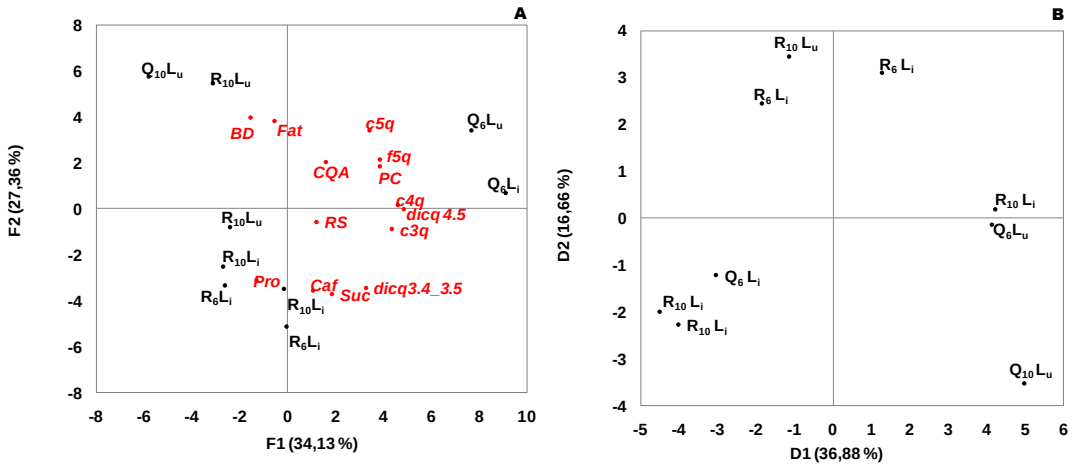

Figure 1. Impact of planting density and pattern and berry positions in plant canopy on: A) chemical composition (PCA) and B) sensorial attributes (FCP).

The PCA and FCP analysis showed the influence of berry position on plant architecture. The berries maturated in the self-shaded layer (L_i) had the chemical composition that resulted in more appropriated beverage attributes than those ones formed and maturated in sun-exposed (L_u) layer.In the second production year, the rectangular PPs originated berries with better chemical and sensory attributes, that could be explained by more competition for light developed in lines of this PPs compared to square ones (see Rakocevic *et al*., in this proceedings).

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Chemical Composition and Sensorial Quality of Coffee Collected in Three Periods from Plants Cultivated under Square and Rectangular Planting Patterns

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SUMMARY

The chemical composition of coffee beans depends on plant genetics, environmental conditions and maturation stage. The aim of this study was evaluate the principal metabolites and sensorial quality of coffee beans dependent on planting patterns and harvest periods. Visually mature berries of *Coffea arabica* L. cv. IAPAR 59 were harvested from plants cultivated in two PPs: rectangular (R) and square (Q), and two high densities: 10,000 plants ha^{-1} and 6,000 plants ha^{-1}, in three harvesting periods (June, July and August). The size of green coffee beans and the principal chemical components were determined from samples dried in sun at concrete yard. The concentration of fat, proteins, sucrose, reducing and total sugars, chlorogenic acids, phenolic compounds and caffeine in green coffee beans were performed by near infrared spectroscopy technique. Principal component analysis (PCA) was applied to associate the coffee bean physicochemical components, harvest periods, densities, and planting patterns. The free-choice profile technique (FCP) was employed to estimate de sensorial attributes of coffee beverage. Significant correlations among various chemical compounds were between various physicochemical parameters. The green coffee beans collected in June had a bigger size and concentration of proteins, chlorogenic acids and caffeine than those collected in July and August. The higher values for reducing sugars, phenolic compounds and fat were associated to the greatest number of samples collected in July and August. The harvest period and PP showed the impact on sensory quality of coffee beverage. Astringency, taste and aroma of immature beans were associated to samples collected in June, while coffee color, strong body texture, bitterness and roasted coffee characterized the collected in July and August. A comparative analysis of PCA and FCP showed that in this case study, the two last harvests had a sensorial quality and it indicates that longer berry permanence at plants could result to chemical composition that lead at a balanced combination of beverage sensorial attributes.

INTRODUCTION

The concentration of principal chemical compounds in green coffee beans depends on plant genetics, environmental conditions and maturation stage of berries. Environmental factors (temperature, light and precipitation) and cultural practices (fertilization, pruning and plant density) influenced the deposition of these compounds during plant development. Pioneering studies demonstrated the effect of shading on the chemical composition of coffee and its interference in the sensory characteristics of the beverage. Cultural practices such as different planting patterns and plant density are proposed to increase production, but also change the light microclimate in coffee canopy. Some recent studies show a direct influence of temperature and irradiation on the accumulation of CGA, lipids and sugar. The presence and concentration of the compounds found in green beans influence the production of numerous volatile and non-volatile compounds responsible for sensory attributes perceived in the

beverage. The aim of this study was evaluate the principal chemical compounds and sensorial quality of coffee beans dependent on planting patterns and periods of berry collection.

MATERIALS AND METHODS

Visually mature berries of *Coffea arabica* L. cv. IAPAR 59 were harvested from plants cultivated in two PPs: rectangular (R) and square (Q), and two high densities: 10,000 plants ha^{-1} and 6,000 plants ha^{-1}, in three harvesting periods (June, July and August) in the first production year (2010) after the pruning (2008). The identifications of 24 available samples for each harvest period from four studied treatments are shown in the Table 1. The samples were available dependant on number of berries collected in each period on plots of $60m^2$.

Table 1. The identifications of available 24 samples referent to three harvest periods and four studied treatments.

**Harvest period	*Treatments									
June	Q_{10-1}	Q_{10-1}	Q_{10-1}	R_{10-1}	R_{10-1}	R_{10-1}	Q_{6-1}	Q_{6-1}	R_{6-1}	R_{6-1}
July	Q_{10-2}	Q_{10-2}	Q_{10-2}	R_{10-2}	R_{6-2}	Q_{6-2}				
August	Q_{10-3}	Q_{10-3}	R_{10-3}	R_{10-3}	Q_{6-3}	Q_{6-3}	R_{6-3}	R_{6-3}		

**Q_{10} - square planting pattern with 10,000 plants ha^{-1}; R_{10} - rectangular planting pattern with 10,000 plants ha^{-1}; Q_6 - square planting patterns with 6,000 plants ha^{-1}; R_{10} - rectangular planting patterns with 6,000 plants ha^{-1}; ** $_{1-3}$ – harvest periods (June, July and August).*

The coffee beans were dried in the sun on concrete yard until reaching 12.5% of moisture. After drying, the beans were benefited (removal of bark and parchment) and all defects have been removed. The beans were frozen with liquid N_2 and were ground a particle size of 0.5 mm (mill Perten 3600). The size of green coffee beans (BS) were determined by the percentage of coffee beans retained on specific coffee sieve with 17/64 holes. Chemical compounds: fat, proteins (Pro), sucrose (SUC), and total (TS) and reducing sugars (RS), chlorogenic acids (CGA), phenolic compounds (PC) and caffeine (Caf) were determined in ground beans, using near infrared spectroscopy technique (NIRSystem 6500 spectrophotometer).

Coffee beans (100g) were roasted at 210-220 °C for 8 to 10 minutes. The coffee beverage was prepared with 70g of roasted and ground coffee in 1000 ml of purified water heated to 96-98 °C. The mixture (water and coffee powder) was filtered through filter paper (Melitta 102) and the beverage was tested in a maximum of 30 minutes after beverage preparation. In each analysis session, panelists evaluated four coffee samples beverage (60 and 65 °C) presented in disposable cups (50 mL) in individual booths under daily light. In the selection test for the panelist team, the candidates were selected based on over the 80% of correct answers referent to the basic taste and flavor recognition test. Repertory grid method was used to generate an individual set of terms for each of 12 selected panelists. The individual score cards were prepared by the descriptors indicated by each panelist. The intensity of individual attributes was marked in an unstructured scale (10 cm), with extreme quantitative expressions, localized on the left and right limits. The XLSTAT statistical software program was used for analyzes of sensory and physicochemical data. Kruskal-Wallis non-parametric test was performed to differentiate the physicochemical properties referent to three harvest periods, density and PP. Correlation and principal component analyses (PCA) were also applied to the physicochemical data. Generalized Procrustes analysis (GAP) was used to analyze the data obtained in the free-choice profile technique (FCP).

CONCLUSION

Coffee beans from different treatments (PP, plant density and harvest period) showed great variability in the concentration of the principal chemical compounds (Table 2).

Table 2. Mean, standard error and p-values for bean size (SB), proteins (Pro), fat, sucrose (SUC), reducing (RS) and total sugars (TS), phenolic compounds (PC), chlorogenic acids (CGA) and caffeine (Caf) in ground coffee beans of three harvests.

				Harvest					
	June	**July**	**August**	**June**	**July**	**August**	**June**	**July**	**August**
Chemical parameter		SB			Pro			Fat	
Mean (%)	70.25	61.84	53.73	14.56	14.43	13.74	14.41	15.41	15.57
St. error	3.05	4.34	3.46	0.17	0.22	0.24	0.18	0.09	0.15
p-value		**0.013**			**0.024**			**0.001**	
Chemical parameter		Suc			RS			TS	
Mean (%)	7.13	6.95	6.99	0.36	0.37	0.40	7.49	7.31	7.39
St. error	0.11	0.15	0.19	0.02	0.04	0.02	0.12	0.18	0.18
p-value		0.640			0.293			0.548	
Chemical parameter		PC			CGA			CAF	
Mean (%)	5.72	5.64	5.74	8.72	8.95	8.06	1.41	1.45	1.42
St. error	0.10	0.10	0.18	0.17	0.23	0.24	0.02	0.02	0.03
p-value		0.989			**0.040**			0.425	

$p < 0.1$ was considered significant and marked in bold.

The size of coffee beans collected in June was significantly bigger than of those collected in July and August. The August berry collection was characterized with lower concentration of proteins, and chlorogenic acids and higher of fat than harvests of June and July. Kruskal Wallis test did not differentiate the density impact on chemical composition (data not shown), while the coffee beans originated from square PP had higher PC content (5.84 ± 0.09 g 100^{-1}) than those ones from rectangular PP (5.58 ± 0.10 g 100^{-1}). The PCA separated the 3rd harvest from 1st at F1 (Figure 1A). This analysis associated the 1st harvest to big SB and high Pro, CAF, SUC, TS and CGA contents. The 3rd harvest was associated to high fat, RS and PC contents. The data suggested the significant influence of harvest period and PP to fruit formation (BS, protein and fat) and their maturation (PC and CGA).

The positive correlations ($p < 0.05$) were observed between coffee bean size and content of proteins (r= 0.68), while the negative ones with concentrations of fat (r= - 0.65) and reducing sugars (r= - 0.43). The protein content was correlated negatively with fat (r= -0.50) and positively with chlorogenic acid (r= 0.48) and caffeine (0.47) contents. TS and SUC were negatively correlated with PC (-0.49 and -0.50, respectively).

The 1st and 2nd dimension (D) of FCP explained 27.89 and 18.46 % of variance, respectively (Figure 1B). Coffee color, roasted coffee, caramel and coffee aroma, astringency, bitterness, acidity, beverage texture, immature beans taste and aroma were employed to describe the coffee beverage by the panelists. The harvest period and PP showed the impact on sensory quality of coffee beverage. The most of samples collected in June (D1+) were associated with

higher astringency, taste and aroma of immature beans than those originated from July and August harvests. The great part of samples collected in July and August, belonging to all PP and densities, resulted in beverage of intense coffee color, strong body texture, bitterness and roasted coffee tastes (D2+).

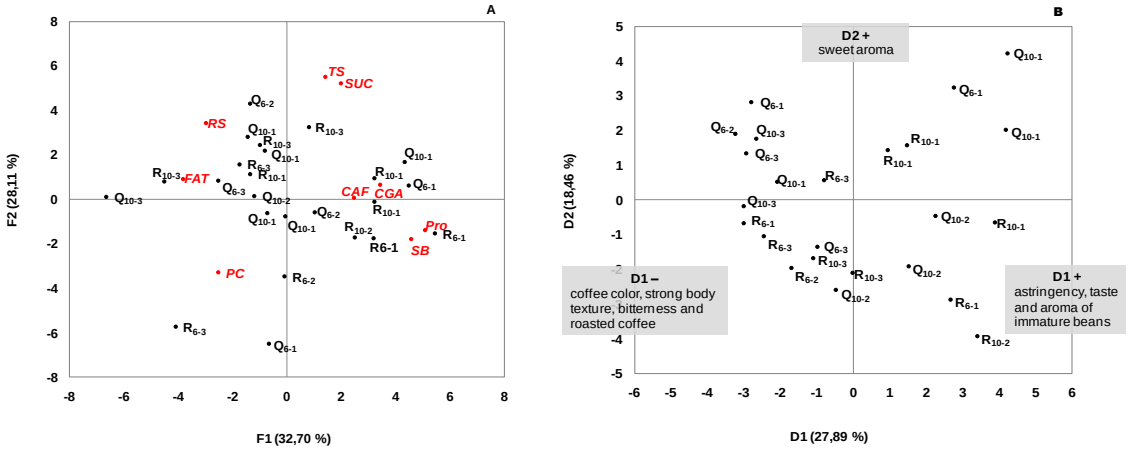

Figure 1. Impact of harvests periods on: A) chemical composition (PCA) and B) sensorial attributes (FCP).

All studied variables that defined the chemical compositions were involved in expression of sensorial attributes. A comparative analysis of PCA and FCP showed that in this case study, the two last harvests had a sensorial quality characterized by numerous positive attributes, due to high concentration of RS, PC and fat, additionally with low protein, chlorogenic acid, TS, SUC and caffeine content. Finally, this indicates that longer berry permanence at plants could result to chemical composition that lead at a balanced combination of beverage sensorial attributes.

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Effect of Processing and Roasting Conditions on the Quality and Chemical Composition of Coffee from Different Regions in Brazil

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SUMMARY

The aim of this work was evaluate alterations in the retention of bioactive components in coffee beans collected from different regions in Brazil, processed by dry and wet methods, in order to identify possible changes in grain composition affecting the quality of the beverage. The roasting of the coffee beans followed the protocol recommended by Specialty Coffee Association of America, but introducing a cooling period of the grains, between the first and second "crack" of the roasting beans. The results showed that the modification introduced in the roasting process contributed to the preservation of bioactive components, rendering an increase these compounds in the extract of the order of 15 to 20%, without apparent differences in the quality of coffee beverage observed in the sensorial scores among the coffee samples.

INTRODUCTION

The increasing consumer demand for specialty coffee has intensified researches able to pinpoint small differences in quality factors as well as to quantify these organoleptic features, leading to appropriated label specifications such as roasting degree, sensorial attributes and origins. Additionally, efforts are being made seeking to correlate the flavor of coffee with its biological properties and attributes as functional beverage. Green coffee grains contain caffeine, a mild stimulant that has been linked to lower risks of Alzheimer's disease and chlorogenic acids, bioactive components related to antioxidant and anti-inflammatory activities. When submitted to high temperature, trigonelline produces vitamin B_3, but roasting process destroys most part of these compounds. The roasting process conditions impact on the different compounds in the coffee beans. During the Maillard reaction the increase in water pressure, along with the large amount of generated gases causes the cell walls to fissure, giving rise to the "first crack". As heating continues, the roasting temperature (around 160-170^{o}C) will darken the coffee grains and a quick popping occurs ("second crack"), in the presence of carbon dioxide, when this accumulation exceeds the strength of cellulose walls of the grains. The roasting of the coffee beans followed the protocol recommended by Specialty Coffee Association of America, but introducing a cooling period of the grains, between the first and second "crack" of the roasting beans. The aim of this study was to evaluate the roasting conditions, sensorial attributes and alterations of bioactive components in coffee collected from different regions in Brazil, processed by dry and wet methods, in order to identify possible changes in the chemical composition and quality of coffee.

MATERIALS AND METHODS

Coffee fruit (Coffea arabica L.), Mundo Novo cv (2012/2013) crop, harvested at different regions in Brazil, mechanically de-pulped immediately after harvest and dried in trays with

layers of 2 cm, revolving 12 times per day, for uniform drying, were processed by the dry and wet method. The roasting methods and the sensorial scoring followed the protocol recommended by the Specialty Coffee Association of America, with the introducing of a cooling period of the grains, between the first and second "crack", in an attempt to optimize the roasting process. The bioactive compounds were determined by extraction with heated water, followed by filtration. The chemical analysis were performed by HPLC (Shimadzu) with a C18 column, isocratic elution with acetic acid and methanol as mobile phase with a flow rate of 1 mL min-1 at 30°C.

The application of PCA techniques was used to correlate the sensorial attributes of the coffee according to its geographical origin, the process and the type of roasting. To statistically process the sensorial results the multivariate analysis was used in order to convert complex data into information with reduced dimensionality to facilitate interpretation.

RESULTS AND DISCUSSION

In Figure 1, samples B, C and F, can be assumed to be similar since they are located near each other and show intense measured attributes. Furthermore, the sample “L” showed the highest values of the attributes in relation to the elements evaluated. There are clusters of data especially regarding the origin and type of processing at the right side of the diagram (samples from the state of Paraná) and at The samples “A and G” are similar and received smaller scores for these attributes, as shown in Figure 1 that they are positioned in the negative direction of the X axis, and this is contrary to the growth of vector X. There is similar variability of the left side (the state of Minas Gerais coffees). The different scores of the attributes differ on the origin of the coffees analyzed. The flavor, aroma and aftertaste variables are also correlated with the positive direction of the Y axis, corresponding to a large proportion of the samples, while the acidity and body variables are correlated with the negative direction of Y axis, presenting “K, E, I” samples .

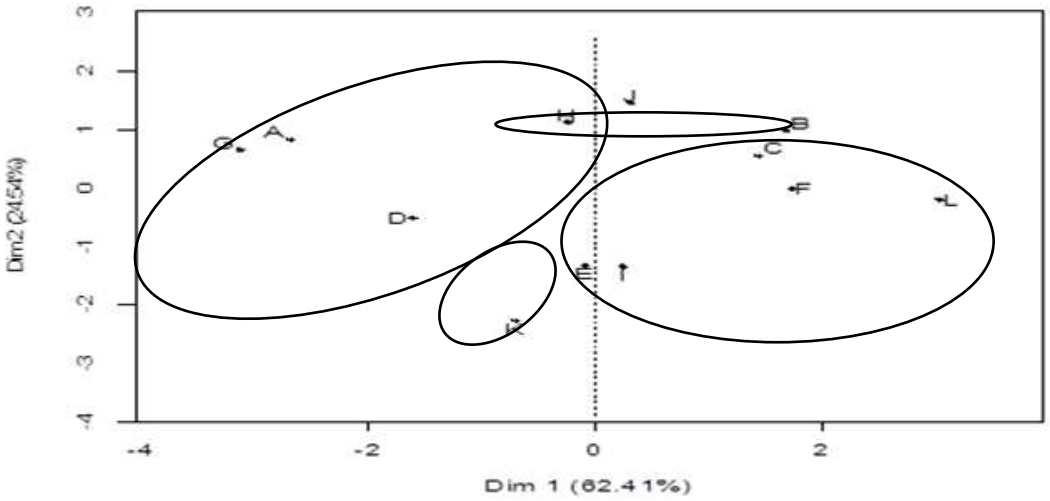

Figure 1. Multivariate analysis - Different process, origin and roasting. A - Natural Minas Gerais – Standard roasting; B - Natural São Paulo – Standard roasting; C - Natural Paraná – Standard roasting; D - De-pulped Minas Gerais – Standard roasting; E - De-pulped São Paulo – Standard roasting; F - De-pulped Paraná – Standard roasting; G - Natural Minas Gerais – Modified roasting; H - Natural São Paulo – Modified roasting; I - Natural Paraná – Modified roasting; J - De-pulped Minas Gerais

– Modified roasting; K - De-pulped São Paulo – Modified roasting; L - De-pulped Paraná – Modified roasting

All sensory variables are represented by vectors in the positive direction of the X axis, except for aroma, in the negative direction of the X axis (Figure 2). The percentage of the total data variation explained by the two first main components is 86.95%, with 62.41% explaining the first component and 24.54% explaining the second component. origin of samples in relation to spatial dispersion. Samples "k and E", located on the negative axis in Figure 1, have similar origin and same wet process, however samples "H and B", have identical origin from the state of São Paulo and dry process. Some samples, independent of type of processing were located further right of the X axis and the other downside of X axis. These samples are the ones that deserve greater emphases, since the measured sensory attributes are the most desirable in a coffee beverage. This means that natural coffee can be as good as de-pulped coffee grains.

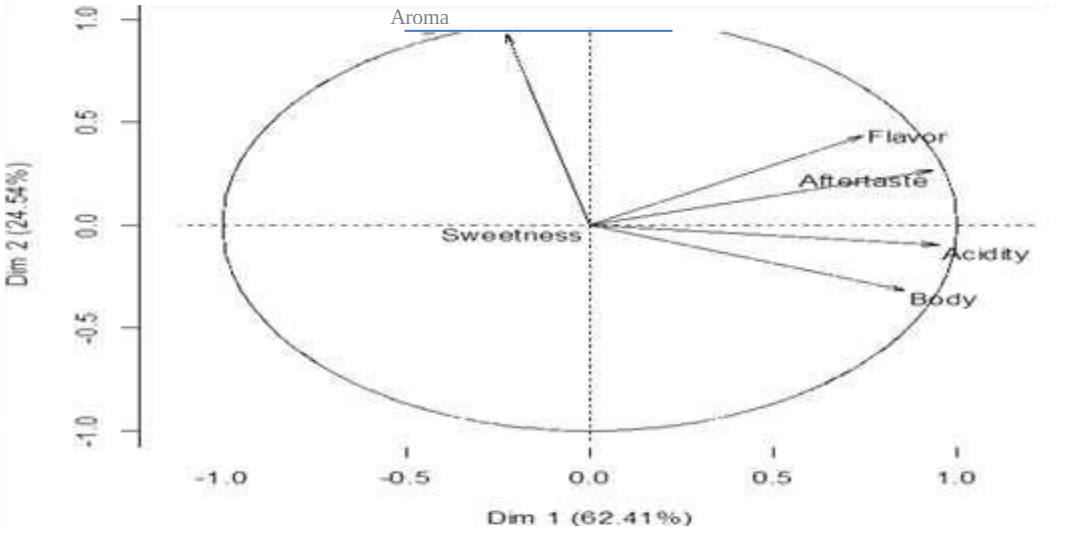

Figure 2. Coffee attributes - Main components analysis.

The results showed significant variations in the sensory scores of the coffee samples, however changes in the roasting process contributes to the preservation of bioactive compounds. The trigonelline, chlorogenic acids and caffeine are components that contribute to the typical flavor and aroma of roasted coffee beans, so that any changes that happens in these compounds during processing and roasting will result in different quantities of flavor precursors. The alteration introduced in the roasting process contributed to the retention of these compounds in the extract in the order of 15 to 20%, improving the functional properties of coffee beverage.

CONCLUSION

The origin of the grain did contribute to score sensorial differences in coffee beans; however, it is known that there are specific locations in a coffee farms with potential to produce grains of unique qualities, and it will be important for the producer to identify them. The modification introduced in the roasting process contributed to increase the preservation of bioactive compounds, promoting an increase in the health-active components, without apparent differences in the quality of coffee beverage.

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Interaction between Environment and Processing in the Enzymatic Quantification of Coffee Beans.

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SUMMARY

This study was conducted with the objective of analyzing the combined effects of genotype and altitude on the levels enzymatic quantification present in green coffee processed using the dry and wet method. This study aimed to assess the relationship of the enzyme ascorbate peroxidase (APX) , with the sensory quality of the drink coffees Samples of coffee (*Coffea arabica* L.) of the genotypes Yellow Bourbon and Acaiá were collected in different environments and evaluated over the course of three harvests. Was found that coffee grown above 1,200 m altitude and processed by the dry method (natural) had higher amounts of the enzyme APX and sensory quality of the drink with total average score around 90 points. Still, there was the distinction of different types of processing (natural and demucilaged) and environment (three ranges of altitude) based on average levels of quantification of ascorbate peroxidase (APX). From the results obtained, it was possible to establish the relation between the enzymatic quantification of the green coffee and the sensorial quality of the roasted coffee beverage as a function of altitude, genotype and processing.

INTRODUCTION

Enzymes are important in cellular metabolism. Enzymatic reactions are very important because they depend on them not only the formation of desirable compounds, such as may have undesirable consequences. In grains, the main changes related to the deterioration process is the degradation and inactivation of enzymes, reduction of respiratory activity and loss of integrity of cell membranes. Noted that to detect the onset of deterioration of the most sensitive assessments are those related to the activity associated with the biosynthesis enzymes new tissue, since the process of deterioration of grains enzymes become less efficient to perform its catalytic activity.

Biochemical events such as oxidation, degradation and inactivation of enzymes, reduced respiratory activity and loss of integrity of cell membranes, are some of the related deterioration process. Enzymes involved changes in the decay process of seed such as esterase, catalase, peroxidase, among others, have the potential to monitor and characterize the quality of the coffee beans and in some cases, help in understanding the causes of qualitative losses of coffee beans that occur due to oxidation of lipids, causing a significant change in taste and odor, namely, as a consequence of loss of quality.

MATERIAL AND METHODS

Samples of coffee (*Coffea arabica* L.) were collected during three harvests (2009/10, 2010/11 and 2011/12) from commercial farms located in the municipality of Carmo de Minas, Minas Gerais, Brazil.

The experimental design was based on the study of the interaction between environmental, genetic, and processing variables. The area of coffee cultivation was stratified into three altitude classes. For each of the environments only mature fruit was harvested and two genotypes were used: Yellow Bourbon (yellow fruits) and Acaiá (red fruits). For all of the combinations involving environment and genotype, three separate samples were collected and processed using the dry and wet method. All of the harvest, processing and drying procedures were completed following. After drying, the beans were ground using a mill, IKA A11 basic analytical, and frozen then stored in "deep-freezer" at -80 ° C until analyzes.

The biochemical analyses were performed on the green coffee using the spectrophotometer Enzyme Linked Immuno Sorbent Assay (ELISA). The sensorial analysis was completed by trained and qualified specialty coffee judges, using the methodology provided by the Specialty Coffee Association of America – SCAA.

To study the joint effects of genotype, environment and processing the quantification of the enzymatic activity of the coffee bean, the principal component analysis (PCA) associated with the technique of biplots was applied, resulting in clusters according to the quantification of the enzymes and sensory analysis of coffee drink. This type of analysis allows to rearrange the distribution of variables, in order to detect significant smaller dimensions to explain their similarities or dissimilarities. Chemoface the statistical software was used.

CONCLUSION

The average values of ascorbate peroxidase (APX) in function of the interaction altitude, processing and genotype are shown in Table 01.

Table 1. Average quantification of enzymes, ascorbate peroxidase (APX) for interaction between genotypes, altitude and processing values.

Altitude (m)	Processamento	Genótipo	APX (umol de AsA min^{-1}ug proteína $^{-1}$)
< 1.000	Natural	Acaiá	0,00015
		Bourbon Amarelo	0,00106
	Desmucilado	Acaiá	8,83E-08
		Bourbon Amarelo	2,82E-07
1.000-1.200	Natural	Acaiá	0,00024
		Bourbon Amarelo	0,00024
	Desmucilado	Acaiá	9,93E-08
		Bourbon Amarelo	1,07E-07
> 1.200	Natural	Acaiá	0,01092
		Bourbon Amarelo	0,00105
	Desmucilado	Acaiá	3,90E-06
		Bourbon Amarelo	5,89E-07

The most prominent is the type of processing, which affects the quantification of all enzymes. The dry processing (natural) features superior to the values of wet processing values for all enzymes. Results that demonstrate the wide variety of metabolites in wet processing compared to the natural. Observed that the highest activity of the enzyme catalase occurs for grain processed by the dry method. In Figure 01, it was verified that quantification of

enzymes has contributed in a significant way in the formation of clusters as a function of altitude, genotype, post-harvest processing and sensory notes. The biplot constructed representing each treatment (genotype, altitude, processing) by a point and each dependent variable (quantification of enzymes and sensory note) by a vector.

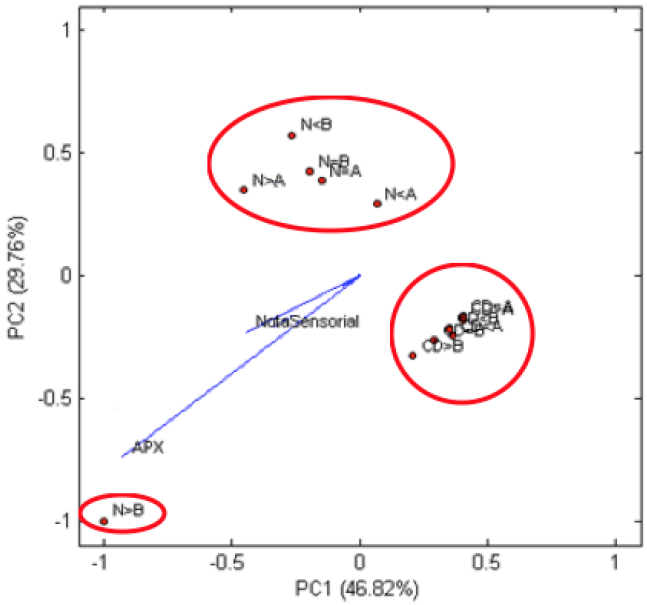

Figure 1. Biplot int he PCA for the enzyme activity peroxidase do ascorbato (APX) e sensory score. N<A (Natural, <1.000 m, Acaiá), N=A (Natural, 1.000 a 1.200 m, Acaiá), N>A (Natural, >1.200 m, Acaiá); N<B (Natural, <1.000 m, Bourbon), N=B (Natural, 1.000 a 1.200 m, Bourbon), N>B (Natural, >1.200 m, Bourbon), CD<A (Desmucilado, <1.000 m, Acaiá), CD=A (Desmucilado, 1.000 a 1.200 m, Acaiá), CD>A (Desmucilado, >1.200 m, Acaiá), CD<B (Desmucilado, <1.000 m, Bourbon), CD=B (Desmucilado, 1.000 a 1.200 m, Bourbon), CD>B (Desmucilado, >1.200 m, Bourbon).

The highest values of ascorbate peroxidase (APX), same with the highest values of sensory score, were responsible for the formation of group III, which contains genotype Yellow Bourbon, natural processing and altitude> 1,200 m. It is noteworthy that the smaller the angle between the vectors, the greater the relative between variables. Therefore, it is inferred that the level of APX enzymes have great potential to express the sensory quality of the drink.
It follows that, the quantification of the enzymes is influenced by the type of processing that the green coffee. The coffee produced by the wet method presented a tendency toward lower levels of all enzymes and the genotype Yellow Bourbon grown above 1,200 m altitude and processed by dry method presented in the raw grain, a trend to higher levels of APX sensory quality higher than the other treatments and beverage enzymes.

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Coffee Arabica from Cape Verde and East Timor. Quality Evaluation from the Grain to the Cup

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SUMMARY

Fogo Island (F) and East Timor (ET) coffee are studied in terms of physical and chemical characteristics of the grains and sensory characteristics of the brew aiming to assess the impact of soil and climatic conditions and post-harvest technology on quality. This work is a part of the first study performed on coffee of Fogo Island.

The evaluation of quality of the green coffee was made through physical analysis (weight of 100 grains, grain size, defects and moisture content). Roasting and related changes on the grains (changes in volume and weight of the grains, color, defects and moisture content of the roasted coffee and pH of the brew) was also studied. Sensory profile was evaluated attending to attributes like flavour, odor, acidity, body, astringency, bitterness, sweetness, salty and aftertaste. The chemical characterization of the green and/or roasted grains was performed by the analysis of ash, pH, titratable acidity, soluble solids, caffeine, trigonelline, total phenols and chlorogenic acids content.

The results showed that almost of the samples can be classified in the higher categories for exportation due to high mass values, coarse grains and low number of defects. Principal component analysis applied to samples show a clear difference of Fogo, comparing to East Timor's samples. Soil composition (minerals) seems to affect the characteristics of Fogo's coffee, in particular the pH, color of roasted coffee and probably the pronounced fermentation flavour of the brew. Future studies of the ash constituents of this coffee will help to understand these results.

INTRODUCTION

Coffee is a major source of foreign currency of East Timor (ET) and is the primary source of income for about one quarter of the population. The post-harvest technology is usually done by wet process that, as the benefit operations, is performed by foreign and National Companies, and farmers' associations [1].

In Fogo Island (Cape Verde), coffee is a product of tradition produced by small farmers through the dry-process. In recent years, it has received the interest by some agents of the international market, without, however, the scientific bases on its quality. This study is therefore the first to be performed on this origin of coffee, which has the particularity of being produced in considerable water deficit conditions, volcanic soils, and very dry weather.

The purpose of this study is to characterize the coffee of Fogo Island and East Timor and to assess the impact of soil and climatic conditions and post-harvest technology on the physical and chemical characteristics of the grains and sensory characteristics of the brew. This is part of preliminary studies on the quality of coffee produced currently in East Timor and Fogo [1-3], which is a subject not well known, although its importance for these countries.

MATERIALS AND METHODS

Material

Samples of parchment coffee and mocha commercial, taken in 2010 in Ermera district (ET) and Fogo's dried cherries harvested in 2011 and 2012 (F11 and F12, respectively) were collected on local production companies and farmers, as described by [1,3].

Methods

Physical analysis of green coffee

Moisture content was evaluated based on hot air oven-drying at 105 ± 1 ºC for 16 h ± 30 min, according to [4]. For determination of the weight of 100 beans, 100 grains of each sample were evaluated, in duplicate, using sensitive weight scale. The weight measured was recorded in grams. The coffee bean size and shape analysis were performed by manual sieving according to [5]. The single defects (from coffee beans and foreign bodies) was determined according to [6] and [7] on a basis of 300 g of coffee beans.

Roasting and related changes on the beans

Two test portions of 200 g were roasted in a sampler Neuhaus Neotec fluidized bed roaster at 235 ºC for 170 s (light/medium roast). For determination of bulk volume and weight of whole beans the test portions were evaluated before and after roasting and the variation was expressed in percentage. The color of ground coffee beans was measured immediately after roasting, with a Colorlest II (Neuhaus Neotec) colorimeter (scale between 56 and 199, corresponding to very dark and very light roasting, respectively). The moisture content of the ground coffee beans was determined with a Toledo Moisture Analyzer. The pH of each brewed coffee was measured with a pH meter Metrohm, in the espresso coffee extract obtained with 7 g of coffee powder and 90 mL of water, cooled to room temperature. The defects in roasted coffee beans were evaluated according to [8].

Sensory analysis of the brew

The coffee brews were prepared using 7.0 ± 0.1 g of medium ground coffee for 100 mL of boiling water, according to [9]. A trained panel with four members scored the brews, using a scale from 0 to 5 ("absent" to "very strong" perception) attending to sensory attributes like flavour, odor, acidity, body, astringency, bitterness, sweetness, salty and aftertaste.

Chemical Analysis

Total ash was determined by combusting dry samples in a muffle furnace at 525 ± 25 °C [10]. The pH and titratable acidity were determined after dispersing the sample in boiling water, according to [2, 11]. For quantification of soluble solids, 25 mL of the coffee drink was dried at 105 ºC until the mass remained constant, according to [10]. Caffeine and trigonelline contents were measured according to [12]. An HPLC system (Beckman System Gold,

equipped with a DAD, model 168 and a column Spherisorb ODS2, 250x4 mm, 5 µm, Waters) was used. Detection was performed at 254 nm. The chromatographic conditions were described in [2]. For identification and quantification, standard curves were built with concentrations ranging between 0.781 and 12.500 mg/100 mL (caffeine) and 0.469 and 7.500 mg/100 mL (trigonelline). Total phenolic content was quantified by Folin-Ciocalteu method according to [13] with slight modifications. Chlorogenic acids were analyzed as described elsewhere [2, 14]. The brew was extracted with methanol/water (40:60, v/v) and Carrez I and II solutions were added for clearing. HPLC analysis was achieved with a Beckman System Gold HPLC, equipped with a DAD, model 168, set at 325 and 330 nm, using a reversed phase column, Spherisorb ODS2, Waters (250x4 mm, 5 µm). The solvent system consisted of 5 % tripotassium citrate buffer solution 0.01 mol L^{-1}, pH 2.5 (solvent A) and methanol (solvent B) with the following gradient: 0-5 min, 20% B; 5-10 min, 20-25% B; 10-15 min, 25-30% B; 15-20 min, 30-40% B; 20-25 min, 40-45% B; 30-35 min, 50-60% B; 35-40 min, 60-70% B; 40-45 min, 70-20% B; flow rate of 1 mL min^{-1}. The identification and quantification of chromatographic peaks were carried out using standard solutions of 5-O-caffeoylquinic acid (5-CQA). To identify the isomers 3-O-caffeoylquinic acid (3-CQA) and 4-O-caffeoylquinic acid (4-CQA), 5-CQA standard was subjected to isomerization with NH_4OH (4 mol L^{-1}) to pH 8 and, thus, with HCl (4 mol L^{-1}) to pH 2.5. Trugo equation [15] was used for quantification.

Statistical analysis

Statistical analysis was carried out using S*tatistica 7.0* Copyright © StatSoft, Inc (2004).

CONCLUSION

Principal component analysis applied to samples (Fig. 1) show a clear difference of Fogo, comparing to the ET's samples. The former presents (1) higher values of ash, pH (in both espresso brew and infusion), 3-FQA, sweetness and size grains homogeneity; (2) lower number of defects, intensity of color of ground roasted grains as well as lower increase in volume and loss of weight during roasting, and lower content of soluble solids, caffeine, trigonelline, 3,5-diCQA, 5-FQA. The sensorial attributes body, odor, flavour and persistence of the brew were more intense in ET's samples.

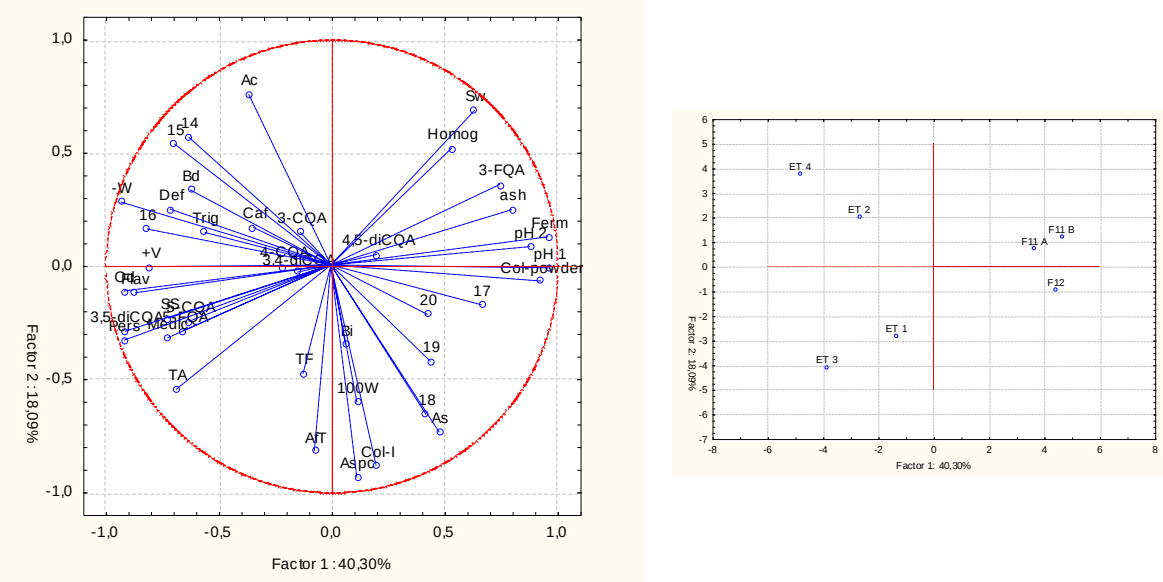

Figure 1. Projection of the variables (left) and cases (right) on the factor-plane (F1xF2). *Variables*: 14-20=grain size (sieves), Homog = grains homogeneity, 100W = weight of 100 grains, Def=number of the green grain defects/300g, -W= weight loss during the roasting, +V= swelling during the roasting, pH 1=pH espresso, Col-powder=Colour of ground roasted grains; TP =Total phenols, pH 2 =pH infusion (lab), TA =Titratable Acidity, SS =soluble solids, Caf =Caffeine, Trig =Trigonelline, 5-CQA=5-O-caffeoylquinic acid, 3-CQA=3-O-caffeoylquinic acid), 4-CQA=4-O-caffeoylquinic acid, 3-FQA=3-O-feruloylquinic acid, 5-FQA=5-O- feruloylquinic acid, 3,4-diCQA=3,4-O-dicaffeoylquinic acid, 3,5-diCQA=3,5-O-dicaffeoylquinic acid, 4,5-diCQA=4,5-O-dicaffeoylquinic acid, Col-I=Colour of the infusion; Aspc =Aspect, Od=Odor, Ac =Acid, Bi=bitterness, Ferm =Fermented, Medic =Medicinal, As =Astringency, Bd=Body, Flav=Flavour, AfT=After taste, Pers =Persistence, Sw=Sweetness; *Cases:* F= Samples of dried cerise from Fogo Island collected in 2011 (F11) and 2012 (F12), ET= Samples of parchment coffee (1-3) and mocha commercial (4) from East Timor.

The high content of ash and its correlation seems to point that the chemical composition of the soil (mineral) affects the characteristics of coffee, like the high value of the pH of the brew and the less intense color of the roasted coffee. A future study about the constituents of the ash of this coffee could help to understand these findings. Also the fermentation taste and smell of the brew need a deep research.

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The Principles of Coffee Extraction from Packed Beds in on-Demand Coffee Systems

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INTRODUCTION

The understanding and manipulation of coffee extraction is core to the whole coffee industry, yet there is limited and sometimes conflicting literature on the basic physical mechanisms of this process. This paper will focus on extraction from a packed bed of roast and ground coffee as performed by the consumer at point of use. A model of flow thru the bed coupled to grain diffusion will be shown to be in good agreement with experiment. The talk will report the time profile of concentration of soluble solids and small molecules inside the coffee bed and their development into the beverage. An extension of the model couples the bed to models of modern OD-Demand coffee systems.

MODEL OF EXTRACTION

A simple model of coffee extraction from a packed bed based on coupled diffusion and flow equations was first reported in a previous ASIC conference Melrose et al (2012). The model is extended here to calculate the pore space concentration profile in packed coffee bed under extracting flow driven by a pump. The axis of the radially symmetric bed, is z, it is modelled as a conical frustum - the pore concentration, C, is treated as uniform in the radial direction.

$$\frac{\partial C}{\partial t} + v(z)\frac{\partial C}{\partial z} + \frac{3(1-\varepsilon)}{\varepsilon R} j\left(C_R^P - C,\ D,\ z\right) = 0 \qquad (1)$$

Dispersion terms are neglected. The equation is solved by dividing the bed into N layers down the z-axis. In each layer the flux into the pore space out of the grinds is found from a pair of representative coarse and fine particles as described in the companion paper at this conference on extraction in slurry (no flow). For simplicity, eq 1. assumes just one grind size of radius R in a bed of porosity ε, j is the flux out of the grain, D the diffusion constant of the extracting species . In the two model (fine and coarse) the flux term is given by

$$J(t) = \frac{3(1-\varepsilon)}{\varepsilon}\left[\frac{j_{fine}(t)\vartheta_{fine}}{R_{fine}} + \frac{j_{coarse}(t)(1-\vartheta_{fine})}{R_{coarse}}\right] \qquad (2)$$

Where ϑ_{fine} is the volume fraction of fine particles. The velocity of the fluid, v, is found from the volumetric flow rate thru the bed, *Q(t)*, and the cross-sectional area at z. The model is a straightforward adaption of ones reported in other context, Zhiguo *et al* (1998). The equations were solved using a code written in Scilab (2012).

The flow, *Q(t)*, out of the bottom layer, *z=0*, of the bed carries the coffee extract into the beverage. The maximal extractable solids measured from a dilute slurry extraction are used to define initial concentrations in the grinds in absolute terms. The brew strength at time t is computed from

$$S(t) = \frac{\int_o^t Q(t')C(t',\, z{=}0)dt'}{\int_o^t Q(t')dt'} \qquad (3)$$

BREWER MODEL

The brewing system provides the volumetric flow rate, *Q(t)*, through the bed. A typical home 'On-Demand' brewer system, involves a pump, heater and capsule holder. The whole system including the coffee bed has a time dependent hydraulic resistance against which the pump works, this increases rapidly as the fluid front moves through the system on start-up but also because the resistance of the coffee bed evolves with time. The time dependent pressure at the pump and flow rate are found by solving the dynamically changing intersection of the pump-characteristic and the system flow-pressure gradient relation with the time varying resistance. Figure 1 illustrates this for the pump characteristic (triangles) of a typical low pressure system, as the resistance increases the system curve (thick black lines) drops towards the pressures axis, the pump pressure and flow follows the intersection of these lines.

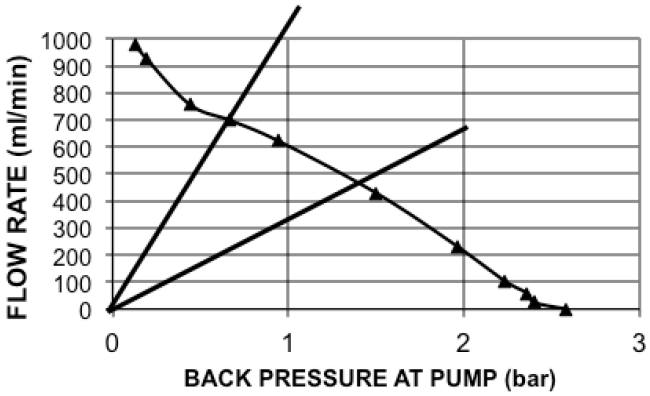

Figure 1.

The resistance of coffee beds was measured using a custom pressure rig and we report this elsewhere, Corrochano *et al* (2014). Under flow conditions of these systems, it increases up to the first 30-60 seconds. We have built models of this evolution. These are input to a brewer model to solve for the flow which is then coupled to the extraction model given above to predict the evolving brew strength.

RESULTS

Figure 2 shows a comparison of experiment and model prediction in a custom built extraction rig for at an average flow rate of 3.7 ml/sec, a bed of bulk density 480 kg/m^3 and coarse grind peak of 360 μm . A single diffusion const. 1.0X10-10 m^2 / s was found to fit the data well.

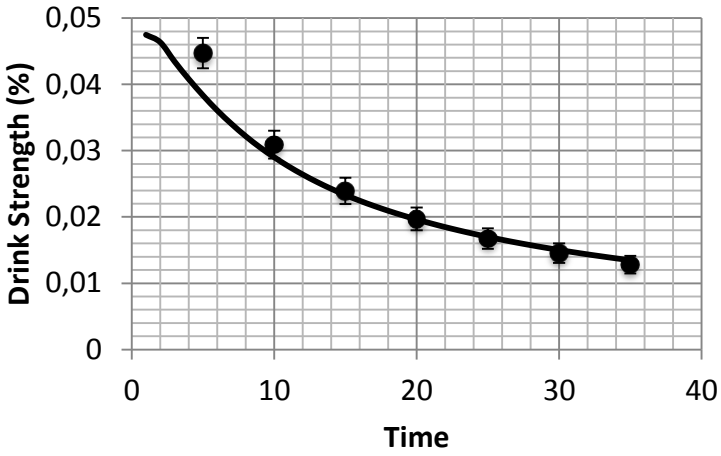

Figure 2.

Figure 2 Experimental data is shown by circles and compared with the model.

Figure 3 shows a model prediction of a pressure time curve of system with a pump that has a higher maximal pressure than that of Figure 1. The rise in pressure and times to extract will vary with the bed resistance, the pump stops when the target volume is reached - two different rates of evolution of the bed resistance are shown.

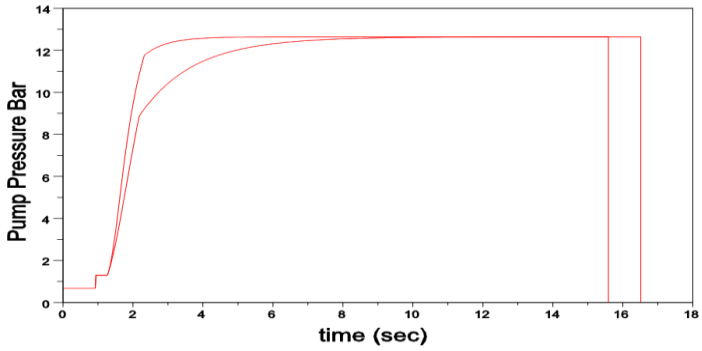

Figure 3.

Figure 4 shows the corresponding brew strength in the extract. The model can be used to understand brew variability.

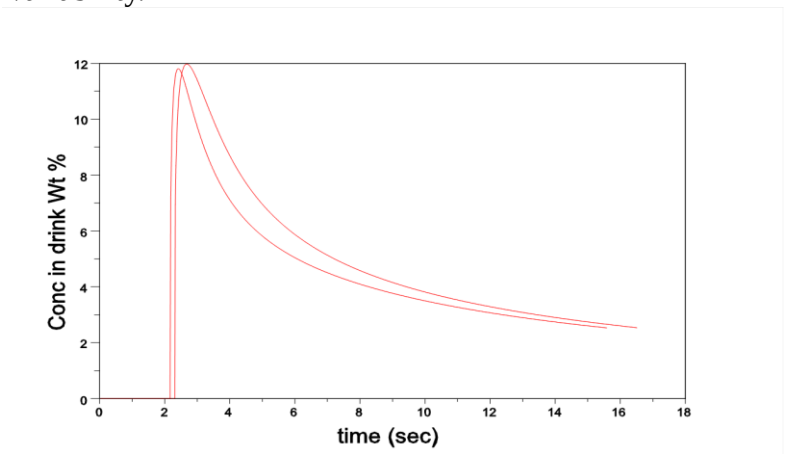

Figure 4.

CONCLUSION

A simple diffusion model coupled to a brewer model gives satisfactory results and insights into system variability and design. The role of pressure in a Brewer system is simply to set the pressure gradient needed to achieve a desired flow rate thru the packed bed (and hence brew time for a target volume) – although extraction properties are coupled to the resulting flow rate they are not *directly* affected by pump pressure.

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A New Approach of Image Analysis for the Evaluation of Espresso Coffee Quality: Study on the Presence of Solid Precipitates in the Coffee Cup Bottom

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SUMMARY

In this paper the effect of grinding grade on the amount of solid precipitates in the espresso coffee cup was studied. Particularly, an innovative method for the determination of volume of the solids precipitates was developed and validated. Moreover, conventional parameters of espresso coffee were measured with the aim to evaluate the optimal grounding grade able to minimize the precipitation of insoluble solids. The amount of insoluble solids didn't show significant differences in espresso coffee samples when produced using different grinding grade while the level of precipitates of the brew was affected by the grinding. Also, the proposed method allowed evaluating the optimal particle size distribution.

INTRODUCTION

Italian espresso is a small cup of concentrated brew prepared on request by extraction of ground roasted coffee beans, with hot water under pressure for short time. The most appreciated properties of espresso are creaminess, body and aroma. Total solid content is the most important characteristic in the chemical composition of espresso, often perceived by lay consumers "strength". Insoluble solids extracted in espresso are considered as an important class of surfactants which play an important role in the foam stabilization [1]. However, a high level of insoluble particles could represent a negative aspect for the quality of brew because their precipitation at the bottom of the cup. The solid content of the brew is mostly controlled by the coffee/water ratio but it is also affected by several variables such as roasting degree and water temperature [2]. Among these, the grinding level is an essential factor for obtaining correct percolation phenomena hence, a good quality of espresso coffee, the so-called 'fineness'. The main effect of the grinding is the increase of the extraction surface (i.e. the specific surface between water and coffee) to improve the transfer of soluble and emulsifiable substances into the brew [3]. Illy and Viani [4] stated as the optimal control of grinding is essential to produce a flavourful coffee brew. During the preparation of the espresso coffee two aims should be satisfied: a short percolation time and a high solids concentration. These requirements can only be attained if a close contact between solid particles and water are achieved. Thus, espresso percolation needs a plurimodal particle size distribution where the finer particles enhance the extraction surface (chemical need) while the coarser particles allow the flow of water (physical need). Few papers were focused on the influence of grinding on the amount of insoluble solids measured after the extraction [5,6] while no scientific data concerning the influence of grinding grade on insoluble solids precipitates on the bottom of espresso coffee. This paper had two main aims: the development and the validation of a method to measure the amount of solid precipitates of espresso coffee; 2. to study the influence of grinding on solid precipitates of espresso coffee.

MATERIALS AND METHODS

Roasted coffee beans (Masini blend) was supplied by ESSSE caffè S.p.A. (Anzola, dell'Emilia, Bologna, Italy). Coffee beans were ground by an automatic flat cutters (model GM 200 Grindonmix Retsch Haan, Germany) at 10 rpm for different time of grinding 8, 10, 12, 14 and 16 seconds obtaining a coarse, medium, medium-fine, fine and very fine powder coffee. A series of sieves stacked (600, 400, 250 μm) (Retsch, Hann, Germania) were used to fractionate 100 g of each ground coffee sample in different particle size (Ø >600 μm; 600 μm < Ø >400 μm; 400 μm < Ø >250 μm; Ø <250 μm). Coffee particles of each sieve were weighed and expressed as percentage.

Espresso coffee preparation

Espresso coffees (ECs) were prepared by using an experimental prototype espresso coffeemaker (ViBiEmme). In all cases, 7 g of coffee ground were used to prepare a 25 mL brew. All experiments were performed using demineralised water at a temperature of 98°C and a relative pressure of 1 bar. Ten ECs for each ground coffee samples were prepared performing the analysis in triplicate.

Analyses

The quality of espresso coffee was evaluated by measuring the following parameters: *Total solids (mg/mL),* determined by drying at 105 °C the brew until constant weight. *No-soluble solids* (mg/mL) and *the total solids of filtrate (mg/mL),* determined according to Severini et al. [7] .*Volume of precipitate (mL),* determined by image analysis through two steps (figure 1).

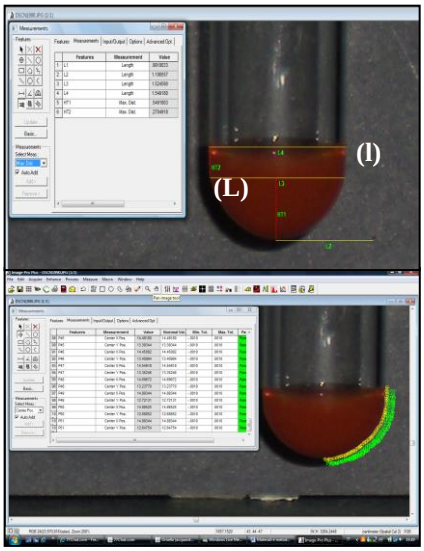

Figure 1. Calibration of the glass tube to determine the volume of precipitate.

Step 1 – Calibration. Different volumes of a reference liquid (water and red pigment) were introduced in a glass tube of 20 mL and digital images were acquired by a camera (Nikon Coolpix 8.0). From the images the following geometric parameters were measured: a) the curvature of the base of the glass tube; b) the distance between the base of the glass tube and the position at which it assume a cylindrical shape **(L**); c) the distance between **L** and the meniscus of the liquid intr**o**duced inside the test tube (**l**). All these measures were performed by the software Image ProPlus ver. 4.5. Then, a series of data representing the curvature of

the glass tube (figure 1) as a function of *z* axis were obtained and fitted with a fourth-degree polynomial, in order to obtain a function *r = f (z)* able to estimates the changes of radius (*r*) as a function of position along the *z* axis: $\boldsymbol{f(z) = r = a+b*z+c*z^2+d*z^3+e*z^4}$ **(Eq.1).** Once the function was obtained, the volume of the liquid on the base of the glass tube (up to the height, L) may be obtained by:

$$V = \int_a^b \pi f(z)^2 dz \qquad \textbf{(Eq.2)}$$

Where **a** = 0, **b** = L, *f(z)* is the equation 1.

Furthermore, the second section of glass tube since has a cylindrical shape, the volume of liquid until the height **l** may be calculated by: $V_{cil} = \pi * r^2 * l$ **(Eq. 3)**. The total volume of the liquid into the glass tube (V_{tot}) was obtained by $\mathbf{V_{tot} = V + V_{cil}}$. All the procedures above described were carried out for 5 different tubes identified as G1 (8/10), G2 (10/10), G3 (12/10), G4 (14/10) and G5 (16/10). A specific program was developed in Matlab ver. 7.0 to perform the estimation. *Step 2 – Validation.* The validation of the method was performed by comparing the estimated values with the experimental ones and studying the results with the values of determination coefficients (r^2), RMSE and the plot of residuals.

Statistical Analysis

All experimental results were analysed by ANOVA with a Tukey post hoc tests (SPSS) by using the software STATISTICA 10.0 (StatSoft, Tulsa, USA).

RESULTS AND DISCUSSION

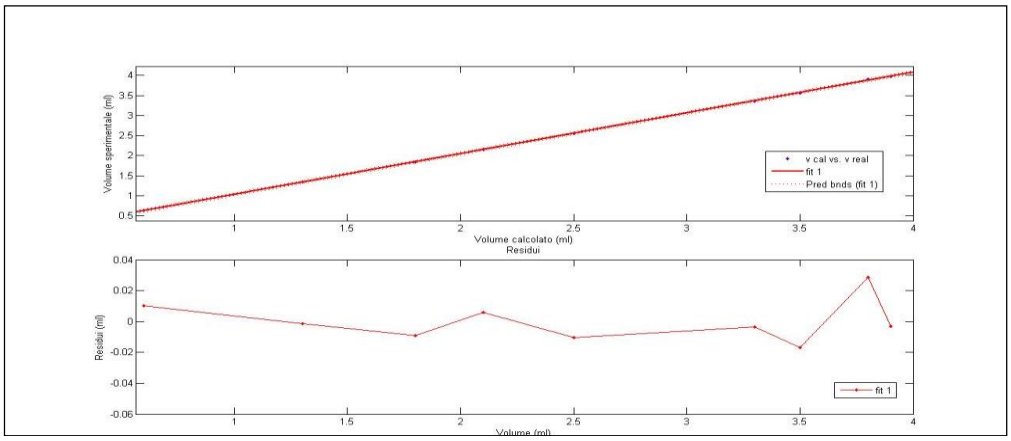

Figure 2. Experimental and estimated volumes of reference liquid. Data obtained with the glass tube G2.

Figure 2 shows the comparison between experimental ad estimated volumes of the reference liquid. Results showed a correlation coefficient greater than 0.98 while the plot of residuals showed errors always lower than 0.02 mL. Grinding grade distribution of ground roasted coffee samples is shown in Figure 3. Similar patterns were observed for all grinding grade for particles size ranged between 600-250 µm (i.e. medium and medium-fine). However, weight fractions of 30% for coarse particles (bigger than 600 µm) and for very fine particles (smaller than 250 µm) were showed for G1 samples (8 s x 10 rpm). Bimodal or plurimodal particles size distributions are shown in figure 3 for all grinding grade of coffee powder with the exception of G1 and G2 samples.

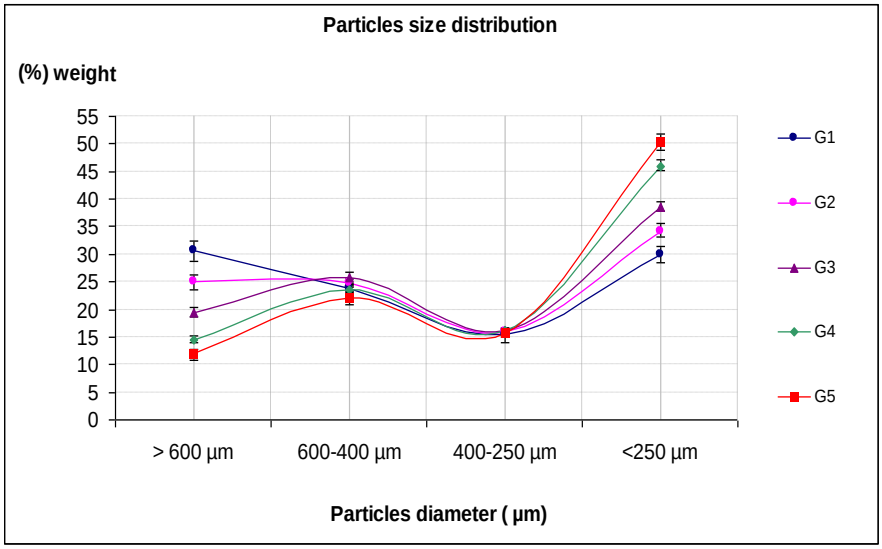

Figure 3. Particles size distribution of ground roasted coffee samples.

EC can be brewed from all of the samples within the percolation time, probably because the peaks were wide enough; this suggests the presence of both coarse and fine particles. A plurimodal particle size distribution is needed, with coarse particles fixing a structure that allows the correct flow through the cake and retains finer particles which facilitate the extraction of large amounts of emulsifiable soluble substances (Illy and Viani 1995).

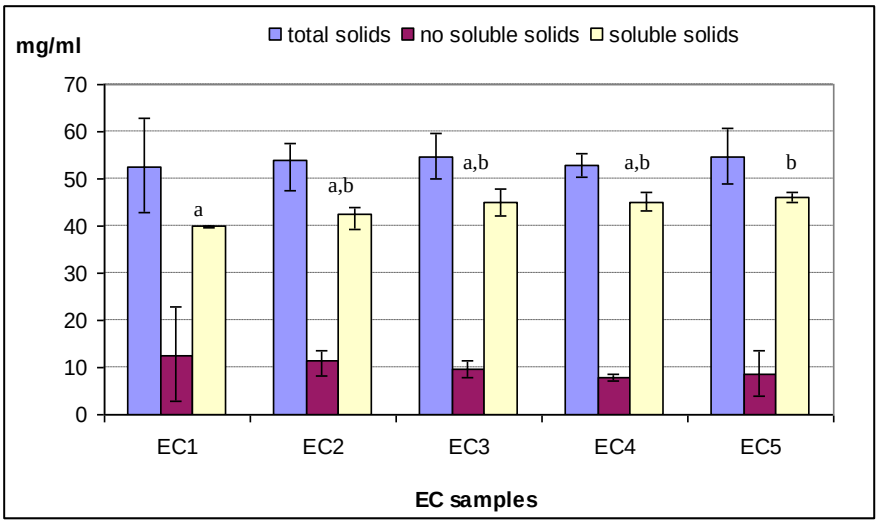

Figure 4. Total, insoluble and soluble solids in espresso coffee brews as a function of the used grinding grade.

As reported in different research on the quality of espresso coffee extraction (total solids/g coffee dose) and concentration (total solids/ coffee volume) yields inversely increased with particle size. Percentages of extraction ranging from 18 to 22% have been proposed as the most acceptable, the coffees below 16% considered to be underdeveloped and those above 24% to be over extracted. The results in terms of total solids did not show significant differences with the increasing of grinding grade (figure 4). In fact, from the ANOVA test, it was shown as the values of extraction and concentration of the brews obtained from the five ground samples (G1, G2, G3, G4, G5) were not significant different (table 1).

Table 1. Extraction and concentration values of EC samples.

EC samples	Extraction (%)	Concentration (%)
EC 1	18.79±3.61 [a]	5.26±1.01 [a]
EC 2	19.26±2.03 [a]	5.39±0.57 [a]
EC 3	19.52±1.72 [a]	5.47±0.48 [a]
EC 4	18.90±0.89 [a]	5.29±0.25 [a]
EC 5	19.48±2.08 [a]	5.45±0.58 [a]

In figure 5 are reported the volumes of precipitate determined by the proposed method. This approach, allowed to highlight statistical differences in the solids precipitated into 25 mL of espresso coffee when different grinding grade were used for the preparation of brews. Particularly, by using the samples G3 (medium fine) the minimum values of precipitate was observed in the brew. The increase of particles fineness did not show significant differences in terms of volume of the solid precipitates.

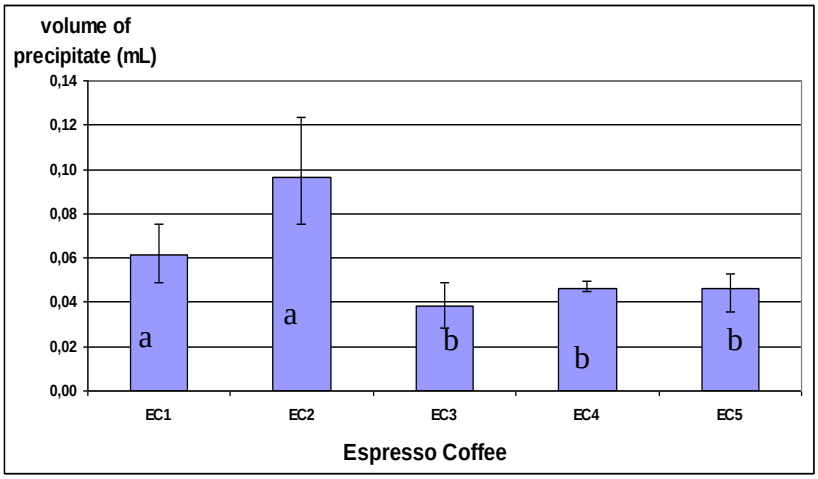

Figure 5. Volume of solid precipitates in ECs samples.

The results did not show a significant reduction of insoluble solids (mg/mL) when ground coffee powder decrease in the 'comminution' grade with a large amount of smaller particle sizes. The volume of solid precipitate significantly decreased as a function of ground coffee fineness. A medium ground coffee showed the better distribution of particle sizes, to obtain the minimum solid precipitate. With the increasing of the ground coffee fineness, the reduction of the precipitate volumes were not significant, on the contrary the percolation time increased. The new approach for determining the amount of insoluble solids precipitates at the bottom of coffee cup resulted effective and presented important advantages in terms of reduction of cost and time of analysis.

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Fortification of Ground Roast Coffees with Iron, Zinc and Calcium: Minerals Recovery in the Beverage

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SUMMARY

The present study evaluated the recovery of iron, zinc and calcium salts from fortified arabica and robusta coffees prepared by different methods. The following salts were used to fortify coffee: iron bis glycine chelate, ferrous sulfate, zinc lactate, zinc bis glycine chelate, tricalcium phosphate and calcium lactate. In control and fortified coffees, beverages prepared by espresso machine (EM) showed higher levels of all the minerals. The mineral salts with better recoveries for arabica and robusta coffees, respectively, in EM were iron bis glycine chelate (86.0% and 89.8%), zinc lactate (98.2% and 93.8%), and calcium lactate (87.1% and 87.0 %). Recoveries for electric coffee maker (ECM) with paper filter were 59.5% and 59.6 % of iron bis glycine chelate, 62.6% and 57.0% of zinc lactate, and 64.9% and 63.0% of calcium lactate, respectively. Average decreases of 0.05%, 1.30% and 0.60% were observed for iron bis glycine chelate, zinc lactate, and calcium lactate, in brews filtered by nylon filter, comparing to paper filter, but only zinc lactate decrease was significant. Despite the higher losses of minerals in ECM using paper and nylon filters, compared to EM, recoveries indicate that coffee may be a promising vehicle of fortification to help fighting deficiencies of iron, zinc and calcium in Brazilian population.

INTRODUCTION

Micronutrient deficiency affects about 2 billion individuals worldwide. Iron deficiency is the most prevalent, reaching about 1.6 billion people. In addition to iron deficiency, data in the literature indicates low intake of foods rich in calcium and zinc by the Brazilian population at different ages.

Nutritional deficiencies are considered major challenges for health policies in Brazil, not only for low income groups of population but for all groups who practice a modern life style. One of the ways to increase minerals intake which has been widely used by the food industry is food fortification, consisting of addition of nutrients to processed foods. The foods chosen for fortification should be habitually consumed by the population and accessible to them.

Coffee is one of the most consumed food products in the world, by all income classes. In Brazil, it is consumed daily by about 78% of the population above 10 years old. Additionally, coffee is rich in bioactive compounds and, therefore, can be considered as a promising vehicle for fortification with iron, zinc and calcium. However, while in the case of soluble coffee the mineral salts are dissolved in the beverage itself, in the case of ground roasted coffee, they have to be extracted and therefore the extraction method must be considered. The aim of the

present study was to evaluate the recovery of salts of iron, zinc and calcium in arabica and robusta coffee beverages prepared by different methods.

MATERIALS AND METHODS

Ground medium roasted samples of arabica and robusta coffees were roasted to medium roast and separately fortified with .4.2mg of iron, 2.1mg of zinc, and 300mg of calcium per 100g coffee powder, amounts equivalent to about 30% of the Brazilian Recommended Daily Intake- RDI, for adults [7]. After preliminary tests, the following salts known to present good bioavailability in humans were selected for recovery and sensory comparison: iron bis glycine chelate, ferrous sulfate, zinc lactate, zinc bis glycine chelate, tricalcium phosphate and calcium lactate. Geometric dilution was applied for homogeneization. Coffees were brewed by electric coffee maker (ECM) equipped with paper or nylon filter and by espresso machine (EM), which are currently among the most used methods for coffee preparation in Brazil. Beverages were prepared from control and fortified ground coffees at 10% (10g coffee for 100mL of ultrapure water). For minerals determination, beverages were digested with nitric acid and analyzed by optical spectrometry with inductively coupled plasma emission (ICP - OES).

RESULTS AND DISCUSSION

The mean concentrations of iron, zinc and calcium in ground roasted control and fortified coffees are shown in Table 1.

Table 1. **Levels of iron, zinc and calcium in ground roasted control and fortified coffees.**

	Minerals (mg/100g)					
CONTROL SAMPLES	**Iron**		**Zinc**		**Calcium**	
Arabica	$5.60^{a} \pm 0.01$		$0.83^{a} \pm 0.02$		$132.82^{b} \pm 0.03$	
Robusta	$5.75^{b} \pm 0.02$		$0.82^{a} \pm 0.03$		$130.66^{a} \pm 0.02$	
FORTIFIED SAMPLES	**Ferrous sulfate**	**Iron bisglycine chelate**	**Zinc bisglycine chelate**	**Zinc lactate**	**Tricalcium phosphate**	**Calcium lactate**
Arabica	$9.82^{c} \pm 0.03$	$9.83^{c} \pm 0.03$	$2.95^{b} \pm 0.02$	$2.94^{b} \pm 0.01$	$432.51^{c} \pm 0.05$	$433.27^{c} \pm 0.05$
Robusta	$9.96^{d} \pm 0.02$	$9.97^{d} \pm 0.01$	$2.92^{c} \pm 0.09$	$2.93^{c} \pm 0.03$	$431.82^{d} \pm 0.05$	$432.15^{d} \pm 0.04$

**Results are expressed as mean of triplicate ± standard deviation. Coefficient of variation < 5% for all samples. Different superscript letters for the same mineral or mineral salt indicate that values differ statistically by Fisher's test ($p < 0.05$).*

The concentrations of iron, zinc and calcium in the control and fortified coffee beverages prepared by different methods are presented in Figure 1.

In all extraction methods the salts iron bisglycine chelate, zinc lactate and calcium lactate presented better recoveries and EM was the extraction method with better performance for all minerals. Recoveries for arabica and robusta coffees prepared by EM were, respectively, 86.0% and 89.8% for iron bis glycine chelate, 98.2% and 93.8% for zinc lactate; and 87.1% and 87.0% for calcium lactate. In our previous study, Costa et al. reported recovery values of

87.6% and 90.4% for iron bis glycine chelate. Reports on coffee fortification with zinc lactate and calcium lactate were not found in the literature.

Recoveries for arabica and robusta beverages prepared using ECM with paper filter were 59.5% and 59.6% for iron bis glycine chelate, 57.0% and 62.6% for zinc lactate, and 64.9% and 63.0% for calcium lactate, respectively. Using nylon filter, recoveries were 59.0% and 60.0% for iron bis glycine chelate, 54.6% and 56.9% for zinc lactate and 63.6% and 65.5% for calcium lactate, respectively. Average absolute decreases of 0.05%, 1.30% and 0.60% were observed for iron bis glycine chelate, zinc lactate, calcium lactate, respectively, in brews filtered in nylon filter, comparing to paper filter. However, only the difference in zinc lactate values was statistically significant ($p = 0.03$). No significant difference was observed in minerals recovery when arabica and robusta samples were prepared using the different methods.

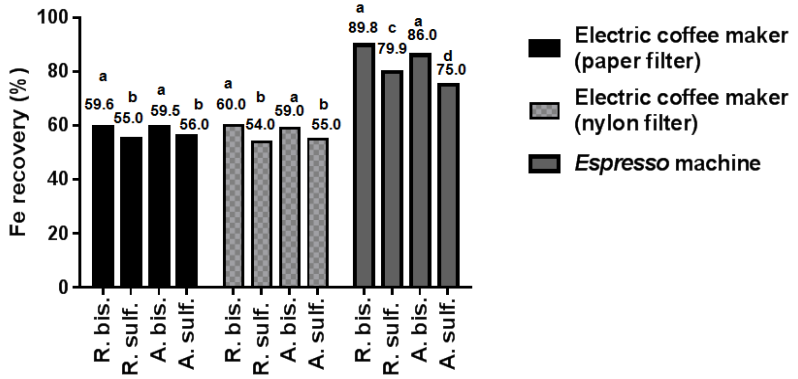

Figure 1a. Recovery of iron (Fe) in beverages prepared from fortified arabica and robusta coffees, using three methods of preparation. Different superscript letters for the same method indicate statistical difference by Fisher's test ($p < 0.05$). Note: R. bis = robusta iron bis glycine chelate; R. sulf = robusta ferrous sulfate; A. bis = arabica iron bis glycine chelate; A. sulf = arabica ferrous sulfate.

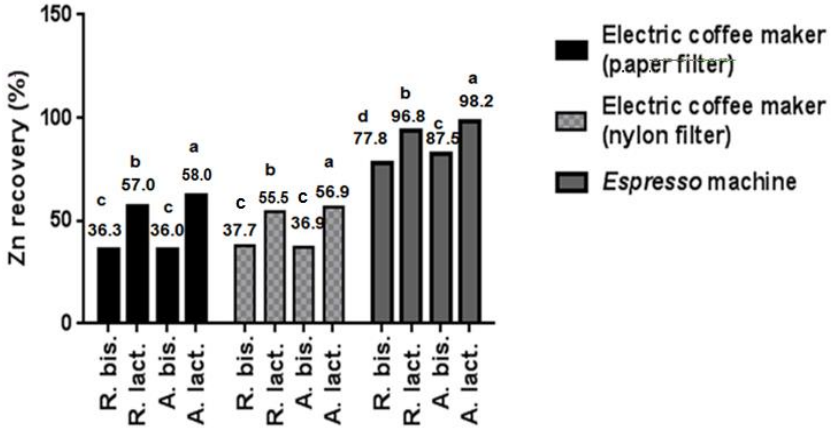

Figure 1b. Recovery of zinc (Zn) in beverages prepared from fortified arabica and robusta coffees, using three methods of preparation. Different superscript letters for the same method indicate statistical difference by Fisher's test ($p < 0.05$). Note: R. bis = robusta zinc bis glycine chelate; R. lact = robusta zinc lactate; A. bis = arabica zinc bis glycine chelate; A. lact = arabica zinc lactate.

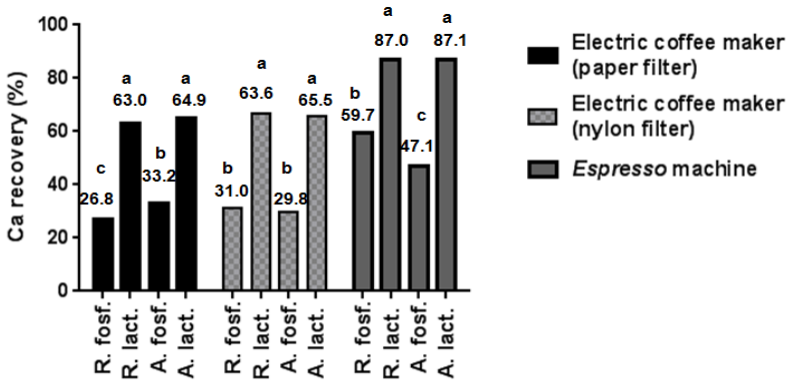

Figure 1c. Recovery of calcium (Ca) in beverages prepared from fortified arabica and robusta coffees using three methods of preparation. Different superscript letters for the same method indicate statistical difference by Fisher's test (p <0.05). Note: R. fosf = robusta tricalcium phosphate; R. lact = robusta calcium lactate; A. fosf = arabica tricalcium phosphate; A. lact = arabica calcium lactate.

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Antibacterial Effect of Aqueous Extract and Bioactive Chemical Compounds of *Coffea canephora* against Microorganisms Involved in Dental Caries and Periodontal Disease

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SUMMARY

Among several plant species presenting antibacterial properties, coffee is the most popular in terms of consumption. This study aimed at evaluating the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *Coffea canephora* aqueous extract (Cc), trigonelline (Tg) and 5-caffeoylquinic acid (5-CQA) against cariogenic microorganisms: *Streptococcus parasanguinis* ATCC 903 (SP), *Lactobacillus rhamnosus* ATCC 9595 (LR); and pathogens related to periodontal disease: *Porphyromonas gingivalis* ATCC 33277 (PG), *Fusobacterium nucleatum* ATCC 25586 (FN), *Prevotella intermedia* ATCC 49046 (PI) and *Prevotella nigrescens* ATCC 33563 (PN). Different concentrations of Cc (0.15625 to 10mg/mL), Tg and 5-CQA (0.005 to 10.24mg/mL) were tested. Chlorhexidine (0.05%) was used as positive control and the substances without the inoculum comprised the blank control. Cc showed bacteriostatic action against SP (MIC = 5 mg/mL) and LR (MIC = 10 mg/mL). Tg showed bacteriostatic action against SP (MIC = 2.56 to 1.28mg/mL), LR (MIC = 2.56mg/mL), PG (MIC = 2.56 to 1.28mg/mL), FN (MIC = 5.12 mg/mL), PN (MIC = 2.56mg/mL), and PI (MIC = 2.56 to 1.28mg/mL). Also, Tg showed bactericide properties against SP (MBC = 2.56mg/mL), PG (MBC = 2.56mg/mL), FN (MBC = 10.24mg/mL), PN (MBC = 5.12 mg/mL), and PI (MBC = 2.56mg/mL). Although 5-CQA has previously shown activity against *Streptococcus. mutans*, in the present study, it showed no activity against all tested microorganisms. *C. canephora* extract only showed antibacterial activity against cariogenic micro-organisms, not presenting action against periodontal pathogens. It was concluded that trigonelline presented the best effect against all pathogens tested, therefore coffee extracts with higher trigonelline content should be tested against these specific pathogens.

INTRODUCTION

Oral health is essential to general health and quality of life [1]. Despite great achievements in oral health of world populations, problems still remain in many communities all over the world, since dental caries and periodontal diseases have been considered the most important global oral health burdens. These two most prevalent oral diseases are dental biofilm dependable [2]. Mechanical removal of the biofilm, performed by appropriate use of

toothbrush and dental floss, has been the main tool in oral hygiene care. However, despite recognition of the effectiveness of mechanical biofilm control, the process may be potentialized by chemical control, especially in individuals at high risk to develop oral diseases [3]. The foremost chemical agents currently available are chlorhexidine, triclosan, cetylpyridinium chloride, and natural products.

Among several plant species presenting antibacterial properties, coffee is the most popular in terms of consumption. In a recent study [4], it was reported that *C. canephora* extracts exerted better performance in relation to inhibition of a *Streptococcus mutans* biofilm, compared to *C. arabica*. Additionally, it was reported that trigonelline and chlorogenic acids, which are bioactive coffee chemical compounds, largely found in *C. canephora*, showed antibacterial activity against this cariogenic bacterium [4]. However, studies clarifying the role of coffee extract and isolated compounds on the growth of cariogenic microorganisms different from *S. mutans* are still needed. Furthermore, the effect of coffee species and its chemical compounds against oral pathogens related to periodontal disease has not yet been investigated. Therefore, we evaluated the antibacterial effect of a *C. canephora* aqueous extract against planktonic forms of some cariogenic bacteria and bacteria involved in periodontal disease.

MATERIALS AND METHODS

Coffea canephora extract

Regular *Coffea canephora* cv. Conillon beans from Espirito Santo, Brazil, were roasted to produce a light roasting degree coffee according to Antonio et al. [4].

Coffee chemical compounds and characterization of trigonelline and chlorogenic acid compounds from *Coffea canephora* extract: chlorogenic acid (5-caffeoylquinic acid or 5-CQA) and trigonelline (Sigma-Aldrich, St. Louis, MO, USA) solutions at 5mg/mL were prepared according to Antonio et al. [4]. The contents of chlorogenic acids were determined by gradient LC-DAD-ESI-MS according to Farah et al. [5] whereas the contents of trigonelline (Tg) were determined by LC-ESI-MS according to Perrone et al. [6].

Bacterial Strains and Culture

An inoculum with 4-5 x 10^6 CFU/mL of the cariogenic bacteria: *S. parasanginis* ATCC 903 and *Lactobacillus rhamnosus* ATCC 9595; and bacteria involved in periodontal disease: *Porphyromonas gingivalis* ATCC 33277, *Fusobacterium nucleatum* ATCC 25586, *Prevotella nigrescens* ATCC 33563 and *Prevotella intermedia* ATCC 49046 were prepared according to Antonio et al. [4].

Minimum Inhibitory Concentration (MIC) and Minimum Bactericide Concentration (MBC) determination.

MIC was performed in 96-well microplates, inoculated with 4-5 × 10^5CFU/mL (final concentration), using Mueller-Hinton (Difco, Franklin Lakes, NJ, USA), when cariogenic bacteria were tested; and Bruscella PRAS medium in the case of periodontal pathogens. The concentrations of Cc and chemical compounds ranged from 0.15625 to 10mg/mL and 0.005 to 10.24mg/mL, respectively. Chlorhexidine digluconate (0.05%) was the positive control and the substances without the inoculum comprised the blank control. An inoculated media without the test compounds was the negative control. The plates were incubated at 37°C with 5% CO_2 for 48 h (cariogenic bacteria), and those with periodontal pathogens were incubated in anaerobic condition (37^0C) for 48 h.

Statistical analysis

SSPS software, version 20.0 was used for statistical analysis. The Kruskal-Wallis test was used for statistical comparison of susceptibility assays results, among the tested substances. A 95% significance level was considered.

RESULTS AND DISCUSSION

The results obtained on the inhibitory activity of the different concentrations of *C. canephora* extract, 5-CQA and trigonelline against the tested strains, can be observed in Table 1. As expected, the positive control (0.05% chlorhexidine digluconate) showed bactericide activity against all oral pathogens tested.

Table 1. Antibacterial (MIC) and bactericidal (MBC) activities of *C. canephora aqueous* extract, 5-CQA and trigonelline.

Bacterial strain (ATCC)	*C. canephora* extract		5-CQA		Trigonelline	
	MIC	MBC	MIC	MBC	MIC	MBC
	mg/mL					
S. parasanguinis	5.0	-	*ND*	-	2.56 to 1.28	2.56
L. rhamnosus	10.0	-	*ND*	-	2.56	-
P. gengivalis	*ND*	-	*ND*	-	2.56 to 1.28	2.56
P. intermedia	*ND*	-	*ND*	-	2.56 to 1.28	2.56
P. nigrences	*ND*	-	*ND*	-	2.56	5.12
F. nucleatum	*ND*	-	*ND*	-	5.12	10.24

In this study, 5-CQA was not able to inhibit the growth of all pathogens tested here, even at higher concentrations. Although G+ species appeared to be more susceptible to the action of phenolic acids than G- bacteria in previous works [7], our results are partially in accordance with these information, since not only G- bacteria (*P. gingivalis*, *F. nucleatum*, *P. intermedia* and *P. nigrescens*) presented resistance to 5-CQA, but also the G+ species tested (*S. parasanguinis* and *L. rhamnosus*). Gury et al.[7] affirmed that phenolic acids are toxic for numerous G+ bacteria under acidic condition. They stated that phenolic acid decarboxylase activity (PAD) in these bacteria is a detoxifying system specifically and strongly induced by these chemicals. Two genes involved in the phenolic acid stress response have been characterized in *Bacillus subtilis* (a G+ bacterium): padA and padR**.** The padA gene encodes the PadA enzyme and padR encodes the PadR transcriptional repressor. Deletion of padA leads to growth inhibition in the presence of phenolic acids, while deletion of padR leads to constitutive over expression of padA and, consequently, to high resistance to phenolic acids. So, we hypothesize that the G+ bacteria tested here could present some similar genetic characteristics to the mentioned *B. subtilis*, resulting in an over expression of some gene responsible for its resistance to 5-CQA. However, this matter should be investigated in future studies.

Despite the negative inhibitory effect of 5-CQA, we observed that the *C. canephora* extract, which is rich in phenolic compounds (2.47±0.734mg/mL; Table 2), showed bacteriostatic activity against *S. parasanguinis* and *L. rhamnosus* (MIC = 5 mg/mL and 10 mg/mL, respectively). Like 5-CQA, other CQA and diCQA isomers are also formed from caffeic and quinic acids, and it is very possible that these compounds in *C. canephora* extract, jointly exert antibacterial activity, contributing to a possible synergistic antibacterial action. In addition, trigonelline contents, and of its derivatives, in *C. canephora* extract may also have

contributed to its positive antibacterial properties against the tested cariogenic bacteria, since this substance showed a strong antibacterial activity for all pathogens.

Trigonelline was bactericidal for all tested bacteria, except for *L. rhamnosus*. Selected vitamins such as d-biotin, pyridoxine, p-aminobenzoic acid, nicotinic acid, thiamine, pantothenic acid and riboflavin enhance the lactic acid production by *L. rhamnosus* [8]. Thus, the present authors hypothesize that these vitamins could consequently improve the survival of these microorganisms. Following this reasoning and considering that trigonelline is a *N*-metyl-betaine, we suggest that trigonelline itself could favored the *L. rhamnosus* resistance to this substance.

Coffee consumption may not be effective on periodontal diseases, since the evaluated extract showed only inhibitory properties against tested cariogenic bacteria and not the bacteria involved in periodontal diseases. However, other important microorganisms also implicated in such illness such as *Actinobacillus actinomycetemcomitan*s, *Tannerella forsythensis* and *Treponema denticola*, which were not investigated in the present study, could be non-resistant to coffee extract. On the other hand, trigonelline from coffee exerts inhibitory activity against the growth of *S. P. gingivalis*, *P. intermedia*, *P. nigrescens*, *F. nucleatum*, which are periodontopathogens. Thus, arabica coffee extract richier in trigonelline and derivatives should be tested in future studies.

Table 2. Contents of cinnamic acid derivatives (chlorogenic acids) and trigonelline in *Coffea canephora aqueous* extract at 20%.[a, b]

Chemical compounds	Mean ± SD
Cinnamic acid derivatives	2.47 ± 0.734
3-CQA	0.53 ± 0.035
4-CQA	0.60 ± 0.036
5-CQA	1.00 ± 0.042
4 + 5-FQA	0.24 ± 0.024
3,4-diCQA	0.03 ± 0.002
3,5-diCQA	0.02 ± 0.001
4,5-diCQA	0.05 ± 0.003
Trigonelline	0.69 ± 0.014

[a] *Results are shown as mean of triplicate analysis, expressed in mg/mL ± Standard deviation.*
[b] *CQA = cafeoylquinic acids, FQA = feruloylquinic acids, diCQA = dicaffeoylquinic acids. SD = Standard deviation.*

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Effects of Coffee High in Chlorogenic acids with Reduced Oxidized Components on Blood Pressure, Body Fat, and Energy Metabolism in Humans.

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SUMMARY

Coffee contains various polyphenols, mainly chlorogenic acids (CGAs), which play a crucial role in the vascular endothelium and in fat utilization. We previously reported health functions of a coffee containing high levels of CGAs as active ingredients, as well as reduced oxidized component hydroxyhydroquinone (HHQ) by filtration. Animal and human studies revealed that CGAs reduced blood pressure (BP) and body fat, and consumption of the high-CGAs coffee helps prevent hypertension and obesity.

These effects of the high-CGAs coffee differ from those of conventional brewed coffee. The purpose of this presentation is to review the health beneficial effects of high-CGAs coffee based on the following studies.

Study A: The hypotensive effect of the high-CGAs coffee was examined in 100 hypertensive men and women. The subjects were randomly assigned to either the CGAs group (n=49) or the placebo group (n=51), and consumed a can of test beverage daily for 12 weeks. Systolic BP was significantly lower in the CGAs group than in the placebo group. Study B: The impact of HHQ on the hypotensive effect was examined in 98 hypertensive men and women. The subjects consumed a coffee product containing CGAs with either low HHQ (n=51) or high HHQ (n=47) daily over 12 weeks. BP was significantly lower in the low-HHQ group than in the high-HHQ group. Study C: The anti-obesity effect of the high-CGAs coffee was examined in 109 obese men and women. Visceral fat area, body weight, and waist circumference were significantly lower in the high-CGAs group (n=53) than in the placebo group (n=56). Our findings confirmed that the high-CGAs coffee has healthy benefits.

INTRODUCTION

Epidemiologic studies indicate that high blood pressure is associated with a greater prevalence of stroke and resulting mortality [1]. Jee et al., in a meta-analysis of 11 clinical trials, reported that coffee consumption is associated with a slight increase in blood pressure [2]. On the other hand, green coffee bean extract reportedly has antihypertensive effects in animal studies [3]. Thus, no definite conclusion has been reached regarding the association between coffee intake and blood pressure.

We recently demonstrated that hydroxyhydroquinone (HHQ), which is produced by roasting green coffee beans, inhibited the antihypertensive effects of chlorogenic acids in an animal study [4-5]. In the present study, we investigated the effect of HHQ-reduced coffee on blood pressure and body weight through three clinical trials in humans.

METHODS

We entrusted three clinical trials to a contract research organization (CRO). Under proper management by the CRO, three double-blind, randomized controlled trials were conducted under careful medical supervision by the principal investigator in accordance with the principles of the Declaration of Helsinki.

Blood pressure (BP) clinical trial

Study A

The purpose of this study was to evaluate the antihypertensive effect of HHQ-reduced coffee containing CGAs in high-normotensive and mildly hypertensive adult men and women. Commonly consumed commercially available coffee products contain CGAs and HHQ. Two test beverages, active (CGAs 299 mg and HHQ 0.05 mg/185 g, n=49) and placebo (CGAs 0 mg and HHQ 0.02 mg/184 mg, n=51), were used in this study. The active beverage was prepared by reducing the HHQ content of a commonly consumed commercially available coffee product using the adsorption method. The Placebo beverage was prepared by reducing both chlorogenic acids and HHQ. Before ingesting the test beverage, subjects were randomly assigned into one of two groups. During the trial period, the subjects were given the following instructions: drink one can of active or placebo beverage daily for 12 weeks; continue usual dietary habits.

Study B

The purpose of this study was to evaluate the effect of coffee with or without HHQ on blood pressure in high-normotensive and mildly hypertensive adult men and women. Two test beverages, active (CGAs 299 mg and HHQ 0.05 mg/185 g, n=51) and control (CGAs 299mg and HHQ 1.69 mg/185 g, n=47), were used in this study. The control beverage was a common coffee product containing HHQ and chlorogenic acids. The protocol and method were same as for Study A except the control beverage was used instead of the placebo beverage.

Body fat clinical trial

Study C: The purpose of this study was to evaluate the anti-obesity effect of HHQ-reduced coffee containing CGAs in 109 obese men and women. Two test beverages active (CGAs 297 mg/185g, n=53) and placebo (CGAs 2 mg/185g, n=56) were used in this study. The active beverage was prepared by reducing the HHQ content of a commonly consumed commercially available coffee product using the adsorption method. The placebo beverage was prepared by reducing both CGAs and HHQ. Body fat, weight, body mass index and waist circumference were measured. The protocol and method were basically same as for Study A except subjects and the test beverage.

RESULTS AND DISCUSSION

Study A

Throughout the 12-week intervention period, SBP was significantly lower in the Active group than in the Placebo group (Group effect, p=0.044, Table 1). A considerable non-significant difference in DBP was also observed between the groups (Group effect, p=0.059).

Table 1. Changes in blood pressure in high-normotensive and mildly hypertensive subjects.

		Active	Placebo	Estimated difference (95%CI)
SBP (mmHg)	Baseline	140.4 ± 1.3	141.1 ± 1.2	
	4wk	135.5 ± 1.5	137.8 ± 1.6	-1.9, (-5.5: 1.7)
	8wk	135.6 ± 1.5	138.0 ± 1.8	-1.9, (-5.9: 2.1)
	10wk	135.9 ± 1.6	140.7 ± 1.4	-4.4, (-8.3: -0.6)
	12wk	135.6 ± 1.5	139.6 ± 1.5	-3.6, (-7.6: 0.3)
Group	*p*	*0.044*		
DBP (mmHg)	Baseline	86.7 ± 0.8	87.2 ± 0.8	
	4wk	84.9 ± 1.0	85.9 ± 0.9	-0.8, (-3.1: 1.5)
	8wk	85.2 ± 1.0	86.2 ± 0.9	-0.8, (-3.2: 1.7)
	10wk	83.4 ± 1.0	86.5 ± 1.0	-2.9, (-5.7: -0.2)
	12wk	84.0 ± 1.0	86.2 ± 1.0	-1.9, (-4.6: 0.7)
Group	*p*	*0.059*		

mean±SEM. n; Active = 49, Placebo = 51. SBP; Systolic blood pressure DBP; Diastolic blood pressure

Study B

Throughout the 12-week intervention period, the active group had a significantly lower SBP than the control group (Group effect, p=0.031, Table 2). The DBP did not differ significantly between the groups (Group effect, p=0.092).

In these studies, we observed that SBP in the active group significantly decreased compared with that in the placebo and control groups during the 12 weeks of ingestion of HHQ-reduced coffee. These findings suggest that chlorogenic acids in coffee have antihypertensive effects and HHQ in coffee inhibits the beneficial effects of chlorogenic acids. Therefore, reducing the HHQ content in coffee can improve blood pressure.

Table 2. Changes in blood pressure in high-normotensive and mildly hypertensive subjects.

		Active	Control	Estimated difference (95%CI)
SBP (mmHg)	Baseline	139.8 ± 1.2	140.6 ± 1.0	
	4wk	135.2 ± 1.4	140.0 ± 1.4	-4.2, (-7.2: -1.3)
	8wk	136.0 ± 1.4	138.4 ± 1.5	-1.8, (-5.2: 1.6)
	10wk	137.5 ± 1.5	140.8 ± 1.3	-2.6, (-6.2: 0.9)
	12wk	139.0 ± 1.5	143.0 ± 1.6	-3.4, (-7.3: 0.5)
Group	*p*	*0.031*		
DBP (mmHg)	Baseline	88.2 ± 0.8	88.2 ± 0.6	
	4wk	85.3 ± 0.9	86.7 ± 0.9	-1.3, (-3.4: 0.7)
	8wk	86.3 ± 0.8	87.6 ± 1.0	-1.2, (-3.3: 1.0)
	10wk	87.0 ± 1.0	88.9 ± 1.0	-1.8, (-4.3: 0.7)
	12wk	88.4 ± 0.9	89.8 ± 1.0	-1.4, (-3.8: 1.1)
Group	*p*	*0.092*		

Mean±SEM. n; Active = 51, Control = 47.

Study C

Throughout the 12-week intervention period, visceral fat area, subcutaneous fat area and total fat area was significantly lower in the active group than in the placebo group (Table 3). Changes from 0wk values in body weight, waist circumference and BMI were greater in the active group than in the placebo group (Data not shown).

Table 3. Changes in abdominal fat areas in obesity.

		Active	Placebo
Visceral fat area (cm^2)	0wk	115.9 ± 3.6	110.6 ± 3.7
	12wk	111.5 ± 3.7	114.2 ± 4.0
	Δ12wk	-4.4 ± 2.1*	3.6 ± 3
Subcutaneous fat area (cm^2)	0wk	231.7 ± 9.8	241.6 ± 10.8
	12wk	226.7 ± 10.0	245.2 ± 10.9
	Δ12wk	-4.9 ± 3.0*	3.6 ± 2.
Total fat area (cm^2)	0wk	347.6 ± 10.8	352.2 ± 12.1
	12wk	338.3 ± 11.3	359.4 ± 12.6
	Δ12wk	-9.3 ± 4.6*	7.2 ± 4.8

Mean±SD. n; Active = 53, Control = 56.
*There was a significant difference between the two groups (*p<0.05).*

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Investigation of *in Vitro* Inhibition of Cytochromes P4501A (CYP1A1 and 1A2) Activities by Phenolic Compounds from Coffee

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SUMMARY

The present work investigated the inhibitory effects of coffee phenolic compounds and metabolites, in free and esterified forms (5-caffeoylquinic, caffeic, ferulic, isoferulic, dihydrocaffeic and *p*-coumaric acids), on the activity of CYP1A1 and CYP1A2 in rat liver microsomes. Additionally, daidzein, hesperetin, naringin, quercetin and resveratrol, representing the major classes of phenolic compounds in foods, were tested for comparison purposes. The effects of the tested substances on the activity of ethoxyresorufin-*O*-deethylase (EROD) and methoxyresorufin-*O*-demethylase (MROD), used as markers of CYP1A1 and CYP1A2, respectively, were determined in hepatic microsomes from untreated (only food and water *ad libitum*) and treated (with β-naphthoflavone) Wistar rats. Results showed that coffee phenolic compounds did not inhibit CYP1A1/2, a finding consistent with the interpretation that these substances are not substrates for CYP1A subfamily enzymes. All other phenolic compounds tested, with the exception of naringin, showed clear inhibition on CYP1A1/2. The inhibitory effect of quercetin on both CYPs was particularly potent (IC_{50} = 2.73 and 6.90 μM), comparable to that produced by α-naphthoflavone (IC_{50} = 2.24 and 2.37 μM), a recognized inhibitor of the CYP1A1/2 enzymes.

INTRODUCTION

Dietary phenolic compounds have been reported to have a protective role regarding development of chronic pathologies such as cancer and cardiovascular diseases, a beneficial effect possibly explained by their antioxidant and anti-inflammatory activities. Chlorogenic acids, which are formed by the esterification of hydroxycinnamic acids with quinic acid, are the most abundant phenolic compounds in the chemical composition of coffee, one of the most appreciated drinks in the world. Despite the recent progress in the knowledge of CGA metabolism, it is unclear whether CGA are substrates for liver cytochrome P450 enzymes, catalysts of xenobiotic phase I biotransformation reactions. In the case they are substrates, it can be expected that they would competitively inhibit the oxidation of other substrates catalyzed by the same cytochrome P450 enzymes, and thereby could also alter the kinetics of xenobiotics, enhancing or attenuating (in the case of pro-drugs) the therapeutic and toxic effects of various drugs. Recently, a number of interactions between bioactive compounds in foods/dietary supplements and drugs have been described and so interest on testing their inhibitory effects has grown.

The present work investigated the inhibitory effects of coffee phenolic compounds and metabolites, in free and esterified forms (5-caffeoylquinic, caffeic, ferulic, isoferulic, dihydrocaffeic and *p*-coumaric acids), on the activity of CYP1A1 and CYP1A2 in rat liver microsomes. Additionally, daidzein, hesperetin, naringin, quercetin and resveratrol, representing the major classes of phenolic compounds in foods, were tested for comparison purposes.

MATERIALS AND METHODS

The following hydroxycinnamic acids and derivatives from their digestion/ metabolism were evaluated: 5-caffeoylquinic, ferulic, isoferulic, dihydrocaffeic, *p*-coumaric acids and gallic and vanillic acids, besides the *trans*-cinnamic acid (Sigma-Aldrich Chem Co). Other compounds representing the main classes of phenolic compounds were: daidzein, hesperetin, naringin, quercetin and resveratrol (Sigma-Aldrich Chem Co). α-naphthoflavone (Sigma-Aldrich Chem Co.) was used as a positive control and the solvent dimethylsulfoxide was obtained from Merck.

Female Wistar rats provided by the Oswaldo Cruz Foundation breeding stock were separated into two groups: treated and untreated control. All animals received a pelleted diet for laboratory rats (Nuvital®, Nuvilab, Curitiba, PR, Brazil) and tap water *ad libitum*, and were maintained in controlled temperature (23 ± 2°C) and humidity (approximately 70%) and dark/light cycle of twelve hours.

After acclimatization, treatment with β-naphthoflavone (Sigma-Aldrich Chem Co.) suspended in corn oil was initiated. Two doses of 80 mg/kg were administered daily to generate a marked induction of CYP1A monooxygenases.

All animals were killed by cervical dislocation on the fourth day, after starvation for 16 hours after the last dose of β-naphthoflavone. Immediately after euthanasia, livers were removed and immersed in a cool 0.25 M sucrose solution. The subsequent steps for preparation of liver microsomal fractions were performed as previously described in De Oliveira *et al*. Microsomal protein concentration was determined by a colorimetric method described by Bradford.

Ethoxyresorufin-*O*-deethylase (EROD) and methoxyresorufin-*O*-demethylase (MROD) activities were determined essentially as described by Burke *et al*., except for the use of NADPH regenerating system proposed by De Oliveira *et al*..

Reactions were initiated by the addition of the NADPH regenerating system and occurred at 37°C in a water bath (Heto®) with stirring for 10 minutes (stopped by adding acetonitrile). The *O*-dealkylation reactions were measured by the fluorescence generated by the accumulation of resorufin; the readings were performed in microplate spectrofluorometer (Devices® Molecular Spectra Max Gemini XS) with excitation at 530 nm and emission at 590 nm (software Softmax Pro version 4.00 for Macintosh® and Windows®). All determinations were performed in triplicate and sample acceptance with a maximum coefficient of variation of 10%.

The IC_{50} was estimated graphically by linear regression of the reciprocal of percentage activities (compared to the control) *versus* concentrations of the inhibitor.

In considering the normal (parametric) distribution of the variables, the statistical comparison was conducted by one-way ANOVA with Dunnett´s post test performed by GraphPad Prism®

version 5.00 for Windows (GraphPad Software, San Diego, California, USA). Differences were considered statistically significant when $p < 0.05$.

CONCLUSION

The inhibitory effects of 5-caffeoylquinic acid and metabolites on EROD activities (with microsomes derived from untreated and treated animals with β-naphtoflavone) were evaluated at concentrations of 1, 10 and 100 μM. As shown in Tables 1 and 2 (EROD activities with microsomes derived from animals treated with β-naphtoflavone), the tested compounds (100 μM) exhibited no significant inhibitory effect against CYP1A1. Results were similar for lower concentrations of tested compounds and therefore are not shown here; in the same way, no inhibitory effect of tested compounds at 100 μM (not shown).

Table 1. EROD activities in the presence of *trans*-cinnamic (*trans*-CinA), 5-caffeoylquinic (5-CQA), ferulic (FA), isoferulic acids(isoFA) (100 μM).

	EROD (pmoles resorufin/mg ptn/min)	ACTIVITY (%) of control
CONTROL	701.85 ± 12.89	100
trans-CinA	723.92 ± 22.17	103.14
CONTROL	427.29 ± 12.98	100
5-CQA	426.79 ± 10.95	99.88
FA	415.47 ± 26.52	97.23
CONTROL	616.09 ± 29.36	100
iso FA	595.64 ± 26.19	96.68

Table 2. EROD activities in the presence of *p*-coumaric (*p*CoQA), dihydrocaffeic (DHCA), gallic (GA) and vanillic (VA) acids (100 μM).

	EROD (pmoles resorufin/mg ptn/min)	ACTIVITY (%) of control
CONTROL	701.85 ± 12.89	100
*p*CoQA	683.40 ± 11.65	97.37
DHCA	662.52 ± 11.60	94.40
CONTROL	819.31 ± 17.53	100
GA	816.49 ± 24.09	99.66
VA	742.03 ± 32.17	90.57

The present results are consistent with recent studies, which indicated that the biotransformation of hydroxycinnamic acids in the human body mainly involves conjugation reactions of phase II mediated by sulfotransferases (SULTs) and UDP-glucuronosyltransferases (UGTs), whereas multiple hydroxyl groups represent potential sites of conjugation. In this regard, it has been demonstrated that SULT1A1 is most active in the sulfation of caffeic, dihydrocaffeic and isoferulic acids, while SULT1E1 is most active in the sulfation of ferulic and dihydroferulic acids.

Among the other phenolic compounds evaluated in the present study, quercetin showed the most remarkable inhibitory effect on EROD activity (Figure 1). With lower potency, represented by $1/IC_{50}$, this compound also inhibited MROD activity (Table 3).

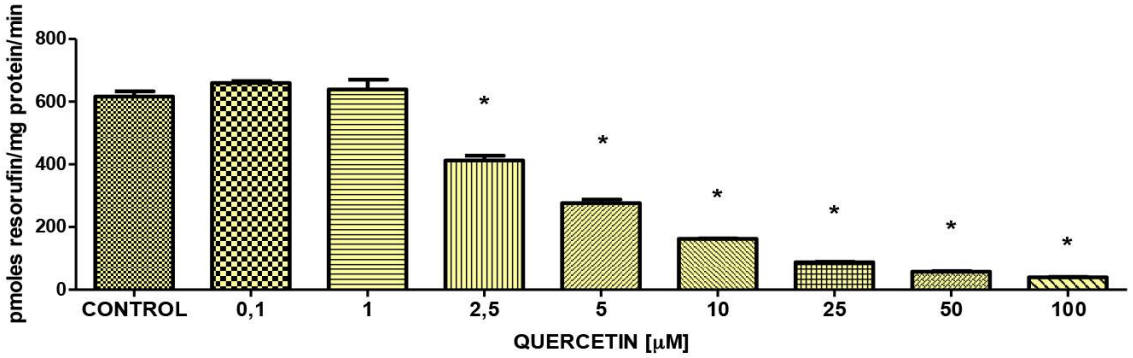

Figure 1. EROD activities in the presence of quercetin.*Values different from control values (P<0.05).

Chaudhary & Willett (2006) tested seven flavonoids in St. John's wort as the inhibitory effect on recombinant human CYP1A1, finding an IC_{50} of 4.10 μM for quercetin. Similar to what was obtained in this study (2.73 μM) (Table 3).

Table 3. IC_{50} values calculated for all the phenolic compounds that inhibit EROD * and MROD ** activities

COMPOUNDS	IC_{50} (μM) CYP1A1	$1/IC_{50}$	IC_{50} (μM) CYP1A2	$1/IC_{50}$	IC_{50} (μM) SEVERAL ISOFORMS	$1/IC_{50}$
α-NAPHTHOFLAVONE	2.24	0.45	2.37	0.42	8.92	0.11
QUERCETIN	2.73	0.37	6.90	0.14	41.50	0.02
HESPERETIN	12.27	0.08	26.50	0.04	24.25	0.04
RESVERATROL	15.22	0.07	18.83	0.05	20.40	0.05

*Note:*microsomes from untreated animals represent several isoforms, while microsomes from animals treated with β-naphtoflavone represent the isoform 1A1; **microsomes from animals treated with β-naphtoflavone represent the isoform 1A2.*

At concentration of 100 μM, naringin did not inhibit the activities of EROD (with microsomes derived from both treated and untreated animals) and MROD (treated animals) presenting the percentages (in relation to control activity) of 98.43%; 94.78% and 97.78%, respectively.
Results obtained in this study showed that coffee phenolic compounds did not inhibit CYP1A1/2, which may indicate that these substances are not substrates for CYP1A subfamily enzymes. All others polyphenols tested, with the exception of naringin, showed clear inhibition on CYP1A1/2, especially quercetin. These findings provide initial data for future studies on risk prediction related to the interactions of drugs with foods/dietary supplements.

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Applicability of Real-Time PCR as a Tool for Detection of Rice (*Oryza Sativa*) in Ground Roasted Coffee

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SUMMARY

The aim of the present study was to use a Real-Time PCR system for detection of rice as adulterant of ground roasted coffee. The specificity of primer pair ARROZ1 was determined by running reactions with genomic DNA from rice as well as other foods such as corn, wheat, coffee, soybeans and barley. The dissociation curve for ARROZ1 demonstrated that this primer pair was specific for rice detection. The absorbance ratio 260:230 in the *in natura* samples was = 1.6, which indicates a good quality isolate. The method was sensitive and specific to detect and quantify down to 0.5% of rice in roasted coffee.

INTRODUCTION

Coffee is a commodity with high commercial value. This makes it a target of fraudulent mixtures with a diversity of cheaper materials, such as rice, among others. Because of that, many methods using different techniques have been developed in order to ensure the quality and authenticity of coffee. However, most of these methods rely on the experience of the analyst, are passive of human error, unspecific or have low sensibility. Currently, Real-Time Polymerase Chain Reaction (PCR) has been applied for food species identification due to its fastness, simplicity, sensitivity and specificity. DNA molecules are highly stable and are present in most biological tissues, what makes of them a good choice for genetic differentiation and identification. While recombinant DNA technology has shown to be a promising tool to determine the authenticity of various processed foods, it has not been used to detect coffee adulteration. Recently, we developed methods for identification of barley and corn in ground roasted coffee. In the present study, we used a Real-Time PCR system for detection of another food product used for coffee adulteration: rice.

MATERIALS AND METHODS

Samples

Fresh samples of rice (*Oryza sativa*) and coffee (*Coffea arabica*) leaves were used as specific target and, barley (*Hordeum vulgare*), corn (*Zea mays*), wheat (*Triticum aestivum*) and soy (*Glycine max*), were used as non-specific targets. All grains were purchased at a local market and were not genetically modified. Ten green coffee beans samples (four *C. arabica* and six *C. canephora*) were obtained directly from producers in São Paulo, Espírito Santo and Minas Gerais, Brazil, and were used as control samples. Coffee was roasted in a fluidized bed roaster (I-roast, USA) at 230°C to give dark color degree (# 35 Agtrom-SCAA); barley and corn were

roasted in a microwave oven to reach the same color as coffee. Samples were ground in a mill (IKA A11basic to pass a 500μm sieve).

To build the five point curve for adulterants quantification, a blend containing 80% of arabica and 20% of robusta roasted beans was used and 0.5%; 1%; 2%; 5% and 10% of rice were added to the coffee blend.

DNA Extraction

Coffee leaves and raw rice were submitted to DNA extraction with CTAB protocol. For roasted beans and rice, DNeasy kit/ buffer CTAB were used. DNA concentrations were determined in all samples by spectrophotometer (Shimadzu UV-1800 Japan) at 260nm.

Primers design

DNA sequences corresponding to the endogenous genes for coffee and rice were surveyed from Genbank (accession number NC008590.1, NM_001049010.2, respectively). Sequences were submitted to the program Basic Local Alignment Search Tool (BLAST) to analyze the similarity with other species. The primer pairs were designed using Genefisher software setting up the size amplicon of 100 pb. Primers were synthesized by Eurofins MWG Operon and their amplification was confirmed using *in silico* PCR runs at BIOINFX (http://www.bioinfx.net/). Coffee primer pair (CAFÉ1): Forward-TTC CGA AGT CCT GGA GAG; Reverse-CGG AGG ATA TCT CAA TCG with a amplicon length of 114 bp. Rice primer pair (ARROZ1): Forward- GTG GAA ATC AGC TCA CTG; Reverse- AAG GCC TAA CTC TGA AGG with an amplicon length 116 bp.

PCR parameters

PCR runs were achieved using SDS-ABIPRISM 7000 (Applied Biosystems). The reaction mixture contained 1 x Power SYBR Green Master Mix (Applied Biosystems) 240nM primers and 50ng DNA in 25μl final volume. Thermal conditions were as follows: 10 min at 95º C, 45 cycles of 15 s at 95 º C and 1 min at annealing temperature (Tm) of primer CAFE1 and ARROZ1.

Limit of detection (LOD) and limit of quantification (LOQ)

The following serial dilutions of DNA template were assayed for each primer pair to obtain standard curves for coffee: 0.0002%, 0.001%, 0.005%, 0.01%, 0.05%, 0.1%, 0.5%, 1%, 5%, 10%, 50%, 100% (= 50 ng). For rice, the dilutions were: 0.01%, 0.05%, 0.1%, 0.5%, 1%, 5%, 10%, 50%, 100% (= 50 ng) Ten replicates for each dilution point were used. The amplification efficiency was calculated based on the standard curves slope, using the following equation: E[%] = [10(-1/(slope)) - 1] x 100. All samples were tested positively with their respective primers, confirming the absence of false negative results that might occur due to PCR inhibition. LOD was considered to be the analyte concentration at which the method detected its presence in at least 95% of the assays (<5% false negative results). LOQ was calculated as follows: MCt-(2 * SD), where MCt = Mean Ct value and SD = corresponding standard deviation.

RESULTS AND DISCUSSION

Specifity of primers and their LOD and LOQ

Regarding the specifity of primers, melting curves from specific (G) and non-specific (H) targets are shown in Figure 1. The melting curve for the specific targets demonstra ted that these primer pairs were specific for rice and coffee detection. The nonspecific amplification peaks were attenuated by increasing annealing temperature.

The method was sensitive and specific to identify and quantify levels down to 0.2 and 0.4pg pg/µL extract of coffee (control) DNA, respectively, and 9.0 and 17.0 pg/µL extract of rice DNA, respectively. The amplification reaction efficiency was 107%.

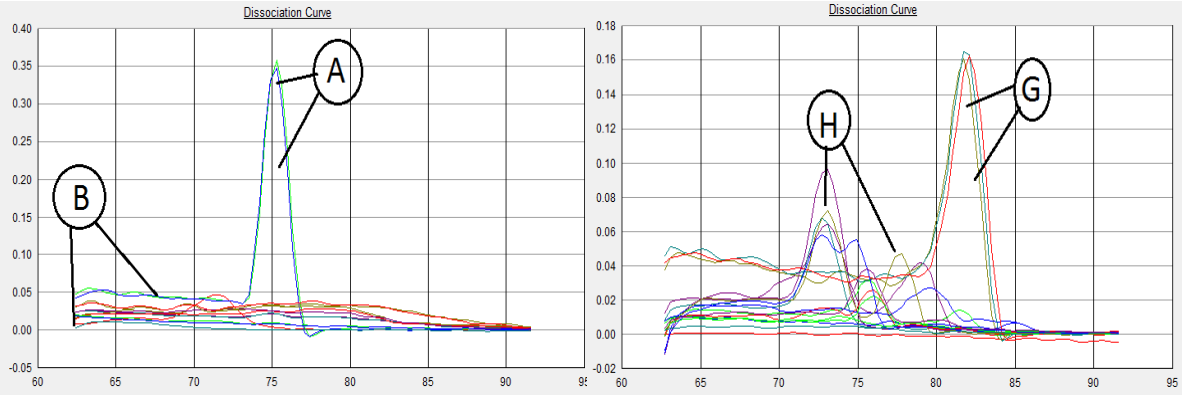

Figure1. Primer Café1 (AB), Arroz1 (GH). Each primer pair was tested with genomic DNA of target (*arabica* / *canephora* coffees and rice) and non-target (wheat, soy, corn, barley).The letters A and G show specific amplifications and letters B and H demonstrate nonspecific amplifications.

Target detection in control samples

Figure 2 shows the Ct values obtained in Real-time PCR analysis from spiked samples (intentionally adulterated) used to build the laboratory adulteration standard curves as well as the equations for percentage estimation. Correlation coefficient (R^2) was 0.998 and the slope -2.9271, indicating amplification efficiencies of 119%. These results are comparable to the parameters of performance data reported in established protocols for validation of analysis for detection and quantification of DNA sequences. The method was sensitive and specific to detect and quantify down to 0.5% of dark roasted rice in dark roasted coffee, which was the lowest percentage tested in the present work. Lower percentages of the adulterant will be tested.

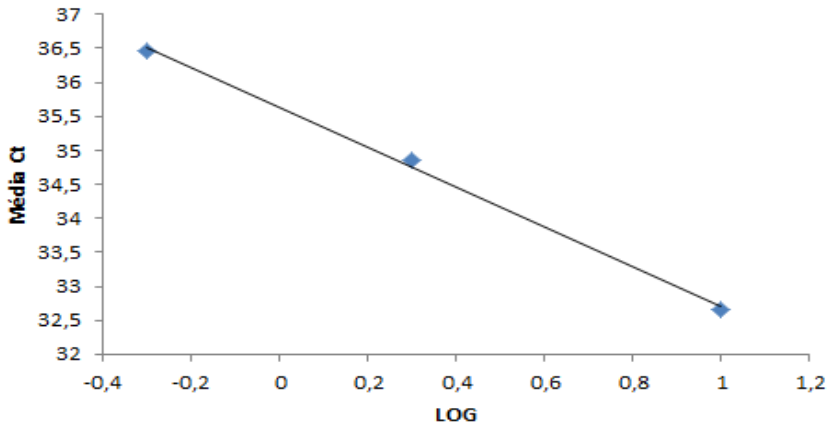

Figure 2. Coffee blends spiked with roasted rice. Value Ct = 8 replicate. Slope: -2.9271 with R2: 0.998.

CONCLUSION

A method was developed for detection of roasted rice in ground roasted coffee. The methodology based on real-time PCR showed to be sensitive and specific to detect small amounts of adulterants, and can be considered a promising tool to be used for adulterants detection by regulatory agencies.

FINANCIAL SUPPORT

FAPERJ and CNPq (Brazil)

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Joint FAO/WHO food standards programme codex alimentarius commission. (2010). Thirty-third Session. Geneva, Switzerland, 5-9 July 2010 Report of the thirty-first session of the codex committee on methods of analysis and sampling. ANNEX II: Validation of a quantitative PCR method.

Antibacterial Effect of Coffea Canephora: Calcium Concentration in a Culture Containing Teeth/Biofilm Exposed to the Coffee Extract

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SUMMARY

Coffee, a popular drink throughout the world, has properties that can fight some diseases, including dental caries. This study evaluated the changes of calcium concentration in a medium containing teeth/biofilm exposed to *Coffea canephora* extract (*CCE*). Enamel fragments were randomly fixed into two 24-well polystyrene plates containing BHI. Pooled human saliva was added to form biofilm on fragments. Specimens were divided into treatment groups (G, n=8 per group) and treated (50µL) daily for 1 min during a week, as follows: G1 - 20% *CCE*; G2 - Milli-Q water (negative control); G3 - antibiotic (positive control). Six fragments represented the blank control (G4). Calcium content was observed at baseline, 4 and 7 days of treatment by atomic absorption spectrophotometry. Cross-sectional hardness value of enamel was a demineralization indicator. Calcium increased in the medium after 4 and 7 days of treatment in G1 (3.80±1.3mg l^{-1} and 4.93±2,1mg l^{-1}, respectively) and G3 (4^{th} day = 5.7±1.8mg l^{-1}; 7^{th} day= 6.7±3.5 mg l^{-1}) (Tuckey test, p=0.136). Calcium from G2 (4^{th} day = 3.75±1.9mg l^{-1}; 7^{th} day= 3.6±1.4mg l^{-1}) decreased after 7 days, which was different from G3 (Tuckey test, p=0.009). The lower calcium content, at the end of the experiment, was represented by G4, 2.16±0.2 mg l^{-1}.Considering that the teeth integrity was not affected by treatments, the increase in calcium after treatment with *CCE* is probably due to its antibacterial effect, which caused the bacterial lysis and consequent release of calcium in the medium.Introduction.

INTRODUCTION

Coffee, a popular drink throughout the world, has properties that can combat some diseases, including dental caries. *Coffea canephora* is rich in polyphenols, which are compounds considered to be potent agents for preventing oral diseases, particularly biofilm-related diseases. A recent study has shown that the *Coffea canephora* extract has an antibacterial effect against *Streptococcus mutans* — one of the bacterial species that causes caries — in addition to showing a preventive effect against demineralization of tooth enamel, which had been previously exposed to mixed biofilm. However, despite the positive results, the authors suggest further research to investigate the anticariogenic ability of *Coffea canephora*.

The aim of the present study was to identify the influence of *Coffea canephora* extract on caries, taking into account the calcium concentration in a culture medium containing

teeth/biofilm that were exposed to this substance. Given that some authors found that calcium is present in the bacterial cell and it is responsible for maintaining the bacterial structure.

MATERIALS AND METHODS

Coffea canephora extract

Regular *Coffea canephora* cv. Conillon beans from Espirito Santo, Brazil, were roasted to produce a light roasting degree coffee according to Antonio et al.

Tooth selection and sample preparation for biofilm plate assay

Fifteen exfoliated primary first molars teeth having no structural alterations were sectioned mesiodistally, resulting in 30 fragments. Each fragment was coated with an acid-resistant varnish leaving a window (22 mm^2) of exposed tooth. All fragments were submitted to ethylene **oxide sterilization prior to the experiment.**

Inoculum to form biofilm on tooth fragments

The inoculum comprised unstimulated whole mixed saliva, collected from three volunteers aged 25–36 years. The saliva (1 mL) from each volunteer was placed into a tube, which was mixed using a vortex, resulting in an inoculum with 2 x 10^8CFU/mL (dilution of 1:200).

Biofilm plate assay and Treatment protocol

The biofilm model was conducted in polystyrene 24-well tissue-culture plates. The tooth fragments were fixed inside the wells, which were completed with BHI media (1485µL/well) already containing the inoculum (15µL/well). The system was incubated in microaerofilia for 10 days so as to produce biofilm. After the biofilm formation, the samples were treated once a day according to the following treatment groups (G, n = 8 for each treatment): G1 - 50 µl of *C. canephora* aqueous extract at 20%; G2 - 50 µl of Milli-Q purified water (negative control); G3 - 50 µl of antibiotics/Clarithromycin (positive control). The treatment protocol was conducted as Antonio et al..

Analysis of calcium content

Culture medium (1000µL) was collected at the following time intervals throughout the study: (1) before starting treatment (baseline); (2) on the 4^{th} day; and (3) on the 7^{th} day of treatment, respectively. The samples were centrifuged and 500µl of 65% nitric acid was added to the supernatant to allow reading by flame atomic-absorption spectrophometry (Analyst 300 - Perkim Elmer, Germany). Lanthanum was used to suppress interference of phosphate.

Cross-sectional microhardness analysis

After the 7th day of treatment, 6 dental fragments from each group were removed from the wells and sectioned in half. The two halves were prepared and polished for cross-sectional microhardness analysis. Two sequences of 5 indentations were made on the exposed and unexposed area (control fragment - CF, representing hardness of the healthy fragment) at a distance of 10, 30, 50, 70 and 90 µm from the enamel surface. The hardness values (expressed in kgf/mm^2) were obtained by a digital hardness tester (HVS-1000, Time Group Inc., China) in accordance to Antonio *et al.*.

Statistical Analysis

From SPSS 17.0 program (SPSS Inc., Chicago, IL), the two-way ANOVA for repeated data was used to find the differences in the calcium content at different time intervals during the study, while Tukey's test was used to find the difference among the groups. The same tests were used to find the differences in microhardness values among the groups. The level of significance adopted in all analysis was 95%.

RESULTS AND DISCUSSION

The results of the calcium concentration in the culture medium containing teeth/biofilm in the different groups are shown in Figure 1.

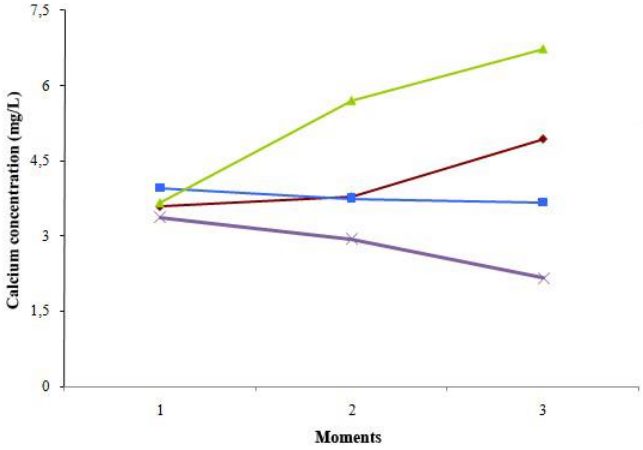

Figure 1. Mean calcium concentration in culture medium containing teeth/biofilm in the different studied groups (G). G1 (♦): *Coffea canephora* extract; G2 (■): Milli-Q water; G3 (▲): Antibiotic; G4: (✖): Blank control.

An increase in calcium concentration was observed after 4 and 7 days of treatment in G1 (3.80 ± 1.3 mg l^{-1} and 4.93 ± 2.1 mg l^{-1}, respectively), on the 4th (5.7 ± 1.8 mg l^{-1}) and 7th days (6.7 ± 3.5 mg l^{-1}) in G3, with no statistically significant difference (p = 0.136) among them at all times evaluated. A decrease in the calcium concentration was found in G2 after the same time interval with a significant difference only when the values were compared with G3 (p = 0.009). As for G4, the lowest calcium concentration (2.16 ± 1.2 mg ml^{-1}) was found after 7 days of treatment in comparison with the other groups. The authors believe that the *C. canephora* extract, as well as the antibiotics, was capable of causing bacterial lysis, and consequently, after 7 days of treatment, an increase in the calcium concentration in the culture medium.

G1 showed no statistical difference compared with G2, despite showing a borderline outcome (p = 0.0531). However, we believe that this result is due to the small number of samples tested in the study. Some researchers state that the presence of an antimicrobial agent causes an increase in cytoplasmic free calcium in the bacterial cell by the influx of ion to maintain its functions. This fact proves that antibiotics inhibited protein synthesis of bacteria (mechanism of action of clarithromycin) causing the death of the microorganisms, and thus probably causing bacterial lysis due to supersaturation of calcium in the cell in G3 which caused the release of ions. Therefore, if only one cell stress had occurred without lysis, the medium

would have been subsaturated with calcium, which was not observed in G3 and G1. Thus, the authors suggest that the coffee extract also caused the death of bacterial cells.

The authors also presume that a possible loss in minerals could have occurred on the dental fragments supersaturated with calcium to the subsaturated medium of the tooth by difference gradients (osmosis). This effect would be due to the acidity of antibiotics (pH = 5.28) and coffee (pH = 5.04). However, both did not differ from water (pH = 5.75) ($p>0.05$). Therefore, if the increase in calcium in the medium were solely a result of mineral loss of the dental fragment due to the acid pH of the coffee extract and antibiotic, a similar concentration of calcium in the medium in G2 should have been found. Furthermore, the cross-sectional microhardness test showed that hardness or mineral loss occurred in all groups that were exposed to biofilm up to 30µm in depth (Table 1), and the highest mineral loss was found in G2 (up to 30µm) ($p<0.05$). Valinotti *et al.* also found higher mineral loss in dental fragments submerged in deionized water when compared with other substances, among them, an antimicrobial agent (clarithromycin).

Table 1. Microhardness of the fragments in each studied group.

Groups studied	Hardness values (mean ± SD) (kgf/mm^2) at each depth				
	10µm	30µm	50µm	70µm	90µm
G1	177.93±79.17^a	224.57±52.32^a	318.00±67.92^b	311.43±47.66^b	322.68±39.06^b
CF in G1	291.78±25.69^b	327.98±32.13^b	334.91±23.97^b	331.56±13.65^b	324.83±16.51^b
G2	102.27±17.54^c	135.75±38.22^c	298.75±64.39^b	312.80±27.87^b	311.60±32.21^b
CF in G2	296.56±37.46^b	310.32±37.25^b	323.60±23.84^b	326.52±28.53^b	345.36±28.72^b
G3	195.87±13.44^a	228.64±42.65^a	315.80±37.91^b	329.72±38.58^b	326.47±31.71^b
CF in G3	289.78±34.12^b	319.16±24.80^b	324.79±14.23^b	322.96±17.52^b	349.42±19.02^b
G4	292.19±26.98^b	313.18±48.96^b	329.42±54.74^b	317.94±42.77^b	331.57±28.46^b
CF in G4	295.23±26.75^b	324.67±35.76^b	319.21±37.63^b	324.53±15.39^b	322.49±43.87^b

Note: SD - standard deviation; CF - control fragment (area protected by the nail polish); G1 - group treated with coffee extract; G2 - group treated with Milli-Q water (negative control); G3 - group treated with antibiotics (Clarithromycin); and G4 (untreated group and without biofilm – blank control). Considering the same depth of enamel (same column) and between the different groups (G1, G2, G3 and G4), values with the same superscript letter are not significantly different ($\alpha = 0.05$).

Considering the results, the authors suggest that the increase in calcium concentration in the culture medium containing teeth/biofilm is due to the antibacterial effect of the *C. canephora* aqueous extract, which probably caused the death of the bacteria and release of ion by supersaturation of the cell. However, further studies are needed to elucidate the *C. canephora* aqueous extract mechanism of action against bacteria.

FINANCIAL SUPPORT

FAPERJ and CNPq, Brazil

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Effect of Caffeic Acid on CAMKII Protein Levels in Glioma Cells at Oxidative Stress Condition

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SUMMARY

Caffeic acid is one of the main primary metabolites in the human body. It is well known that this compound exerts concentration-dependent antioxidant activity in *in vitro* but it is not well known whether this activity actually occurs inside the cells. In the present study, we investigated the modulatory effect of caffeic acid on CaMK II protein levels in increased oxidative stress conditions and its relevance for apoptosis by using cytochrome C. Results indicate that caffeic acid, can actually act preventing cell oxidative damage.

INTRODUCTION

Phenolic compounds are secondary metabolites in plant foods that play important antioxidant role in plants and humans. Hydroxycinnamic acids are phenolic compounds widely distributed in plants but coffee is one of the major sources in nature, especially of caffeic acid (Figure 1), which can be found in these seeds mainly in the form of mono and diesters with quinic acid. In consequence, after coffee intake, caffeic acid is one of the main primary metabolites in the human body. It is well known that caffeic acid exerts concentration-dependent antioxidant activity in *in vitro* but it is not well known whether this activity actually occurs inside the cells. Oxidative stress remodels Ca(2+) signaling in several cells. Ca(2+)/calmodulin-dependent protein kinase II (CaMKII) is activated by oxidative stress, and therefore its expression increases upon oxidative stress. CaMK II mediates phosphorylation of a wide range of target proteins involved in cellular processes such as cell growth, and apoptosis (programmed cell death), particularly in the the brain.

It is well known that apoptosis can be induced by oxidative stress and results in brain damage. The concept of apoptosis appear with its unique and dynamic morphological features that are discernible from senescence or necrosis, such as cell shrinkage, blebbing of cell membrane, chromatin condensation, nuclear membrane breakdown, and formation of small cell parts from the cell surface also known as apoptotic bodies. At the end of apoptosis, the apoptotic bodies are rapidly swallow up by phagocytic cells, and thus a potential inflammatory response is abstained. During apoptosis, cytochrome C is released from mitochondria to the cytosol to activate a caspase cascade, which commits the cell to the death process.

In this study, using human C6 Glioma cells, we investigated the modulatory effect of caffeic acid on CaMK II protein levels in increased oxidative stress conditions and its relevance for apoptosis by using cytochrome C.

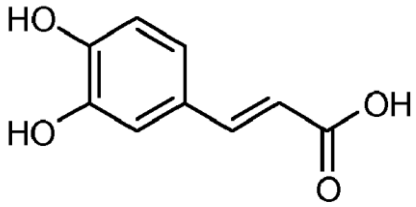

Figure 1. Chemical structure of caffeic acid.

MATERIALS AND METHODS

C6 Glioma cells were grown with DMEM in a 75 cm^2 cell flasks. Culture flasks were divided into 4 treatment groups: 1) negative control; 2) H_2O_2 ($5X10^{-4}$) (oxidative stress control); 3) caffeic acid ($5X10^{-4}$); 4) caffeic acid+ H_2O_2. Flasks were incubated for 72 hours at 37ºC in CO_2 atmosphere. After incubation, protein extraction was performed with Trizol (Invitrogen Life technologies, USA). Evaluation of CaMK II protein expression was performed by Western Blotting. Image J program was used for densitometry analysis.

Cells were homogenized in 5 ml hypotonic medium (50 mM Tris/10 mM MgCl2), using a polytron for 2 s and centrifuged at 13 K rpm for 20 min at 4°C. The pellet was suspended in homogenizing buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% Na orthovanadate, 1.5 mM pepstatin, 2 mM leupeptin, and 0.2 U/ml aprotinin. Equal volumes of membrane fraction were subjected to 10% polyacrylamide gel by using a gel electrophoresis apparatus. The proteins were subsequently transferred electrophoretically to nitrocellulose membrane using a protein transfer unit. Membranes were washed for 10 min and blocked with 3% milk powder in PBS. Then the blots were incubated with primary monoclonal antibodies (anti-CAM II and anti-Cytocrom C, diluted at 1:200 in the blocking solution overnight at 4ºC. To reduce the interblot variability, membranes were also incubated with anti-actin antibody at a concentration of 1:1000 in the same conditions. The membranes were then thoroughly washed and incubated with alkaline phosphatase-linked secondary antibody for 60 min at room temperature. Bands on the autoradiogram were quantified using Image J image analysis system (NIH), and the optical density of each sample was corrected by dividing by the optical density of the corresponding actin band.

Data was processed by one way ANOVA, followed by Tukey's Multiple Comparison Test, using Graph Pad Prism (USA, version 5.0). Data were express as Mean ± SEM.

RESULTS

As expected, a 1.8 fold increase in the levels of CaMK II protein was observed after treatment with H_2O_2 (Treatment 2), compared to negative control group. Although there was no difference between caffeic acid administrated group (Treatment 3) and control group, in group receiving Treatment 4 the increase in CaMK II protein expression was attenuated by the presence of caffeic acid to 1.3 fold comparing to control ($p < 0.05$) (Fig. 2).

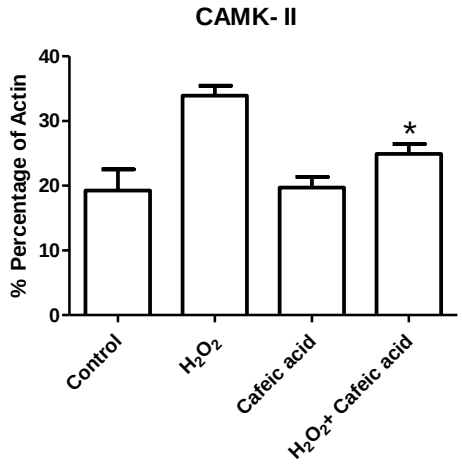

Figure 2. Modulatory effect of caffeic acid on CaMK II protein from human Glioma cells. 4 treatment groups (from left to right): 1) negative control; 2) H_2O_2 ($5X10^{-4}$) (oxidative stress control); 3) caffeic acid ($5X10^{-4}$); 4) caffeic acid+ H_2O_2. Error bars represent SEM, * represents $p < 0.05$ compared to H_2O_2 treatment).

To investigate the effect of caffeic acid on apoptosis we also performed Western blott analysis for cytochrome C with the same proteins. The increase in CaMK II protein expression by H_2O_2 was decreased in the presence of the caffeic acid (Fig. 3).

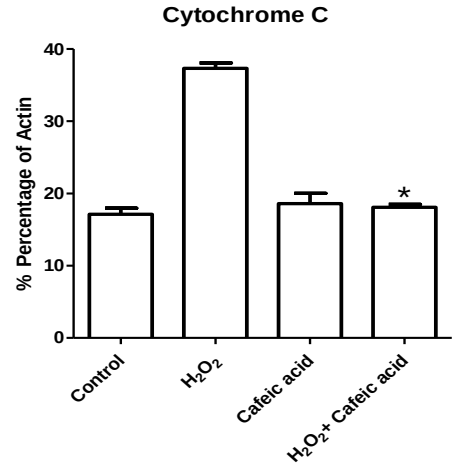

Figure 3. Modulatory effect of caffeic acid on cytochrome C protein from human Glioma cells. 4 treatment groups (from left to right): 1) negative control; 2) H_2O_2 ($5X10^{-4}$) (oxidative stress control); 3) caffeic acid ($5X10^{-4}$); 4) caffeic acid+ H_2O_2. Error bars represent SEM, * represents $p < 0.05$ compared to H_2O_2 treatment).

CONCLUSION

The present results indicate that caffeic acid, a coffee metabolite, can actually act preventing cell oxidative damage.

FINANCIAL SUPPORT

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Mathematical Modeling of the Volumetric Expansion of Coffee Beans during Roasting

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SUMMARY

During the roasting, coffee beans are dehydrated and undergoes a series of physical and chemical changes that give to final product the features listed for consumption (color, aroma, taste). One of these changes is the volumetric expansion, mainly occurring by the generation and release of CO_2 and steam from the inside of the grain. This expansion is an important variable in studies of heat and mass transfer. The aim of this study was to develop a mathematical model to explain the real volumetric expansion of the coffee bean depending on moisture loss during roasting. It was used benefit coffee beans size 18, *Coffee arabica* L. red catuai variety, containing initial moisture of 0,129 $kg_a\ kg_{ms}^{-1}$. Rotary drum roaster with gas direct combustion at 45 rpm was used with internal temperatures of 200, 220, 240, 260 and 280 ^oC. The grains were roasted unitarily. Their volume and humidity were stated every 20 seconds during the roasting time (10 minutes). To represent the variation of volume respect to grain moisture, classical mathematical models were used: Bala and Woods (1984), exponential, linear, polynomial, Rahman (1995), Corrêa et al (2004) and Copace (2014). The fit of the models was performed by analysis of non-linear regression by Gauss-Newton and Simplex & Quase-Newton methods using the Statistica 7.0®. To check the degree of fit of each model were considered the coefficient of determination (R^2), mean relative error (P) and mean estimated error (SE). The model provided a better fit was Copace 2014:

$$y=\frac{1}{1+a(X_0-X)+b(X_0-X)^2}$$

Where ψ: real volumetric expansion index, dimensionless. Xo: initial moisture, decimal b.s. X: moisture at a given time, decimal b.s. a and b: The parameters of the model.

INTRODUCTION

Although Brazil is the largest coffee producer in the world and the second largest consumer market, it is still far from reaching industrialized coffee exporting countries such as Italy and Germany. To achieve the generation of value added in the coffee production chain, it is necessary to know and study the operations involved in processing. Roasting is a very important step because it can only maintain a good quality coffee, and it does not improve. During the roasting, coffee beans are dehydrated and undergoes a series of physical and chemical changes wich give to final product the features listed for consumption (color, aroma, taste). One of those changes is the volumetric expansion, mainly occurring by the generation and release of CO_2 and steam from the inside of the grain. This expansion is an important variable in studies of heat and mass transfer because it directly affects the surface area, the particle radius and the mass especific. The heat transmitted to the bean is the most important parameter of the roasting process and can be determined from the bean temperature and

roasting time. Several studies have been made to develop mathematical models to predict the temperature increase inside the grain, moisture loss and mass loss. This as a tool for on-line process control and optimization. These models consider constant bean volume and physical properties (specific heat, thermal conductivity, diffusivity). The aim of this study was to develop a mathematical model to explain the real volumetric expansion of the coffee bean depending on moisture loss during roasting, trying to bring the models and simulations as close as possible to the reality.

MATERIALS AND METHODS

The experiment was developed in the laboratory of physical properties and quality of agricultural products from the National Training Center in Storage (CENTREINAR), Department of Agricultural Engineering, at Federal University of Viçosa, Viçosa, Minas Gerais, Brazil. Was used benefit coffee beans size 18, Coffee arabica L. red catuai variety, containing initial moisture of 0,129 $kg_a\ kg_{ms}^{-1}$. Rotary drum roaster with gas direct combustion at 45 rpm was used with internal temperatures defined by the literature on the levels of light, medium and dark roasts (220, 240 and 260 ° C respectively) and two extreme temperatures (200 and 280 ° C) to improve the fit of the model. The grains were roasted unitarily, supported inside the roaster by a type K thermocouple, being convection the main mechanism of heat transfer. Their volume and humidity were stated every 20 seconds during the roasting time: 10 minutes (this time exceeds the time required to obtain a dark roast level, and was established only for the purpose of mathematical modelling).

Bean volume before and after each time interval was determined by measurements of the axes l, e and c (approximation to the shape of a half ellipsoid, equation 1), of ten grains using a digital caliper. Real volumetric expansion index was calculated for each bean in each time step using the equation 2. The average index for each time step was then calculated. Moisture was determined in triplicate by drying in air circulating oven at 105 ^oC for 24 hours. To represent the variation of volume respect to bean moisture, classical mathematical models were used (table 1). The fit of the models for each temperature was performed by analysis of non-linear regression by Gauss-Newton and Simplex & Quase-Newton methods using the Statistica 7.0®. To check the degree of fit of each model were considered the coefficient of determination (R^2), mean relative error (P) and mean estimated error (SE).

$$V = \pi \frac{l\,e\,c}{6000} \tag{1}$$

$$\psi = \frac{V}{V_0} \tag{2}$$

Where ψ: real volumetric expansion index, dimensionless. Vo: initial grain volume, ml. V: grain volume at a given time, ml. l: longitudinal diameter, mm. e: ecuatorial diameter, mm. c: thickness, mm.

Table 1. Mathematical models used to model volumetric shrinkage/expansion of grains

Corrêa 2004	Exponencial	Linear
$\psi = \frac{1}{a + b\exp(X)}$	$\psi = a\exp(bX)$	$\psi = a + bX$
Polinomial	**Rahman**	**Bala and Woods**
$\psi = 1 + aX + bX^2$	$\psi = 1 + a(X - X_0)$	$\psi = a\{1 - \exp[-b(X_0 - X)]\}$

Where ψ: real volumetric expansion index, dimensionless. X_0: initial moisture, decimal b.s. X: moisture at a given time, decimal b.s. a and b: the parameters of the model.

CONCLUSION

Only the linear model acceptably represented the experimental data but the parameters values showed no relation to the roasting temperature, for which the authors proposed to use the model Copace 2014 achieving an acceptable fit (table 2). Expansion kinetics at 200 °C depending on the humidity difference (X_0-X) is shown in figure 1a. Parameters a and b showed a linear relationship (R^2=97%) with the roasting temperature, therefore it's possible to infer a general model to estimate the real volume of bean roasted anytime, depending on roasting temperature, initial moisture and inicial volume (equation 3).

Table 2. Parameter values and degree of fit Copace 2014.

Copace 2014	T (°C)	*a*	*b*	*P*	*SE*	R^2
$\psi = \frac{1}{1+a(X_0-X)+b(X_0-X)^2}$	200	-5,5660	19,7352	2,1471	0,041	0,9713
	220	-6,1173	22,5839	2,2461	0,045	0,974
	240	-7,3371	32,4195	2,5884	0,054	0,9613
	260	-7,8687	35,5246	2,8654	0,069	0,9337
	280	-8,4289	40,0709	1,3014	0,032	0,9856

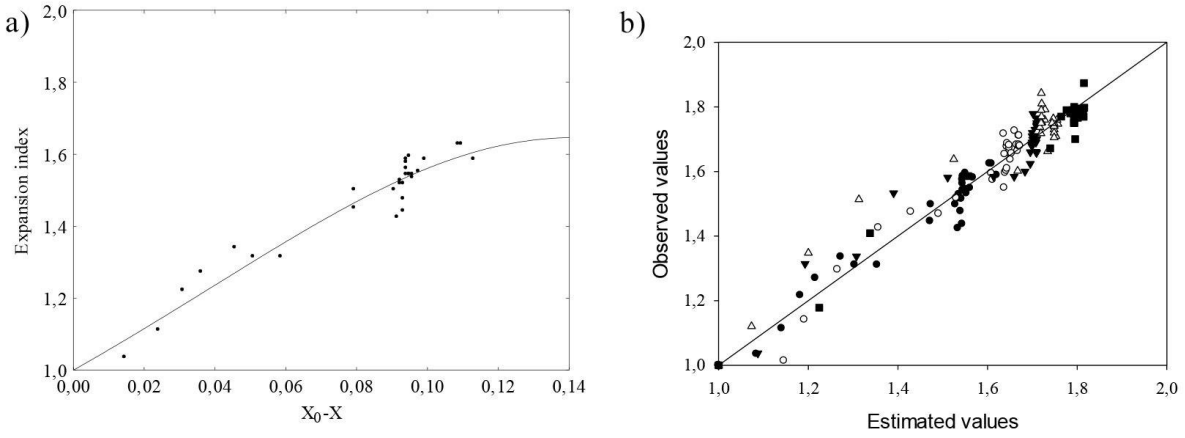

Figure 1. a) Observed and estimated values by Copace 2014 model for real volumetric expansion index at 200 °C b) Correspondence of the observed and estimated values by the Copace 2014 model •: 200 °C; ○: 220 °C; ▼: 240 °C; Δ: 260 °C; ■: 280 °C.

$$V = V_0 \left[\frac{1}{1 - (0{,}0374T_a - 1{,}9091)(X_0 - X) + (0{,}2681T_a - 34{,}268)(X_0 - X)^2} \right] \quad (3)$$

Expansion index reach values from 1,6 to 1,86 for roasting temperatures between 200 and 280 °C respectively. This is explained by the highest cumulative pressure within the grain (due to rapid water evaporation and CO_2 generation in high temperatures) and is consistent with the results obtained by Vargas, who analyzed the apparent expansion in four roasting temperatures, and Schwartzberg, who found a volumetric expansion of between 50% and 120% at air temperatures of between 270 and 550 °C. As observed in the figure 1b, the model has an acceptable fit with experimental values. The figure 2 shows the evolution of axis *l*, *e*

and *c* during the roasting time at two temperatures. Data showed an exponential behavior type *f=a*(1-exp(-b*t)) (R^2 > 91%).* A non-isotropic expansion is observed at low roasting temperature (200 °C), with the *e* and *c* axes show the greatest variation. As the process temperature increases, the bean expansion tends to be isotropic with a maximum increase of 18% in each axis. Hernández et al considered isotropic expansion to develop a model to predict bean surface area (computed from the projected area) using artificial neural networks.

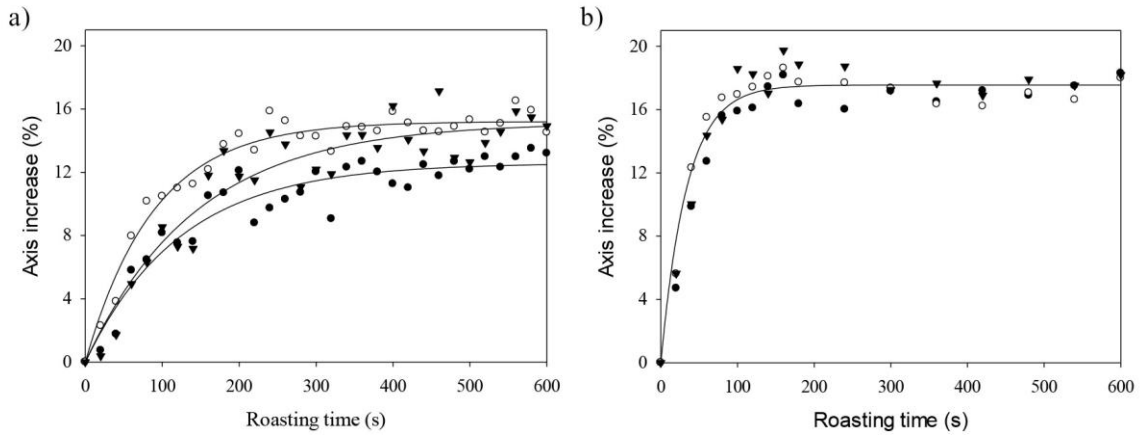

Figure 2. Average axis increase (%) during roasting time. a) 200 °C b) 280 °C. •: observed *l* axis; ○: observed *e* axis; ▼: observed *c* axis; ---: estimated.

By studying changes in the dimensions of the coffee bean and moisture loss during roasting, it can be concluded that the real expansion of coffee beans during the process, exhibits a non-isotropic behaviour at roasted temperatures below 220 °C. At higher temperatures, the expansion can be considered isotropic. This study provided an increased understanding of physical changes undergone by the coffee bean during roasting. Expansion model proposed (Copace 2014) can be used to improve the fit of the models that attempt to predict the temperature evolution and mass loss of the coffee bean during the roasting process.

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Development of Controlled Fermentation Processes to Add Value to Coffee Quality

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SUMMARY

To add value to the coffee quality, new fermentation and washing processes were developed. By controlling temperature, time and system of coffee fermentation different specialty aromas and flavors were obtained.

INTRODUCTION

In Colombia, the temperature in coffee farms varies between 10 °C and 34 °C. Most coffee growers use fermentation to remove mucilage from the bean, in this way, the pulped coffee beans are deposited in a tank, where it is allowed that fermented mucilage drains during the time of processing. Besides, there are no systematic methods for controlling this process. In order to improve coffee quality by fermentation, several new controlled fermentation systems that include the effect of temperature, time, way of fermentation, way of washing and degree of roasting were proved and developed. In these new fermentation processes, the fermented mucilage remains with the coffee beans until time of washing.

MATERIALS AND METHODS

The effect of the external temperature, the fermentation time and the fermentation system on coffee quality was assessed. Blocks of open fermentations of pulped coffee beans were carried out in Cenicafe in solid substrate as well as in submerged systems. Coffee beans were obtained from Arabica varieties by selective mature harvesting followed by hydraulic classification, pulping, mechanical as well as manual removal of pulps from the mass. In submerged systems 30 L of clean water was used per each 100 kg of pulped coffee beans. The external temperatures, 23°C and 15°C were achieved by air conditioning and refrigeration devices. Coffee fermentation times between 12 and 66 hours were evaluated. A systematic washing method was used for completing coffee processing. The coffee beans were roasted at 45 and 55 Agtron color scale. The percentage of coffee cups with good qualification, percentage of flavors and percentage of defects were measured, using a quantitative descriptive scale for coffee quality (Table 1).

Table 1. Scale of quantitative and descriptive qualification for coffee quality (Source: Puerta, 1996; Puerta, 2013).

Special and higher quality			Average quality			Rejection		
9	8	7	6	5	4	3	2	1
The best	Very good	good	tolerable	average	Just tolerable	rejection	rejection	rejection
Mild, hazelnut, fruity, sweet Almond, citrus, malt, blackberries, chocolate, caramel, cloves, vanilla Wine, toast			sisal	Grass, astringent, Banana, tasteless	Low body, Low acidity	Corn, wood Cereal, burnt, very bitter	Sour, floral, pulp, pepper, dirty, rough, fat, onion, wet	Vinegar, Spicy burnt, earthy, smoky, leather, mold, rotten Stinker, acrid, phenolic.

RESULTS

Through changes in the way of coffee fermentation differences in coffee qualities were obtained, by processing the same variety and origin of coffee, (Figure 1). By solid substrate coffee fermentation process a complex cup with fruit and citrus flavors was the most frequent; while in submerged 30%, the coffee beverage presented more uniform flavors with chocolate and toast notes.

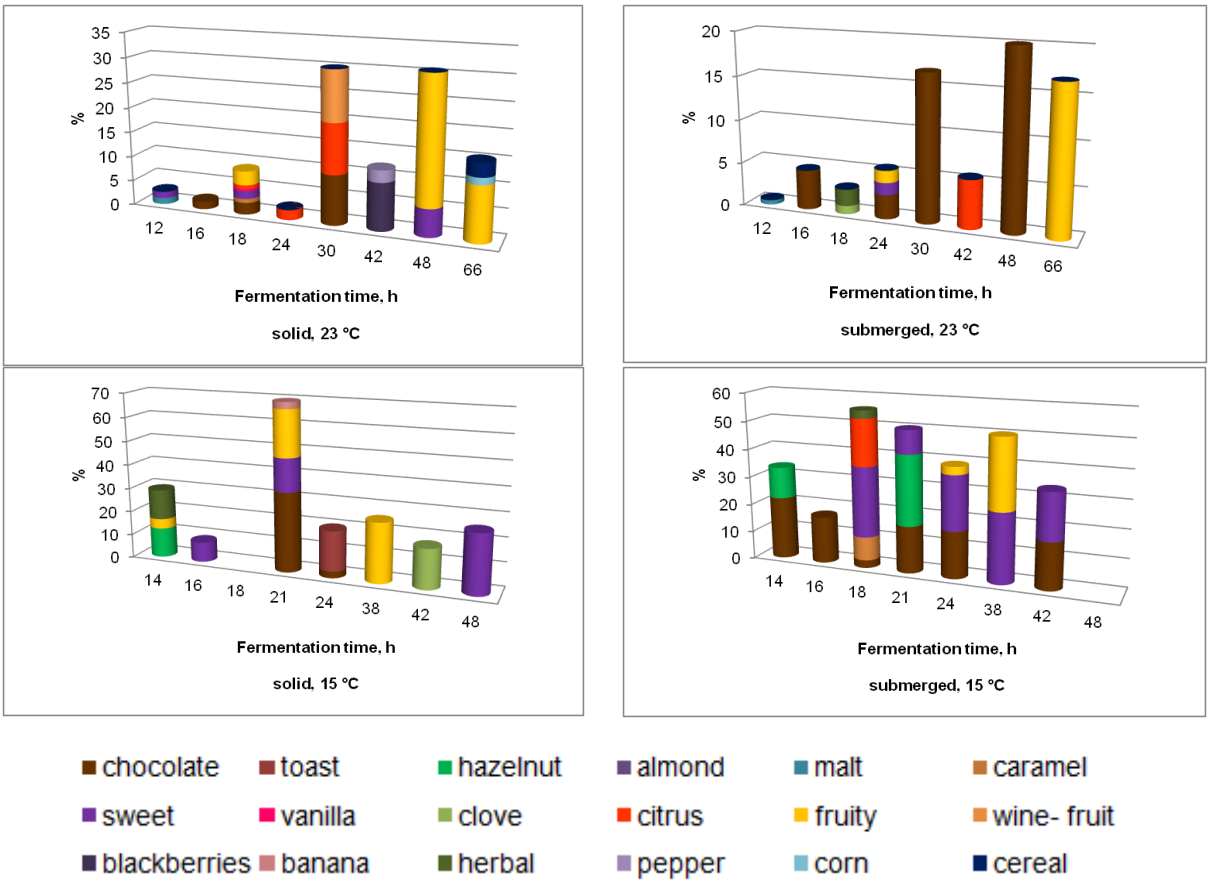

Figure 1. Specialty coffee flavors in open coffee fermentation systems

There was an effect on the coffee quality and in the frequency of flavors of the beverage according to the outside temperature of fermentation. At 15°C the percentage of specialty

coffees was higher, up to 60% of specialty flavors compared to room temperatures, 30%. After 2 nights of coffee fermentation some defects could appear although these were lower at lower temperature as well as in submerged systems.

In order to follow the conditions of the coffee fermentation process pH and Brix values of coffee mucilage could be measured (Table 2), this allow to fix the wanted time of fermentation in a farm according to desired coffee flavors and system of fermentation. To complete the controlled coffee fermentation the following systematic washing method must be used, which includes, first agitate the mass after chosen time of fermentation, then drain the fermented mucilage from the beans, then add 30% of the required quantity of water then mix and leave run, and so on in the next steps it is used 20%, 20% and 30% of the required quantity of water. As control of coffee washing operation, the final waste water should show a Brix value of 0,2 to 0,3 % and a pH value of 5,5.

Table 2. Summary of best conditions for coffee controlled fermentations

Coffee Fermentation system	Quantity of water by fermentation	Fermentation time h	Initial pH	Final pH	Initial Brix %	Final Brix %	Quantity of water by washing
Solid substrate	No water	12-18	5.0 - 5.3	3.7 - 3.9	17- 19	12 - 14	2 L water /kg pulped coffee beans
Submerged	+ 30%	18 -30	5.3 - 5.6	3.9 - 4.2	4.2- 5.8	8.0 - 9.0	1,7L water /kg pulped coffee beans

CONCLUSION

With the same variety and origin of coffee it is possible to obtain different coffee flavors through controlled fermentations. According to the outside temperature of coffee farm, coffee grower may choose the system of fermentation, fix the duration of fermentation and produce the desired coffee quality. To ensure the flavors developed during fermentation, it is essential to wash the coffee beans with enough clean water and it is necessary to use clean air for coffee drying. It is recommended a medium roast degree at 210 °C.

The aroma and flavors developed and perceived by coffee cupping should correspond to chemical and volatile compounds developed by microorganisms such as bacteria and yeast during coffee fermentation.

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To coffee growers, personnel of Experimental Stations, Gustavo Echeverry M. and Hernando Garcia O.

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FERMAESTRO®: a Simple Method to Determine the End of the Fermentation Stage

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INTRODUCTION

In the wet processing of coffee, fermentation is a natural process that involves biological, chemical and physical factors, which purpose is to degrade the mucilage structure, in order to ease washing. Due to the complexity of the process it is difficult to identify its finalization. Therefore, in practice, the control is achieved by the growers following practices based on traditional knowledge, which most used is the elapsed time, generating uncertainties about the final coffee quality.

In order to help coffee growers to know when mucilage degradation by natural fermentation has ended, dynamics of several variables such as pH, temperature, viscosity, puncturing force and cohesion were measured. But none of these variables showed a reliable behavior to be part of a method to mark the right time.

Nonetheless, when the research was taking place, it was observed a very stable volume reduction when passing from pulped coffee to coffee with degraded mucilage. This observation allowed to develop a control-volume method to determine in a reliable and exact way the finalization of mucilage degradation by natural fermentation, which is described in this poster.

DEVELOPMENT

Mucilage is a layer between 0,4 and 2,0 mm thick adhered to the coffee beans, which represents between 20 and 30% of the pulped coffee mass. When a mass of freshly pulped coffee is introduced into a perforated reservoir, it is observed that the volume reduces while fermentation is in progress, because degraded mucilage places into the pores or flows throughout the perforations, and promotes a bulk density change between these two coffee states (Table 1). The moment when the volume reduction becomes stable marks the end of the mucilage fermentation process and the time when coffee must be washed. Figure 1 shows the volume reduction during the fermentation process in a cylindrical perforated reservoir.

In order to obtain a reservoir to magnify the volume reduction, it was run an optimization process to obtain important technical specifications of the perforated reservoir: minimum volume and maximum height. The results pointed to 500 mL as the minimum volume that gives appreciable volume changes in a reliable way and that 250 mm is the maximum height to fit in all the fermentation tanks. In that sense, the four different shapes shown in Figure 2 were studied in order to define the maximum height change (*dh*) with respect to the total height (*H*), which is directly related to the sensitivity of the method.

Table 1. Bulk density change between pulped coffee and washed coffee.

Parameter	Pre-selected coffee	Raw Coffee
Pulped coffee (kg/m^3)	823,71	803,40
Washed coffee (kg/m^3)	701,87	693,66
Volume reduction	11,9%	13,1%

It was determined that porosity of washed coffee is 36,5% (3), which gives the idea all this space can also be filled by degraded mucilage during the fermentation process.

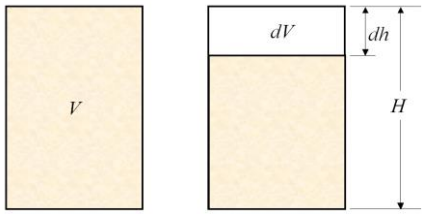

Figure 1. Volume reduction during the state change during the fermentation process.

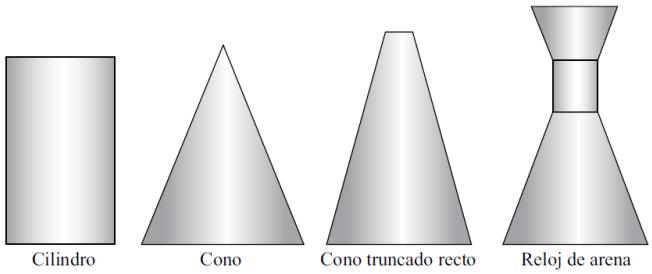

Figure 2. Evaluated shapes.

Figure 3 shows the results of a theoretical study of the three reservoirs. The reservoir in which it is observed least differences at the interest range (between 11,9 and 13,1%) is the cylinder or regular prism. The geometry that magnifies most the measurement is the cone, closely followed by the truncated cone.

Even though the theoretical results for the cone were better than the ones obtained for the truncated cone, in practice, the sharp section at the top of the cone is unable to contain coffee beans and therefore the regular cone behaves as a truncated cone with granular material. This reason led to select the truncated cone as the best geometry to determine volume reduction.

For the final design of the reservoir (Figure 4), it was also considered the following characteristics:

- Maximum size of the perforations: 6 mm
- Minimum perforated area: 55%
- Rigid bottom to avoid deflection

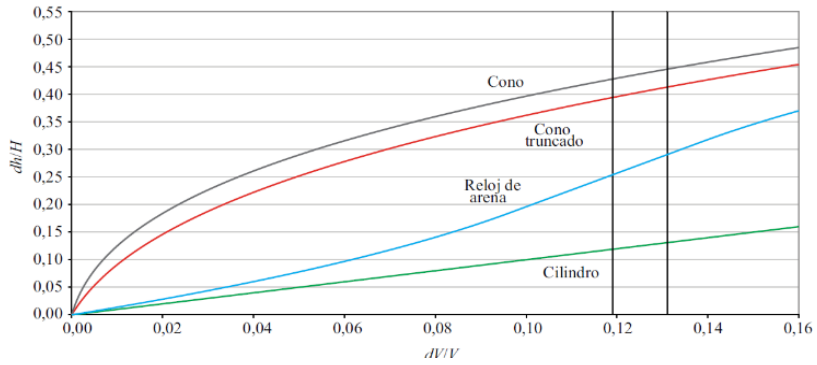

Figure 3. Relative height change with respect to relative volume change.

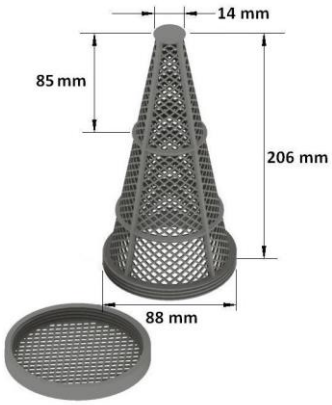

Figure 4. Perforated reservoir with truncated cone shape to determine the end of the fermentation process.

LABORATORY EVALUATION

This phase was conducted in the experimental coffee processing plant of Cenicafé, with coffee of the variety Castillo®, coming from Central Experimental Station, Naranjal. The method was used to follow the fermentation process of 45 coffee batches up to mucilage was degraded. The variables measured were mucilage removal (%) and height of the empty space (mm).

In Figure 5 it is observed a lineal relationship between the two variables with a determination coefficient of 99%. When the height of the empty space was in 85 mm, the mean mucilage removal was 96,7%, with a standard error of only 0,22%, which demonstrates it is an accurate method.

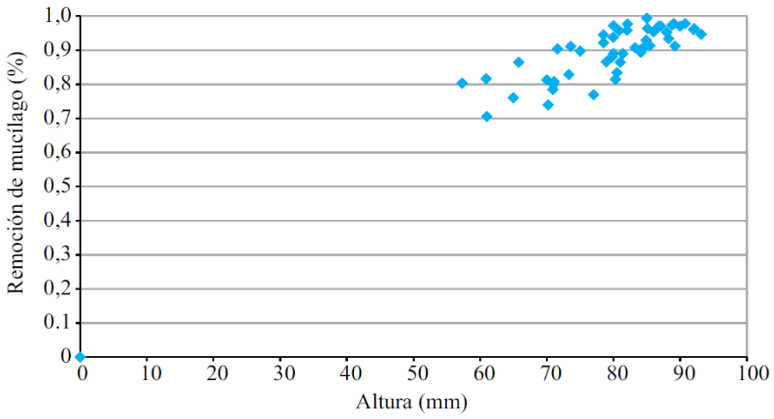

Figure 5. Graph of height of the empty space versus mucilage removal for the runs in laboratory.

VALIDATION

The method was evaluated in 70 coffee farms placed in zones with different environmental conditions and different crop seasons and processing conditions, simultaneously with the traditional methods used by the growers.

FERMAESTRO® gave correct answers the 97,1% of the times, which makes it very reliable to determine the right washing time. The wrong answers were due to bad use, such as working with very low quality coffee or with coffee coming from different days, which are not recommended practices.

CONCLUSION

FERMAESTRO® is and accurate, objective, easy to use and simple method to determine the end of the fermentation process. In this sense, the growers count with a method that helps them to improve quality without adding complexity to the farm labors.

FERMAESTRO® is a valuable tool for the growers with interest in specialty coffees because its use helps them to control not only the fermentation process but the process of selection, pulping, classification and good practices.

With FERMAESTRO® the "washing point" is not determined by time in a process that is affected by numerous factors such as coffee maturity, environmental conditions, variety and processing practices.

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Coffea Arabica Ability to Mechanical Harvesting in Different Genotypes

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SUMMARY

Mechanical harvesting of coffee is growing daily and becoming an irreversible process, thus the force required to release the coffee fruit is a useful parameter in the scaling and adjustment to equipments of mechanical and selective harvesting, which gives a better use to these equipments. The objective of this study was to evaluate the variation on the force of release over the period of fruit maturation in cultivars and progenies to determine which are able for mechanized and selective harvesting. The study was conducted in the city of Três Pontas (Minas Gerais - Brazil), where five plants were sampled from each treatment, this treatment was represented by cultivars that have already been overthrown and other progenies in testing. In each treatment the variable force of release was measured by digital dynamometer developed for this purpose.

INTRODUCTION

The use of agricultural mechanization in the various field operations is one of the great tools that drive up global production, according to the system of mechanized harvesting has lower operating costs and better fruit quality in coffee production compared to manual harvesting system.

The force required for the detachment of the coffee fruit is useful in sizing and adjustment of mechanical harvesting equipment, or in the development of machines for pre-harvest and selective harvesting of coffee fruits where the force for removal of green fruit coffee is generally greater than the force required to remove mature fruits.

The difference of the force of detachment from fruits and cherries differ between cultivars and over the period of ripening. This difference in strength between the stages of ripening green and cherry can be a parameter for the management of harvest.

The assessment of the strength of detachment of the fruits has allowed a better understanding and determination of mechanized harvesting operations due to the good results obtained.

According to the strength of detachment along with the maturation stage of fruits are important parameters for the definition and management of mechanized selective logging, aiming lower losses by natural fall.

Within this context, this study aims to evaluate and compare the strength of detachment of different genotypes of coffee, a parameter which is strongly correlated with the ability of mechanized harvesting, over the period of maturation.

MATERIALS AND METHODS

The experiment was conducted in 2013 at the Experimental Farm EPAMIG - Agricultural Research Company of Minas Gerais, located in the city of Três Pontas, southern Minas Gerais State, 900 m altitude, latitude 21º22'01" S and longitude 45º30'45" W. The average annual rainfall is 1670 mm and the average annual temperature 20.1º C. The soil of the experimental area is classified as oxisol yellow-red, medium texture.

As evaluated material, cultivars and progenies were used in the final stage of selection as: Catiguá MG 2, Topázio MG 1190, Catuaí Vermelho IAC 99, Catuaí Amarelo IAC 62, Araponga MG 1, MGS Travessia, Sacramento MG 1, Pau-Brasil MG 1, Paraíso H 419-1, and Catucaí 2 SL, while for high size were selected Mundo Novo IAC 376/4, Acaiá Cerrado MG 1474, and the progenies in selection phase 29-1-8-5, 32-3-15-20, 32-11-17- 4-2, and 136-1-13-5-3 29-1-5-5-4-2 (Icatu X Catimor), 1189-12-52-2 (R3 PL6), 1189-12-52-2 (Catuaí X Mundo Novo), 464-5-12-22 (Híbrido de Tímor x Mundo Novo).

The evaluation of cultivars was held on June 5, 2013 and on July 2, 2013, to determine the strength of detachment of the fruit was used digital portable dynamometer DD-500 model, five plants of each cultivar, performing measured in Newtons (N) of two cherry fruit and a green fruit in the upper third of the plant, a fruit and a green cherry fruit in the middle third and two cherry fruit and a green in the lower third, according to the methodology proposed.

The experimental design was a randomized block with three replications. Data were tabulated and after, when significant, statistically and means of Scott-Knott at a significance level of 5% probability test analysis.

RESULTS AND DISCUSSION

Table 1 refers to the characteristics of strength of release medium to green fruits and cherries for these assessments. According to the results, the strength of detachment from fruits of the first assessment showed two distinct groups, represented by the lowercase "a" and "b" where the means followed by the letter "b" had lower detachment force, highlighting the growing Catucaí 2 SL, smaller force of detachment with 7.29 (N) and piglets 464-5-12-2-2 that found strength 11.28 (N), for fruit cherry three groups was observed, with the cultivars highlighting the smaller force of detachment Catucaí 2 SL with, 4.76 (N), Topázio MG-1190, 4.77 (N), IAC Catuaí Amarelo 62, 5.29 (N) and the progeny 32-3-15-2 5.60 (N), with higher forces to green fruits compared to cherries.

With respect to the second assessment Catuaí Yellow IAC 62, Araponga MG1, Topázio MG-1190 and Pau Brasil MG1 showed the best results while the progeny, with 29-1-5-5-4 12.35 (N) showed a higher force detachment to green fruits. Analyzing the cherry fruit can notice the decrease in mean strength of detachment from the first evaluation, especially cultivars Topázio MG-1190 and Araponga MG1 tightly below 4 (N), which characterizes cultivars with higher suitability for mechanical harvesting.

Table 1. Release Force Average of green fruits and cherry fruits in different genotypes sampled on two times of evaluations.

Genotypes	Evaluation 1		Evaluation 2
	Green	Cherry	Green
Acaiá Cerrado MG 1474	8,26 b	7,08 a	9,43 b
Pau Brasil MG1	9,62 a	7,04 a	7,75 c
Araponga MG1	8,35 b	6,39 b	7,37 c
Paraiso MG H 419-1	8,15 b	6,42 b	8,16 c
Topázio MG-1190	8,99 b	4,77 c	7,73 c
Sacramento MG1	8,95 b	6,14 b	9,81 b
Mundo Novo IAC 376-4	8,56 b	6,02 b	9,14 b
Catuaí Vermelho IAC 99	9,71 a	6,78 a	8,43 c
Catuaí Amarelo IAC 62	8,83 b	5,29 c	7,34 c
Catucaí 2 SL	7,29 b	4,76 c	8,15 c
Catiguá MG 2	8,89 b	6,32 b	8,50 c
MGS Travessia	9,59 a	6,39 b	9,30 b
32-11-17-4-2	9,77 a	7,30 a	9,89 b
136-113-5-3	10,50 a	7,76 a	9,17 b
29-1-5-5-4	10,41 a	8,41 a	12,35 a
29-1-8-5	9,61 a	6,98 a	9,15 b
32-3-15-20	7,71 b	5,60 c	8,48 c
1189-12-52-2	8,84 b	7,32 a	9,89 b
1189-12-52-2 (R3 Pl 06)	9,59 a	6,79 a	9,36 b
464-5-12-2-2	11,28 a	7,58 a	10,34 b
CV (%)	12,94	14,23	15,29

**average followed by the same letter isn't different itself in the Scott Knott test with 5% of probability.*

Comparing strengths of detachment from fruits and cherries progenies studied, it is possible to verify that there was a decrease in the mean values from the first to the second assessment, these results corroborate those found by, evaluating that the force of detachment found that the strength of cherry fruits decreases as a function of maturation, becoming up to 66% less than that required for green fruits.

In general it can be seen that the force required to cherry fruits were on average about 30% smaller than green fruit during the evaluation period.

evaluated the behavior of the force of detachment throughout the harvest period found that on average the strength to green fruits was higher than the ripe stage, which reinforces the results obtained in the present study. According to this concept to cultivate Topázio MG-1190 has higher average difference between the forces 4.19 (N) which can happen more easily detachment for cherry fruit, unlike what happens with greens, featuring selective harvest .

Another factor is the difference in the behavior of the force of detachment from the color of cherry fruit, where the group of cultivars smaller force is formed by cultivars of fruit, yellow, except the cultivar Araponga MG1, which corroborates where studying the forces of detachment of Catuaí Amarelo IAC 62 and cultivar Mundo Novo IAC 379-19, found lower values for the cultivar with yellow fruits.

CONCLUSION

Based on it, can be concluded that the cultivars Topázio MG-1190, Catuaí Amarelo IAC 62, Araponga MG 1 had lower release force with better trend to mechanization. Another remark was the difference in the behavior of the release force concerning the color of cherry fruit.

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Quality Analysis of Home Based Hand-Pulper Washed Coffees to Unveil Inter-Farmer Gains or Losses Accrued by Averaging Quality Parameters

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SUMMARY

Kenya primarily produce fully washed Arabica coffee (*Coffea arabica* L.) under two distinct production systems; the small scale (co-operative sector) and the large scale (estate) sector. The small scale growers are organized into over 500 co-operative societies in which they are members and deliver their cherries to coffee factories (wet mills) for processing. These co-operatives have management committees that oversee the running of the factories. Coffee farmers in areas where there are no conventional wet mills use hand pulpers to pulp their cherries which they ferment and dry using innovative methods. They deliver the parchment coffee to marketing co-operatives, whereby dry parchment is bulked and delivered to dry mills without prior quality analysis. The purpose of this study was to characterize the quality of coffee from home based hand pulper washing stations owned by members of parchment marketing societies. This study demonstrated existence of high diversity in moisture content, coffee grade proportions and overall cup quality translating to inter-farmer gains or losses. The recommendation derived from this study was to build capacity in all coffee growing regions where coffee from small holders can be pre-screened for quality before bulking for milling and marketing purposes.

INTRODUCTION

Coffee in Kenya is mainly wet processed with a small proportion being dry processed (commonly known as “buni”). The primary unit operations involved in the wet processing of coffee include; harvesting, sorting, pulping, fermenting, washing and grading, drying, storage and conditioning. Each of these steps has an influence on the final quality of the coffee (Mburu, 2004). In the dry mills, parchment coffee is hulled and graded into seven grades according to size, shape and density by use of mechanically agitated sieve graders. Coffee farmers in areas where there are no conventional processing facilities use hand pulpers to pulp their cherries which they ferment and dry using innovative methods (Kathurima et al., 2006). The coffee is marketed through parchment marketing co-operatives, whereby dry parchment is collected, bulked and delivered to dry mills without prior quality analysis. Grading of Kenyan coffee by size, density and shape is a way of adding value to the coffee and each of the grades is marketed independently (Chege, 2012). Apart from the grades, liquor quality also referred to as beverage quality, determines the desirability of coffee for consumption purposes and acts as a yardstick for price determination (Agwanda et al., 2003). The overall objective of the study was to evaluate the grade proportions and cup quality of coffee produced by farmers practicing home based innovative wet processing.

MATERIALS AND METHODS

Sampling, roasting and sensory evaluation

Dry parchment one (P1) samples approximately of one kilogram were collected from 44 farmers practicing home based wet processing of coffee in the west of the Rift Valley region in Kenya. The samples were transported in odorless khaki paper bags to the cupping laboratory at Coffee Research Foundation headquarters. The samples were assessed for moisture content and hulled using a sample huller. The green coffee beans were graded by size and shape to obtain six grades (AA, AB, E, PB, C and T). The coffee beverage produced from roasted coffee (AB grade) was classified as by the method of Devonshire, (1956). The quality assessment was done by a panel of three liquoreres.

CONCLUSION

The moisture content of the samples ranged from 8.5 to 12% (Table 1) The results showed that 73% of the samples were over dried while only 27% had moisture within the recommended levels as stipulated in the Kenyan Code of Practice, (KEBS, 2013). The premium grade AA proportions among the samples ranged from 1- 62% while AB ranged from 26 -88%. (Table 1). A T-test showed that the differences between the minimum and the maximum grade proportions were significant ($P<0.042$). The data on the grades was subjected to principle component analysis (PCA) which indicated that the first two principal components explained 76.84% (PC1 54.86 and PC2 21.68) of the total variation (Figure 1). Most of the samples clustered in PC1 had higher proportions of AA grade. Significant differences at ($P<0.05$) were observed in the overall cup quality (class) as shown in Table 1. Coffees which scored class 4 to Class 5+ were significantly different from those that scored class 5- and class 6. The coffees that were clustered in PC1 were not significantly different in cup quality. Bulking of coffees with similar quality characteristics (grades and cup quality) would increase the percentage of coffee with potential for sale at higher prices giving an advantage to the farmers in terms of proceeds. The summary of the minimum, maximum and mean prices of a 50kg bag of different green coffee grades at the Nairobi Coffee Exchange between 1st Oct 2013 - 31st May 2014 are shown in Figure 2. During that period the grade AA fetched the highest prices. Usually, the differences observed in prices of a particular coffee grade (minimum and maximum) are attributed to the differences in cup quality.

RECOMMENDATION

This study demonstrates the advantage of prescreening coffee for quality before bulking as a means of separating coffee lots of similar quality characteristics for value addition. It is therefore recommended that capacity be built in all coffee growing regions to facilitate coffee from small holders to be pre-screened for quality before bulking for milling and marketing purposes.

ACKNOWLEDGEMENTS

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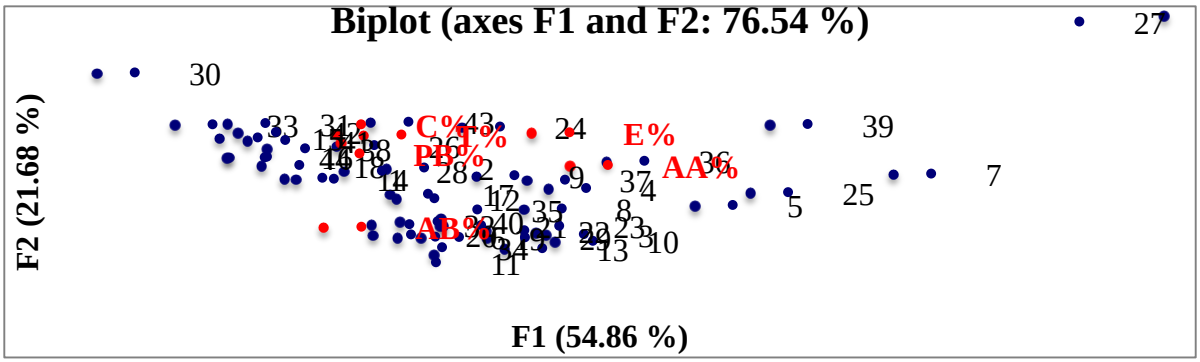

Figure 1. Principle component (PC) analysis plot of first two principle components, illustrating relationship among the coffee samples by grade proportions.

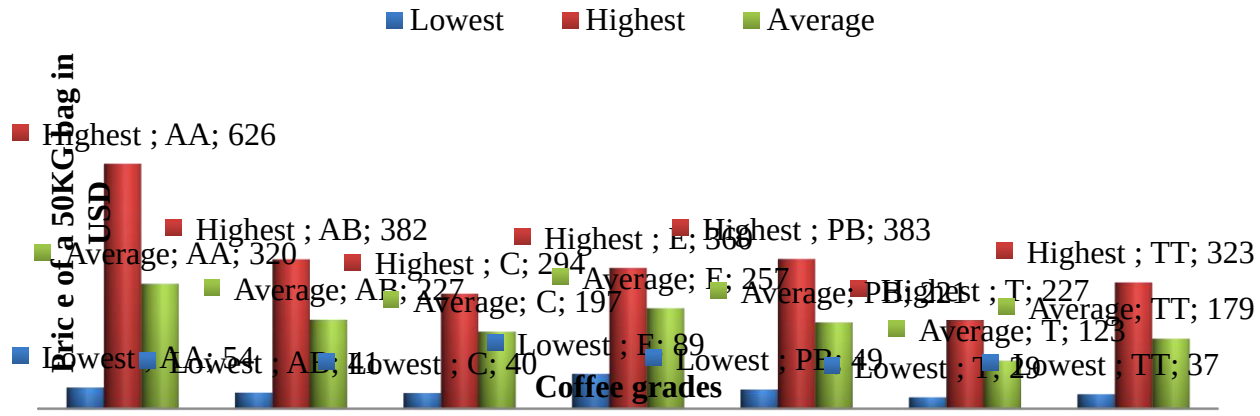

Figure 2. Lowest, highest and mean prices of different coffee grades at the Nairobi between 1st October 2013 - 31st May 2014 (Source: Nairobi Coffee Exchange: 4th June 2014).

Table 1. Percent moisture content, % grade proportions and average overall cup quality analyzed in forty four samples

Sample Code	MC	Grades						overall cup quality
		AA	AB	E	PB	C	T	
1	9.5	9	81	0	3	6	1	4^{a}
2	9.5	17	74	0	2	6	1	4^{a}
3	11.2	23	73	0	1	4	0	4^{a}
4	9.6	28	67	0	1	3	1	4^{a}
5	10.0	39	57	0	1	2	0	4^{a}
6	9.6	9	85	0	1	3	1	4^{a}
7	9.3	60	38	1	0	1	0	4^{a}
8	9.2	24	70	0	1	4	0	4^{a}
9	9.4	15	75	2	2	5	0	4^{a}
10	9.0	22	73	0	1	4	0	4^{a}
11	9.4	10	86	0	1	3	0	4^{a}
12	9.7	13	78	0	1	7	1	4^{a}
13	10.5	17	78	0	1	4	0	4^{a}
14	10.5	6	82	0	2	9	1	4^{a}
15	10.5	1	76	0	1	21	1	4^{a}
16	11.1	2	81	0	2	15	1	4^{a}
17	9.9	14	79	0	1	4	1	4^{a}
18	10.0	7	80	0	3	10	1	4^{a}
19	10.0	11	82	0	1	6	0	4^{a}
20	11.1	7	88	0	1	3	1	4^{a}
21	10.1	15	80	0	1	3	1	4^{a}
22	10.4	17	78	0	1	4	0	4^{a}
23	9.2	22	74	0	1	2	0	4^{a}
24	9.1	27	64	1	1	5	2	4-^{ab}
25	9.4	45	52	1	1	2	0	4-^{ab}
26	10.6	18	72	0	3	7	1	4-^{ab}
27	9.3	62	27	9	0	1	0	4-^{ab}
28	8.5	11	79	0	1	7	2	4-^{ab}
29	12.1	17	79	0	1	3	1	4-^{ab}
30	10.7	2	74	0	4	19	2	4-^{ab}
31	9.2	5	77	0	1	14	2	4-^{ab}
32	10.0	9	84	0	2	6	0	4-^{ab}
33	10.3	3	77	0	3	16	1	4-^{ab}
34	10.8	13	81	0	1	5	0	$5+^{ab}$
35	9.6	16	78	0	1	4	0	$5+^{ab}$
36	9.7	35	57	1	1	5	1	$5+^{ab}$
37	8.9	26	68	1	1	3	1	$5+^{ab}$
38	11.8	7	75	0	3	15	0	5^{ab}
39	10.1	44	46	2	1	6	0	5^{ab}
40	8.8	10	82	0	1	6	1	5-^{b}
41	11.3	6	78	0	2	11	2	5-^{b}
42	9.6	7	78	0	2	11	2	5-^{b}
43	8.8	22	65	0	2	10	1	5-^{b}
44	9.0	4	83	0	3	9	1	6^{b}

Overall cup quality means along the column not sharing a letter are significantly different ($P<0.05$) using Student-Newman-Keuls test. KEY:MC: Moisture content

Kinetics of Coffee Extraction and Particle Microstructure: Numerical Modelling and Experimental Validation in Slurry Extractions

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SUMMARY

Coffee extraction is a key operation for both coffee industry and consumers, which is still not fully understood. As the majority of biological materials, coffee grains show a complex porous microstructure that significantly hinders soluble solids extraction. In addition, ground coffee shows a natural occurring bimodal particle size distribution. In order to deliver high quality brews to the consumer and properly scale up energy-efficient processes, coffee extraction optimisation must rely upon the fundamental understanding of extraction mechanisms.

The main objectives of this work are to (i) develop a multi-particle extraction model to describe the kinetics of coffee extraction; and (ii) evaluate the prediction capacity of effective diffusion coefficients (D_{eff}) values estimated according to measurable and estimable microstructure characteristics of the grains.

SLURRY MODEL EQUATIONS

The model proposes Fickian diffusion (Eq. (1)) in a homogenous medium as the mass transfer mechanism. The model considers the diffusion of a single compound (i.e. all the extractable coffee soluble solids within the particles). The naturally occurring continuous bimodal particle size distributions are approximated to discrete bimodal distributions formed by spheres of a measured sphericity.

$$\frac{\partial C_S}{\partial t} = D_{eff}\left(\frac{\partial^2 C_S}{\partial r^2} + \frac{2}{r}\frac{\partial C_S}{\partial r}\right) \quad (1)$$

$$t = 0\ ;\ C_s = C_{s,0}\ ;\ (0 \pounds r \pounds R_i) \quad (2)$$

$$t = t\ ;\ D_{eff}\frac{\P C_s}{\P r} = 0\ ;\ (r = 0) \quad (3)$$

$$t = t\ ;\ C_s = \frac{C_b}{K}\ ;\ (r = R_i) \quad (4)$$

$$J_i = -D_{eff}\left.\frac{\P C_s}{\P r}\right|_{r=R_i} \frac{u_i m}{V_i r_{particle}} \quad (5)$$

$$C_b(t) = \frac{\int_0^t \sum_{n=1}^{j} J_i 4\rho (R_i f)^2}{V_w - V_{wp}} \quad (6)$$

$$D_{eff} = D_b \frac{\varepsilon}{\tau} f(\lambda) \quad (7)$$

C is the concentration ion the grain, D_{eff} is the diffusion constant of the diffusing species, D_b is the bulk diffusion constant, R is the radius of the particle, K is a partition coefficient,

Eq. (2), (3) and (4) represent the initial and boundary conditions of the model respectively. Eq. (5) calculates the mass flux coming out of each particle size class, the last factor scales for the fraction of mas per unit volume of the given particle size. Eq. (6) integrates over time the flux multiplied by the particle area and calculates the bulk concentration of soluble solids, the particle radius is scaled by a sphericity, the volume of water in the slurry is corrected for that adsorbed in the particle . Eq. (7) estimates the effective diffusion coefficient as a function of measured microstructural characteristics. The model was solved with COMSOL Multiphysics®.

RESULTS

Figure 1 shows the estimated D_{eff} as per Eq. (7) for three species present in coffee soluble solids form high to low D: caffeine, galactomannans with a degree of polymerisation (DP) of 20, and galactomannans DP = 45 (Nunes & Coimbra, 2001) and for four particle sizes. Particle porosity (ε) and tortuosity (τ) were derived from mercury porosimetry experiments as in del Valle et al. (2006).

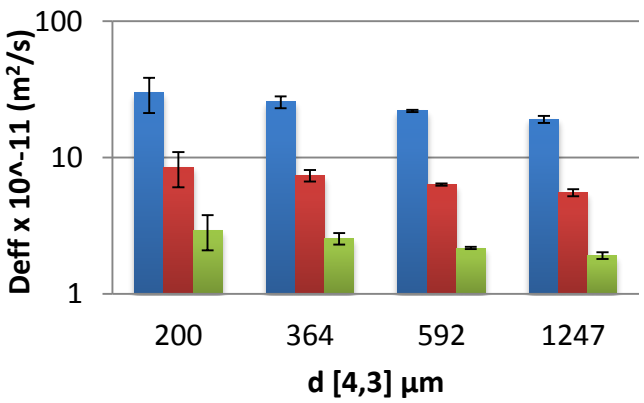
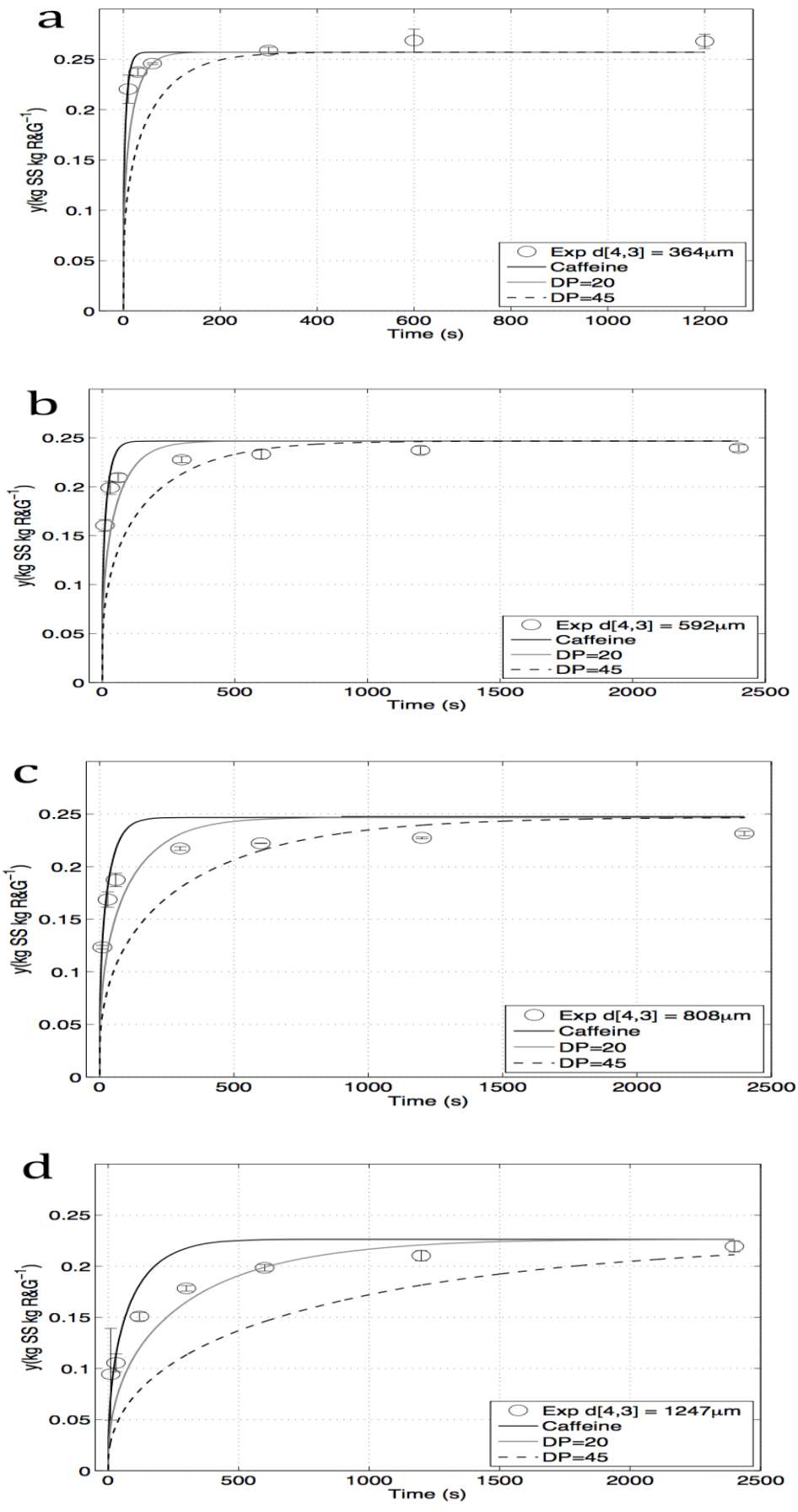


Figure 2 shows the simulated extraction yields (lines) under the assumption of all the coffee soluble solids being either caffeine, galactomannans DP 20 or DP 45. Experimental extraction yield (points) were obtained in slurry extraction system.


a
b
c
d
y(kg SS kg R&G^{-1})
Time (s)
Exp d[4,3] = 364μm
Exp d[4,3] = 592μm
Exp d[4,3] = 808μm
Exp d[4,3] = 1247μm
Caffeine
DP=20
DP=45

Earlier time points are better by D_{eff} caffeine whereas, at longer times, D_{eff} DP 20 better describes the data. The different diffusion rate of species and their associated flavour attributes, presumably underpins how extraction affects coffee brews sensory profiles.

CONCLUSION

A novel mathematical model to describe coffee extraction in a slurry system was developed and validated against experimental data. The process can be modelled with a single D_{eff} with an acceptable error (Mean Percentage Error ~ 9-18%). Bimodality of ground coffee and particle microstructure are accounted for in the model.

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Evaluation of Capacity Expansion of Moca Grains and its Comparison with the Conventional Ones

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SUMMARY

The moca coffee has grains with ovoid shape, caused by lack of fertilization of a shop in the ovarian fruit. During roasting physical and chemical changes occurring in grains, releasing gases that form the aromatic principles responsible for the aroma and flavor of roasted coffee and driving the expansion of the grain. The objective of this work was to develop mathematical model to explain the unit volumetric expansion of grain moca, due to loss of moisture during roasting. Moca grain (*Coffee arabica L.*) benefited was used. Roasting temperatures were 260, 300 and 340 °C. Samples were removed every minute. Volume of the grains was determined using a caliper. The moisture was determined by drying in an oven with air circulation at 105 ° C for 24h. The unit volume increase ranged from 58.16% (340 ° C) to 92.54% (260 ° C) for the moca, and the "b" axis showed the greatest increase during the roast (22.40 to 32.62%). For conventional ones, the increase ranged from 58.16% (340 ° C) 92.54% (260 ° C), wherein the axis "c" showed the largest increase during roasting (22.40 to 32 , 62%).

INTRODUCTION

The moca grains differ from flat ones because they are rounded, longer than wide, the tips are more honed and having a central groove in the longitudinal direction. The moca coffee came from of the not fertilization of an eggs from the fruit that typically has two stores. Thus, only one grain grows, filling the void left by the other and taking the rounded shape (TEIXEIRA, 1999). The most demanding markets generally tolerate up to 10% of moca grains for lots classified as flat grains. Marketing of seeds are tolerated at most 12% of moca beans (BRASIL, 1992).

According Matiello et al. (2002), the presence of large number of moca grains indicates that there is some deficiency in fertilization, related phenomenon basically a genetic problem with interference also of climatic factors and nutrition. At the base and even among the productive branches, the fruits are larger and have a higher percentage of flat beans. Already on the tip of the branches, the grains are smaller and larger percentage of moca grains occurs**.**

Roasting is the most important and crucial process in coffee quality. In this step the inside of the grain is being subjected to elevated temperatures (between 200 and 250 °C) for a period of 6 to 30 minutes, according to the type of coffee. During this process, a dry distillation (pyrolysis) occurs with continual increases in temperature accompanied by changes in destruction, formation and transformation of compounds from the grain. Whereas the physical are essentially volatile in nature and give the characteristic flavor of roasted coffee (FERRÃO, 2009).

According Sivetz and Desrosier (1979), the roasting process can be divided into three consecutive stages: drying, roasting and cooling. At first, the weight loss is due to water elimination and liberation of volatile compounds present in the grains. At this stage, the beans change from green to yellow. The second is characterized by exothermic pyrolysis reactions that result in the modification of the chemical composition of the grains, due to the release of large amounts of carbon dioxide. The colors of the beans change from light brown to dark due mainly to the caramelization of sugars. The third step is required to promote the immediate injection of cold air or water spray cooling to avoid charring of the product.

The last stage of roasting is characterized by the expansion of the grains whose volume doubles. The increased volume of coffee during roasting can be quantified by measures of its three principal axes (PITTIA et al., 2001), but there are no reports in the literature of direct volumetric measurements. During these three processes which include roasting, the study of the consequences (changes in water content, color and expansion of the grains) becomes necessary and mathematical modeling is important for prediction analysis of these data. This assessment provides for modeling parameters for the study of heat and mass transfer during the drying process to scale roaster and enable more practical, the prediction regarding the quality of the final product.

The objective of this study was to evaluate the ability of volumetric expansion unit grain moca coffee, submitted to different roasting temperature conditions: 260, 300 and 340 °C, and its comparison to conventional grains, subjected to the same experimental.

MATERIALS AND METHODS

The experiment was carried Laboratory of Physical Properties and Quality Evaluation of Agricultural products belonging to CENTREINAR, located at the Federal University of Viçosa, Viçosa-MG. Moca coffee beans, retained in the sieve 12, and conventional coffee beans retained on sieve 16, both of *Coffea arabica L*., red Catuaí, from a coffee betterment located in Viçosa were used.

In the roasting process, samples of 350g were placed into a roaster of the direct combustion gas rotating at 45rpm with four cylinders. We used the infrared thermometer, Mult-Temp portable tag, which provides readings between -50°C and 500°C with a response time of 1 second. Roasting temperatures were 260, 300 and 340°C. These temperatures are used to represent light, medium and dark roasts respectively.

Caliper was used to measure unit volume.

The experiment was performed with five replicates for each temperature.

RESULTS AND DISCUSSION

The grain of conventional coffee retained on sieve 16 was chosen to compare to the moca retained on sieve 12, because these grains have similar values of specific mass, and therefore, comparisons become feasible.

The results show that the roasting of the beans was considered uniform. The roasted grains had moisture between 1.5 and 2.2%. The results regarding the loss of mass and unit volumetric expansion are shown in the following table (Table 1).

Table 1. Results from the roasting of coffee beans moca and conventional ones.

Results from moca grains					
Temperature (˚C)	Mass lost (%)	Unit volumetric expansion (%)	Expansion by axis (%)		
			a	b	c
260	18.1069371	92.6	22.51	32.619	25.26
300	16.38524614	82	19.982	27.521	19.78
340	19.58291673	65.9	14.65	22.405	11.61
Results from convencional grains					
260	18.0674682	81.9	a	b	c
300	17.11326615	82.6	16.58	26.57	33.25
340	19.88273574	89.9	19.77	22.89	31.58
			18.95	21.46	34.25

Can be seen that the grain moca showed a mean weight loss of 18%; greater volumetric expansion at lower temperature roasting, because under these conditions, the roasting process was more time consuming when compared to the other conditions studied. Moreover, among the axles, which was expanded over the "b", in relation to others.

Already, over conventional grains, it appears that the largest expansion occurred at higher temperature, and among the axes measured, which increased the most was the "c".

The above results are in agreement with those obtained by other researchers such as France, 2002; and Vargas-Elias, 2011 and 2013.

This behavior of differential expansion between the grains is mainly due to each format. Once the moca grain has a more rounded shape (approaches an ellipsoid), different from the conventional grain having a flat face. These morphological differences allow each grain expands differently during the roasting process.

CONCLUSION

It was concluded that the capacity expansion of grain moca was higher in the lower temperature because these conditions roasting, the process was slower when compared to the others. This was seen both in the apparent volumetric expansion as in the unit.

Furthermore, we found that the "b" axis showed the greatest expansion.

Moreover, conventional grain showed higher expansion capacity and higher temperature in the axis with the highest expansion was "c".

These differences in behavior related to expansion during roasting, is due to morphological differences of grains analyzed.

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Application of Multivariate Statistical Techniques for Data on the Costs of the Post-Harvest Processing of Coffee

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SUMMARY

The choice of method of processing coffee is decisive on the profitability of the coffee activity, and will depend on several factors. Thus, due to the existence of many variables is common producer questioned the viability of certain types of processing. Thus, the objective of this study was to conduct a study of the major influencing factors in the cost of post-harvest coffee. Forty-six farms in the regions of the Cerrado, Matas de Minas and southern Minas Gerais answered a questionnaire in order to enable this analysis. The application of multivariate techniques of cluster analysis, factor analysis and principal component analysis, allowed us to conclude that farms with higher costs simulated were those with the highest percentages of wet coffee production.

INTRODUCTION

In view of the rapid recent expansion of the specialty coffee market with increasingly demanding consumers, it is essential to improve the quality of the beverage in order to meet market demand and increase the income of the grower (Resende, et al., 2011). One of the determining factors for this situation is the post-harvest processing (Abrahão et al., 2010). Borém (2008) affirm that most agricultural issues related to Brazilian coffee production had a very favorable evolution. However, there is lack of information on the management of technology knowledge. Such information could help us ensure high quality and low cost for the post-harvest processing of coffee.

According to Valente (2011), determination of costs is relevant for this analysis and essential for decision makers.

Pós-Café consists of a support system aimed to facilitate decision making regarding the most cost-effective post-harvest processing, that is, it uses human knowledge to solve problems that require the presence of a specialist in this stage of coffee processing.

The multivariate techniques make it possible to measure a particular set of characteristics, considering the existing correlations, so that inferences on the set of variables are made at a given significance level (Mardia et al., 1997).

In multivariate analysis of variance, a wide range of multivariate methodologies is used, which makes it possible to complement the results obtained and assist with the practical application of the information (Benin et al., 2009).

Since many variables are associated to the costs of the post-harvest processing of coffee, the present paper is aimed to study the major variables that influence the referred cost, by means of a decision support system (Pós-Café) and multivariate statistical techniques.

MATERIALS AND METHODS

Application of the questionnaires to coffee growers

Forty-six farmers in the regions of Cerrado, Matas de Minas and Southern Minas Gerais answered the questionnaire. These regions were selected because of their use of several types of post-harvest processing of coffee. This ensured a more consistent study of the variables related to the costs of the post-harvest processing of coffee.

Simulations of the computer program

Data from each questionnaire answered by the 46 farmers were used as inputs in simulations processed in the support system aimed to facilitate decision making regarding the most cost-effective post-harvest processing of coffee, called Pós-Café.

Statistical analysis

The software used to perform the analyses were: Statistica and R. At this stage, multivariate statistical techniques for an in-depth analysis of data from the questionnaires applied to the 46 formers were discussed.

Cluster analysis of farms

With the use of cluster analysis it has been possible to classify quantitative variables into relatively homogeneous groups and to verify the similarities between them (Mardia et al., 1997). Data were standardized and the type of distance chosen was the squared Euclidean distance due to the lack of repetitions in treatments, as suggested by Cormack (1971). The cluster method used was the complete linkage method that favors compact clusters.

Factor analysis

Used to explain the behavior of a relatively large number of response variables, regarding a small number of factors (Malhotra, 2001). Besides, correlations between the variables were also studied, especially in relation to the simulated post-harvest cost. For the selection of the number of components, the Kaiser's criterion was used, cited by Mardia et al. (1997), i.e., eigenvalues greater than one.

Principal components analysis

Had confirmatory character, both with the aim of confirming the groups of variables formed through the cluster analysis, and regarding the correlations identified in factor analysis. (Jackson, 1980).

Correspondence analysis

Proposed to verify the correspondence between different categories concerning the observed variables (Greenacre, 2007). The R software was used, more specifically with the FactoMineR library.

RESULTS AND DISCUSSION

Multivariate statistical analysis for the cost of the post-harvest processing of coffee

46 farms responded to the questionnaires for some of the input variables. Although other variables have also been used in the simulation costs Postgraduate Café , these were selected for multivariate analysis directly influence the cost of post-harvest coffee:

QS - Number of bags of coffee beans picked (bags); QDC - Number of days required for coffee harvest (days); PCVU - Percentage of coffee produced by the wet processing method (%); CMO - Cost of labor per working day (R$); TER - Declared area of the patio (square meters); SEC - Declared drier capacity (liters); CSIM - Post-harvest simulated cost per coffee bag (R$).
The answers related to the six first variables are the data informed by the interviewed farmers. The data related to variable number 7, namely CSIM, were originated from software simulations using the Pós-Café tool.

The following multivariate analysis techniques: cluster analysis, factor analysis, principal components analysis and correspondence analysis were used for the data on the costs of the post-harvest processing of coffee.

Cluster analysis for the costs of the post-harvest processing of coffee

In cluster analysis for standardized data, using the complete linkage method and squared Euclidean distance, the result was the dendogram of Figure 1.

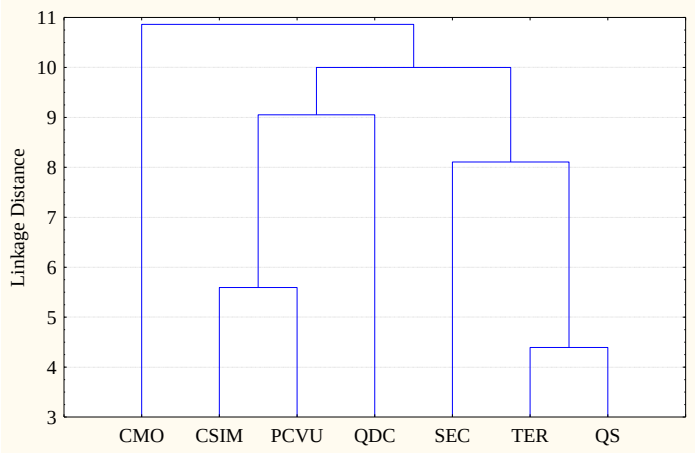

Figure 1. Vertical dendogram of data on the costs of the post-harvest processing of coffee.

In the referred dendogram, based on the distance 5.60, the formation of 4 different groups is observed: a group composed of variable CMO; a group composed of variables CSIM and PCVU; a group composed of variable QDC; and a group composed of variables SEC, TER and QS. Once these groups are formed by the similarities between the variables, it should be said that CSIM and PCVU have high homogeneity, that is, they are correlated variables.

Factor analysis for the costs of the post-harvest processing of coffee

In addition to conducting a study of correlations between variables measured the adequacy of the data through the KMO test, which gave a value of 0.61 and the Bartlett Test value of 2669.629 and significance level of p = 0.000 . Through these values demonstrates that the factor analysis could be conducted, obtaining a degree to near reasonable, according to the classification of KMO (Bezerra and Corrar, 2006).

This analysis explained the behavior of the seven response variables regarding two factors. The response variables were grouped by their correlations.

Considering the seven variables and analyzing the results obtained, it can be seen that the 1st and 2nd eigenvalues are greater than one and explain 65.83% of the variance, as shown in Table 2. The other eigenvalues are smaller than one. Thus, the data will be summarized by the 1st and 2nd principal components.

Table 1. Eigenvalues Extraction: using the method of principal components.

Value	Eigenvalue	% Total Variance	Cumulative Eigenvalue	Cumulative %
1	2.984130	42.63043	2.984130	42.6304
2	1,624432	23,20617	4,608562	65,8366
3	0,983149	14,04498	5,591711	79,8816
4	0,796313	11,37590	6,388024	91,2575
5	0,286391	4,09130	6,674415	95,3488
6	0,261929	3,74184	6,936344	99,0906
7	0,063656	0,90937	7,000000	100,0000

CONCLUSION

The farms can be grouped in relation to yields on bags of coffee (QS), yard areas declared (TER) and declared capacity dryers (SEC). Furthermore, the simulated farms with higher costs (CSIM) are those that have the highest percentages of wet coffee production (PCVU).
The cost simulated by bag of coffee in the post-harvest processing of coffee was characterized for being independent from the influence of the many studied factors. Since only the variable percentage of coffee produced by the wet processing method showed a relatively significant correlation with cost, it is concluded that the influence of the other factors occurs independently for the studied farms.

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Advances in Semi-Mechanized and Mechanized Coffee Harvesting in Colombia

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INTRODUCTION

Manual harvesting is the most expensive labor in coffee production, responsible for about 40% of the production costs. A significant rise of harvesting capacity is the most effective way to reduce coffee production costs, because massive harvesting allows to establish new payment methods to benefit coffee growers and coffee pickers. In that way, semi-mechanization and mechanization of coffee harvesting are options to meet the objective, however, the extreme Colombian conditions and the rigorous quality standards make this work a big challenge.To overcome the problem, Cenicafé has developed and tested several technologies to improve coffee pickers capacity while meeting quality standards. Semi-mechanized harvesting is framed in the use of motorized portable tools to selectively detach fruits from the trees, and mechanized harvesting refers to the use of self-propelled or remote-powered machines for massive detachment of fruits.

SEMI-MECHANIZED COFFEE HARVESTING

Engineering approaches to achieve massive harvesting with portable-motorized tools is divided in two: own developments and adaptation of technologies. And the second one is divided in technologies for coffee harvesting and technologies for coffee-like products.

Own Developments

The own developments began with motorized machines driven by small power plants, then they used IC engines as power source, and finally batteries and DC motors. The most advanced equipment is called Alfa (Figure 1), which has two rechargeable batteries to power a 15W DC motor with a striker coupled to its shaft. The batteries are carried out in the operator waist. This tool, complemented with plastic meshes on the ground to receive the detached fruits, has allowed pickers to rise 100% their capacity. The harvested coffee meets the standards for immature fruits content. However, the capacity increase was lower than expected because extending and collecting the plastic meshes take around 33% of the harvesting time.

Technologies for Semi-mechanized Coffee Harvesting

Derriçadoras are portable machines developed in Brazil (Figure 2) to simultaneously vibrate and strike coffee branches and fruits, throughout two oscillating fingered strikers, out-of-phased 180°. In spite they were developed to harvest coffee, the results with this kind of machines were not satisfactory for the Colombian conditions. Only in exceptional cases, when the crop presented high load and concentration of mature fruits, the device rose the capacity around 100%, with an immature fruit content above 10% and leaving around 15% of the mature fruits in the trees.

Figure 1. Alfa device for semi-mechanized coffee harvesting.

Figure 2. Derriçadora for semi-mechanized coffee harvesting.

Technologies for Coffee-like Products

Portable stem shakers (PSS) are machines driven by IC engines (Figure 3), used to detach olives, which have showed good performance in coffee harvesting. The use of PSS with plastic meshes to receive the detached coffee fruits has allowed to increase pickers capacity up to 300%. However, the immature fruit content and the amount of fruits left on the trees have been far away from the acceptable range. PSS is considered a promising technology for coffee harvesting because of the high capacity, but it is aimed to conduct much more research in order to put the two mentioned parameters into the acceptable range.

Another technology developed for other kind of fruits that has had good performance with coffee harvesting are Electric Strikers (ES) (Figure 4). This technology combines the advantages of the high efficiency of electric DC motors and the portability and autonomy of modern batteries, which make it more viable than the Derricadoras and the PSS in economical terms. However, the results obtained with the ES are similar to those obtained with the *Derricadoras*.

Figure 3. Portable Stem Shaker in coffee harvesting.

Figure 4. Electric Strikers in coffee harvesting.

MECHANIZED COFFEE HARVESTING

Several attempts have been done in order to obtain the mechanical coffee harvester suitable for the Colombian conditions. The first attempt consisted in the construction of a machine similar to the coffee harvesters developed in Brazil. The machine, called COVAUTO, presented low harvesting quality, low efficacy and problems related to the combination of heavy weight, low flotation tires and wet soil which caused the vehicle to get stuck, even in flat terrains.

Another approach, called Ergatis, consists in a machine and a concept to massively harvest coffee. The machine is a four-wheeled vehicle that follows the terrain contour with a control to keep the center of gravity in the middle of the wheel base, in order to allow working in slopes up to 100% without tipping over. The concept consists in the use of cable to pull the machine up and let the machine to go down while harvesting the rows of trees running parallel to the terrain slope. The machine and concept are still in developing status [1].

Figure 5. Coffee harvester Covauto.

With the lessons learned from the previous work, a more adjusted harvester was designed and built, which name is Covautico. The machine has a lighter structure and keeps the oscillating strikers developed for coffee and other fruits. The machine can be pulled by several sources such as a small tractor, a motocultor or even a mule. The machine is following design loops in order to overcome problems related to mobility, fruit reception and stiffness.

Figure 6. Covautico for massive coffee harvesting.

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Management of Leachates and Waste Waters Coming from Wet Coffee Processing

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SUMMARY

Among the developments of Cenicafé it is found the technology for eco-friendly wet coffee processing with byproducts management – Becolsub, which utilizes small amounts of water and avoids more than 90% of the water contamination. The technology has been such impacting that more than 90% of the potential users have adopted it and processed more than 30% of the Colombian coffee in the last years. Besides these great results, the leachates continue being a problem because they are very difficult to treat before throwing them to water streams. In this paper we present an option to take advantage of the thermal energy that is lost during mechanical coffee drying, to reduce the environmental impact. The alternative consists in using the hot gases that exit throughout the chimney to evaporate leachates put in metallic trays. With the arrangement it was allowed to evaporate a mean of 71,42% of the water in the leachates and it was obtained a global evaporation rate of 0,61 L/h. In addition to the leachates coming from coffee processed with the Becolsub technology, solar dryers were used to dry waste waters coming from traditional wet coffee process with low water consumption. In solar tunnels it was evaporated 92% of the water and the evaporation rate was 2,74 L/day/m^2, while natural evaporation of water in the same conditions is 3,47 L/day/m^2. The resulting matter can be used as organic fertilizer in order to achieve a total control of the water contamination by wet coffee processing.

INTRODUCTION

Mucilage of coffee is removed in Colombia by natural fermentation and washing, or by mechanical means with the Becolsub technology, which name stands for the term Eco-Friendly Wet Coffee Processing With Byproducts Management. Studies achieved in Cenicafé showed 72% of the contamination generated by the wet coffee processing, corresponding to 82.080 mg of chemical oxygen demand (COD) per kg of fruits, are due to the use of water in pulping and conveying of the fruits skin. The resting 28%, corresponding to 31.920 mg of COD per kg of fruits, are due to mucilage. The Becolsub technology basically consists in pulping without water, mechanical demucilaging and mixing of the by-products (fruit skin and mucilage) in a screw conveyor. With the Becolsub technology the specific water consumption is reduced to only 0,7 L/kg of DPC and more than 90% of the water contamination is controlled within the process by using the right water needed for mucilage removal and washing. Lixiviation takes place when degraded mucilage contacts pulp and takes out the phenols in it. When phenols contact air change their color to black, which is a characteristic of leachates of coffee. Studies achieved in Cenicafé showed that when the Becolsub technology is used, around 65% of the mucilage is retained in the pulp and 35% of the volume is drained as leachates.

MATERIALS AND METHODS

This reseach work took place in the National Research Center of Coffee of Colombia – Cenicafé in the municipality of Chinchiná (Colombia), at an altitude of 1.310 m, 21,5ºC of mean temperature, 79,5% of mean humidity and a mean annual precipitation of 2.662 mm. The work had two phases. The first phase consisted in verifying the amount of leachates obtained when using a machine Becolsub with a capacity of 300 kg of fruits per hour, operated according to the recommendations established in Cenicafé. The second phase consisted in the design, construction and evaluation of a device to take advantage of the exhausting gasses to heat up and evaporate leachates put in trays.

The Becolsub equipment (Figure 1), consisted of a pulping machine, a cylindrical screen, a mechanical demucilager Deslim and a screw conveyor to transport and mix mucilage and pulp. The byproducts were stored in perforated reservoirs and the drains were collected and measured.

Figure 1a. Becolsub equipment to process 300 kg of coffee fruits per hour. Figure 1b. Mechanical dryer.

A commercial mechanical dryer (Figure 1b), with a total capacity of 56 kg of DPC was modified to use the gasses flowing up in the chimney, to heat up metallic trays containing leachates. The trays were designed taking into consideration the volume of leachates and the capacity of evaporation of the exhaust gasses.

Leachates evaporation in a mechanical dryer

The heating system consisted in an isolated duct, with six openings matching six metallic trays, through which the exhaust gasses were conducted out. Some partitions were included in the duct with the purpose of increasing the time of the hot gasses contacting the trays in its way out. (Figure 3) shows the design of the duct.

Figure 3. Evaporator of waste waters of coffee in mechanical drying.

Leachates evaporation in a solar dryer

A solar dryer with a transparent plastic cover (Figure 4), known as parabolic dryer, was also used to dehydratate leachates. In the first phase 24 runs were conducted using the mentioned Becolsub equipment processing an experimental unit of 300 kg of coffee fruits of the variety Castillo®. The response variable was the volume of leachates.

Figure 4. Solar parabolic dryer.

In the second phase six runs were conducted using the try dryer with an experimental unit of 50 L of leachates. The response variable was the volume of water evaporated. The information was analyzed taking the mean and standard deviation, coefficient of variation and minimum and maximum for each of these variavles in the process and stages evaluated.

RESULTS AND DISCUSSION

Six runs were carried out and the mean drying time for the coffee was 30,5 hours at a mean air temperature of 50,4ºC. The mean temperature of the gasses in the chimney was 176,6ºC, which indicates the high heating power they still have. The mean temperature of the leachates in the first tray was 53,4ºC and for the leachates in the sixth tray was 31,8ºC, (Figure 5). The mean temperature of the gasses after passing the trays was 37,7ºC, which indicates an efficient process. The mean evaporation after 30 hours was 61,16%, with a range varying between 48,98% and 84,35%. As expected, the greatest rate of evaporation was obtained in the first tray. The global evaporation rate was 0,61 L/h.

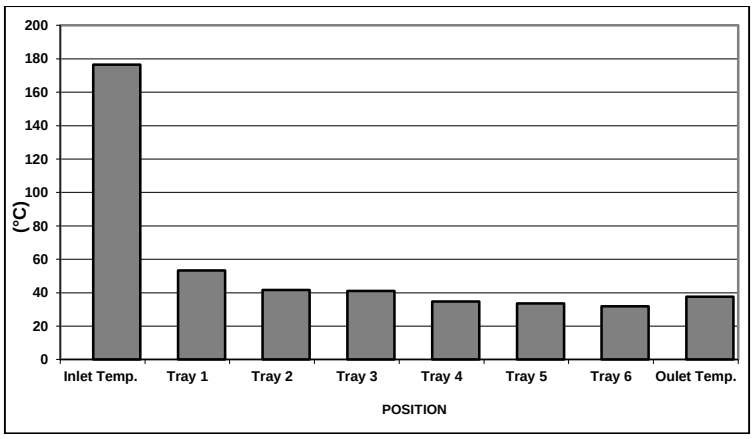

Figure 5. Temperature of the exhaust gasses in the evaporator.

Wastewaters evaporation in a solar dryer

Wastewaters resulting of the traditional wet process of coffee with natural fermentation and a specific water consumption of 0,4 L/kg of DPC, were obtained in order to dry them in a solar dryer. The effluent was put into a 26-square-meter parabolic dryer which floor was previously covered with a plastic film to avoid filtration. These results gave an evaporation rate of 2,74 L/day/m^2 in this kind of dryer, which is very high compared to the evaporation rate of 3,25 L/day/m^2 obtanied in this research work with water in a class A tank and to the evaporation of 3,47 L/day/m^2 obtained by Jaramillo. (Figure 6) the specific evaporation rate for the wastewaters in the solar dryer and for water in a class A tank.

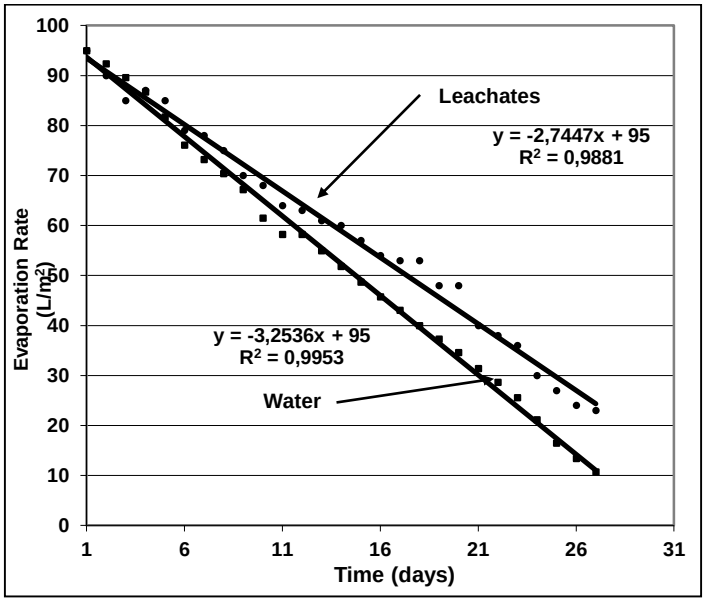

Figure 6. Evaporation rates of wastewaters in the solar dryer and for water in a class A tank.

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Assessment of Coffee Picking Process Indicators

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SUMMARY

The assessment of efficiency, effectiveness, quality and losses indicators is required to establish the actual performance of the coffee picking process. Analysis of these indicators was conducted in the present research that provides the baseline for picking process indicators. Various factors related to the characteristics of the picker, the plantation and harvesting method were evaluated. The traditional picking method and the Canguaro 2M – a tool designed by CENICAFE to assist the picking process- were analyzed. Study results support the conclusions that the picker´s experience, the load of ripe fruits on the tree and the picking method influence significantly the harvesting indicators.

INTRODUCTION

Colombian coffee is recognized worldwide for its excellent quality, this is a result of a selective harvesting process, fruit by fruit, considering only the ripe coffee berries. The harvest is done manually due to the topographical difficulties, the non-uniformity in fruit ripeness and the lack of automatic technologies adapted to local conditions. Consequently, the harvest is a key stage in the coffee industry. The assessment of harvesting indicators is required to establish the actual performance of the coffee harvesting. Analysis of these indicators was conducted in the present descriptive research that provides the baseline for picking process indicators, which could be used by farmers to control and monitor the coffee farm.

METHODS

The harvest process was assessed using the traditional picking method and the Canguaro 2M, a tool designed by CENICAFE to assist the picking process. The Canguaro 2M consists of a bag that is supported on the waist and shoulders of the picker, with two legs that carry the picked fruits to the container or bag as shown in Figure 1. The analysis considered the gender and the experience of the picker, the slope and the load of ripe fruit on the coffee tree. The ANOVA was employed to identify significant differences (α=0,05) between factors.

Figure 1. Picking process with Canguaro 2M.

The study was developed at the Central Station of CENICAFE called Naranjal, located in Chinchiná, Caldas, Colombia. Lots of second and fourth harvests, variety Castillo, were evaluated. The unit of analysis was the pickers (13 persons in total). A picker monitoring was carried out during the workday, considering the traditional harvesting method and the Canguaro 2M. During the monitoring, variables for the assessment of harvest indicators on efficiency, effectiveness, quality and losses were registered. The yield in kg per hour was considered for the efficiency indicator. The amount of mature unharvested coffee cherries was recorded for calculation of effectiveness indicator. The percentage of mature harvested coffee cherries was related to the quality indicator. The amount of the fruits fallen on the ground was considered for losses indicator.

RESULTS

Effectiveness, efficiency, quality and losses were evaluated as Coffee picking process indicators, which were affected by various factors as shown Figure 2.

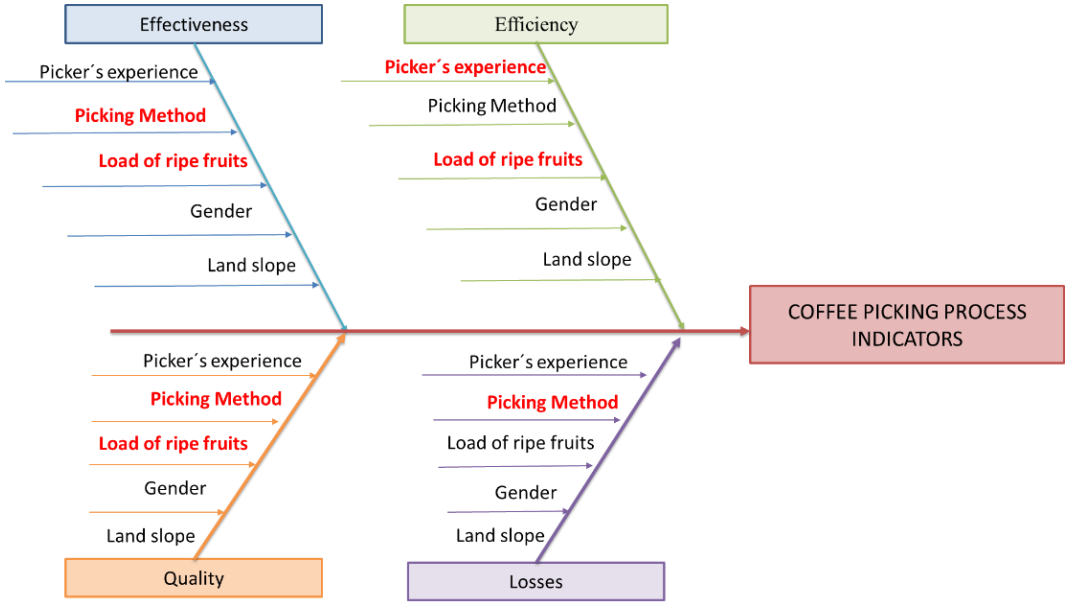

Figure 2. Factors in coffee harvesting.

ANOVA results are shown in Table 1. There are no significant differences between harvest indicators considering picker´s gender. Nevertheless, there are significant differences in efficiency and losses indicators related with the picker´s experience. Regarding to the land variables, the slope did not affect harvest indicators, but the load of ripe fruits on the tree influences significantly the effectiveness and losses indicators.

Table 1. Multivariate ANOVA results.

Factors	P-Value- Indicators			
	Efficiency	Quality	Effectiveness	Losses
A:Load of ripe fruits	0.0000	0.0002	0.0175	0.9290
B:Picker´s experience	0.0104	0.3173	0.1239	0.1247
C:Picking Method	0.5514	0.0159	0.0001	0.0000
D:Gender	0.4480	0.0613	0.6150	0.8610
E:Land slope	0.5954	0.5230	0.6001	0.2024

In addition, significant differences in harvest indicators were observed with regards to the picking method. According to Figure 3, better performance in quality, losses and effectiveness indicators was achieved with the Canguaro 2M.

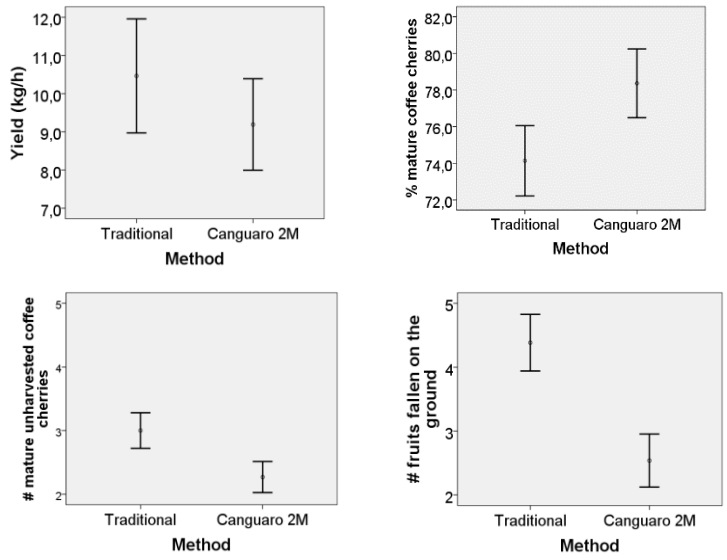

Figure 3. Coffee picking process indicators.

CONCLUSION

Picker´s experience, load of ripe fruits and picking method are the factors that affected the performance of coffee picking process indicators. Better performance in quality, effectiveness and losses indicators are obtained with the Canguaro 2M in relation to the traditional method. Although, higher yield was obtained with the traditional method, there were no significant differences between the yield considering picking method.

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Sensory Analysis of Stored Coffees Subjected to Different Processing and Drying Procedures

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SUMMARY

The aim of this study was to verify the effect of drying in the shade, in the sun, and in a mechanical drier on the quality of wet process and natural coffees. The coffee cultivar used in this experiment was Catuaí Amarelo IAC 62 produced at the Fundação PROCAFÉ (PROCAFE Foundation) in Varginha, MG, Brazil. After selective harvest of the mature coffee fruits, they were immediately sent for processing to the Universidade Federal de Lavras, Lavras, MG, Brazil. Three forms of processing were assessed: natural coffee, semi-washed coffee, and fully washed coffee. After processing, the coffees were then subjected to drying in sieves in the shade, in the sun and in fixed bed dryers with temperature control of the drying air at 35ºC until reaching moisture content near 11% (w.b.). After drying, the coffees were stored and subjected to sensory analysis at 0, 4, 8, and 12 months of storage. According to the results, a harmful effect from drying is seen on the quality of natural coffees dried in mechanical dryers, which was seen throughout the period of storage. The coffees obtained by wet process exhibited greater tolerance to drying than unwashed/natural coffees.

INTRODUCTION

After being harvested, coffee may be processed in two manners, dry process and wet process. In dry process, the fruit is dried in its whole form, giving rise to coffees called dry cherry or natural coffee. Preparation through wet process consists of removing the husk, pulp, and/or mucilage of the mature fruit, which are substrates prone to the development of microorganisms that may lead to the occurrence of fermentation harmful to coffee quality. Wet process may give rise to pulped, fully washed, or semi-washed coffees. To obtain pulped coffee, the fruits are mechanically dehusked and part of the mucilage still adheres to the parchment of the fruits. In obtaining fully washed coffee, after dehusking, the part of the mucilage that still adheres to the fruit is removed in biological fermentation tanks. If removal of this remaining mucilage is carried out mechanically, then the coffee is known as semi-washed.

Various studies indicate that the chemical composition of the coffee beans are dependent on the form of processing used, contributing to distinct characteristics in coffee quality. Recent studies have shown variations in glucose and fructose content, as well as in free amino acids in the green coffee beans depending on the form of processing, without, however, describing the interferences on the drying conditions.

During drying, physiological changes may occur, compromising the quality of the coffee beverage. Various studies have been carried out for the purpose of correlating physiological aspects with the sensory quality of the beverage during this process. Poorly structured,

disorganized and damaged membrane systems due to high temperatures leach a greater quantity of solutes, exhibiting higher values of electrical conductivity.

The drying rate has a significant effect on the quality of the coffee bean. High rates may lead to physical damages, discoloring of the product, stains, and other defects. The coffee drying rate is affected by various factors, such as the temperature and flow of the drying air, the humidity and temperature of the ambient air, and the initial and final moisture content of the product.

Thus, the aim of this study was to verify the effect of different processing and drying conditions on coffee quality over the storage period.

MATERIALS AND METHODS

The coffee cultivar used in this experiment was Catuaí Amarelo IAC 62 produced at the Fundação PROCAFÉ (PROCAFE Foundation) in Varginha, MG, Brazil. After selective harvest of the mature coffee fruits, they were immediately processed at the Universidade Federal de Lavras, Lavras, MG, Brazil.

Three forms of processing were assessed: natural coffee, semi-washed coffee, and fully washed coffee. To achieve these different forms of processing, mature fruits were selectively harvested. Part of the harvested coffee already gave rise to the natural coffee process. The semi-washed coffees were obtained after passing the fruits through the washer, through the dehusker of the coffee cherries, and, finally, through a mechanical mucilage remover for removal of the remaining mucilage. The fully washed coffees were obtained in the same manner as the semi-washed coffee, along with removal of the remaining mucilage through fermentation in water. After processing, the coffees were then subjected to three methods of drying: in sieves in the shade, in the sun, and in fixed bed dryers with temperature control at 35ºC until reaching moisture content near 11% (w.b.).

After drying, the coffees were stored in a cold chamber at a temperature of 10ºC and 60% relative humidity and were assessed after 0, 4, 8, and 12 months of storage. After each period of storage, the coffees were processed and subjected to sensory analysis according to the protocol of the Specialty Coffee Association of America.

RESULTS AND DISCUSSION

The results of sensory analysis of stored coffees as a function of different processing and drying methods is shown in Table 1. At the beginning of storage, significant differences were not observed in sensory analysis among the forms of processing and drying studied. It should be noted that at the beginning of storage, all the coffees were classified as specialty coffees because they achieved a score greater than or equal to 80 points, which, according to the Specialty Coffee Association of America (SCAA), classifies them as specialty coffees.

Nevertheless, significant differences are observed in the coffees assessed at four months of storage, with the natural coffees dried in the mechanical dryers exhibiting the lowest scores in sensory analysis. This behavior was observed in the other assessment periods, i.e., at eight and twelve months.

The harmful effect of drying on natural coffees when they were dried in mechanical dryers may thus be perceived, and this was seen throughout the storage period. In relation to the type of drying, it is believed that the high rate of drying normally used in mechanical dryers

may have a negative effect on coffee quality due to the high temperature used. In this experiment, the drying temperature in the mass of coffee beans, in the fixed bed mechanical dryers used, did not go beyond 35ºC. It is believed that this temperature of the drying air did not negatively affect coffee quality, with the exception of the natural coffees.

In accordance with the results analyzed until now, it may be suggested that natural coffees are more sensitive to drying in mechanical dryers due to the greater drying time, which may lead to possible thermal damage to the beans.

Malta et al. observed that the natural coffees exhibited higher values of electrical conductivity and leaching of potassium when subjected to quick drying, i.e., in mechanical dryers, which denotes a greater probability of these coffees losing quality. Afonso Júnior, studying physical and physiological aspects and coffee quality as a function of drying and of storage, affirms that the quality of pulped and fully washed coffees is less affected in relation to that of the natural coffees. According to this author, the variation of the temperature of the drying air is largely responsible for this loss, while the variation of the relative humidity of the drying air has little effect.

These results are in agreement with recent studies which confirm that the fully washed coffees are more tolerant to drying than natural coffees, regardless of the drying method they are subjected to. According to Taveira, fully washed coffee is more tolerant to drying than natural coffee, regardless of the drying method, exhibiting better physiological quality. Similar results were also observed by Oliveira, who observed higher values of electrical conductivity and of potassium leaching in natural coffees when compared to fully washed coffees, which, according to the author, is the result of the maintenance of the cell structures and the quality of the fully washed coffee. According to Prete, greater tolerance of fully washed coffees to drying in relation to natural coffees is related to the lower time of exposure to high temperatures, due to the removal of the exocarp and of the mucilage.

Table 1. Sensory analysis of stored coffees subjected to different processing and drying methods.

Storage (months)	Drying	Processing		
		Natural	Semi-washed	Fully washed
0	Dryer	82.25 Aa	81.56 Aa	80.69 Aa
	Sun	82.81 Aa	82.25 Aa	82.44 Aa
	Shade	82.06 Aa	80.00 Aa	81.06 Aa
4	Dryer	75.75 Bc	81.50 Aa	81.62 Aa
	Sun	81.00 Aa	80.50 Aa	80.37 Aa
	Shade	78.00 Bb	80.75 Aa	80.00 Aa
8	Dryer	76.25 Bb	82.12 Aa	81.00 Aa
	Sun	80.75 Aa	80.00 Aa	82.15 Aa
	Shade	81.62 Aa	80.62 Aa	80.00 Aa
12	Dryer	78.50 Cb	84.25 Aa	81.38 Ba
	Sun	81.12 Aa	82.50 Aa	81.62 Aa
	Shade	82.12 Aa	82.00 Aa	82.12 Aa

Mean values followed by the same uppercase letters in the rows and lowercase letters in the columns do not differ among themselves by the Scott-Knott test at 5% probability.

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Coffee Certification and Verification in Tanzania: Challenges and Opportunities

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SUMMARY

Certification and verification schemes are growing in popularity due to consumers increasing interest in the economic, social and environmental aspects of coffee production. Based on the public commitments of major roasters, the share of certified coffees in the world trade is projected to grow to 18% by 2015. However, this growth trend is hampered by increasing concerns of high costs of compliance with certification schemes, which majority of smallholder producers are unable to meet. The high costs of certification and verification are seen as barrier, if not exploitation, to small scale coffee farmers to enter niche markets. Evidence on producer benefits from implementing sustainability programmes is mixed thus posing high levels of uncertainty as to whether the benefits of implementing a certain standard outweigh the costs. In Tanzania a number of certification schemes such as organic, fair-trade, rainforest alliance, Utz certified and common code for the coffee community (4C) exist and are being implemented for smallholder coffee producers through coffee co-operatives and on the other hand by large producers (estates). Tanzania Coffee Research Institute (TaCRI) in collaboration with African Fine Coffees Association (AFCA) implement a pilot project on "*Building capacity in coffee certification and verification for specialty coffee famers in AFCA member countries*" to enable farmers to improve the skills to meet certification and verification standards so as to increase the quantity and quality of verifiable and certifiable coffees in the member states. We have trained 1,000 farmers in the Northern part of Tanzania to meet certification and verification standards who will later be linked to sustainability programmes of their choices. This paper, assess the opportunities and challenges smallholder farmer get toward complying with certification and verification schemes.

INTRODUCTION

Certification and verification schemes are growing in popularity due to consumers increasing interest of certified product in the traditional and emerging markets (ITC 2011). Fig. 1 indicates the percentage growth of different certification schemes in the world market as documented by Daniele (2010). The study conducted by ITC (2011) documented that, certified coffee is showing strong growth and higher retail prices, particularly in mature markets. Since then little is documented on the opportunities and challenges that small producers and other actor along the chain encounter in certification and verification standards. According to ICC (2012) public commitments of major roasters, the share of certified coffees in the world trade is projected to grow up to 18% by 2015. However, this growth trend (Fig. 1) is hampered by increasing costs of compliance which majority of smallholder producers are unable to meet. Meanwhile producer benefits from implementing sustainability programmes is mixed thus posing high levels of uncertainty as to whether the benefits of implementing a certain standard outweigh the costs. In Tanzania a number of certification schemes such as organic, fair-trade, rainforest alliance, Utz certified and common code for the coffee community (4Cs) exist and are being implemented for smallholder coffee producers through coffee co-operatives and on the other hand by large producers (estates).

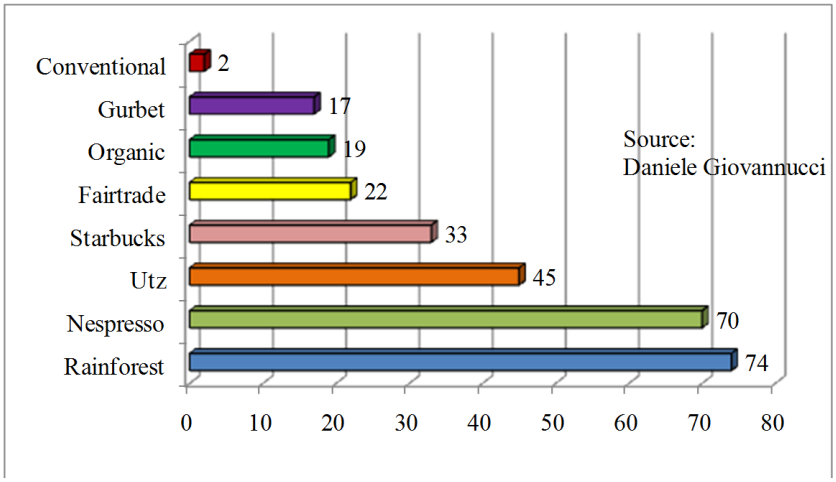

Fig. 1: Growth of certification schemes in the world market

Figure 1. Percentage growth of different certification schemes in the world market.

Tanzania coffee research institute (TaCRI) in collaboration with African Fine Coffees Association (AFCA) implement a pilot project on "*Building capacity in coffee certification and verification for specialty coffee famers in AFCA member countries*". The aim of this project is to improve the skills to meet certification and verification standards so as to increase the quantity and quality of verifiable and certifiable coffees in the member states. The project was implemented through training smallholder farmers to different types of standards that offer specific conditions for their market incorporation. Therefore need to assess the opportunities and challenges toward coffee certification and verification along the value chains.

MATERIALS AND METHODS

The study employ purposive sampling technic to select 20 primary co-operative and randomly sampling technic to select 300 farmers who produce coffee under Fair Trade, Organic and noncertified coffee in Northern Tanzania. Both primary and secondary data were collected. Primary data were collected using structured questionnaire, observation and focus group discussion with key informant while secondary data were collected from various reports and from International Coffee Organization (ICO) website. The information collected stick much on the opportunities and challenges on coffee certification and verification standards in Tanzania in line with the implementation of CFC/ICO/45 project. The study measure awareness of farmers certified by fair trade and organic and compare the effect of these standards against the income collected also production in terms of yield and compliance with the standard criteria both social, economic and environment. Moreover, the implications of CFC/ICO/45 training on different certification and verification standard requirements as impact on behavior changes in farming practices toward adopting sustainable farming management.

RESULTS AND DISCUSSION

Profile of the study area

Data were collected in 78 primary co-operatives of which 84.6% were fair-trade certified while 15.4% were organic coffee certified. The average number of registered co-operative members both male and female was 422 with average land size of 0.5 hectares which is closer

similar to that of Hella (2005). The average coffee production (Fig. 2) in the study area from 2008/09 to 2010/11 production season was 43,412 kgs followed by 33,317 and 31,971 kgs respectively. The trend of production declined by 26% from 2008/09 to 2010/11 due to prolonged drought while the price trend in Moshi coffee exchange (Clean coffee) according to Tanzania coffee board increased by 186% from 2.56 USD per kg of clean coffee in 2008/09 to 4.77 USD in 2010/11.

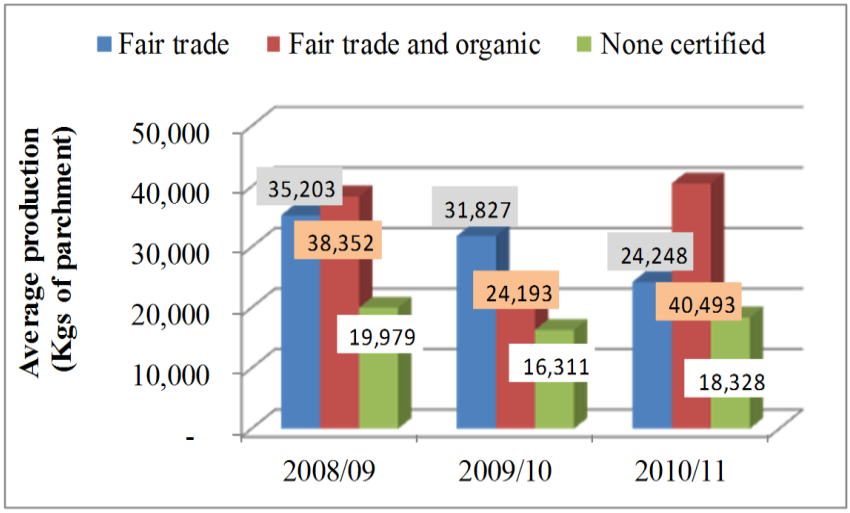

Figure 2. Average trend of coffee production in the study area.

Result in (Fig. 3) indicates the average trend of coffee quality by grades in the study area. The result implies that, coffee quality increased from 2008/09 production to 2010/11 due to increased training on implementation of good agricultural practices and reward of increase in coffee price. Meanwhile no quality data recorded for individual co-operatives due to mixing of coffee from one cooperative to another as they produce low volume.

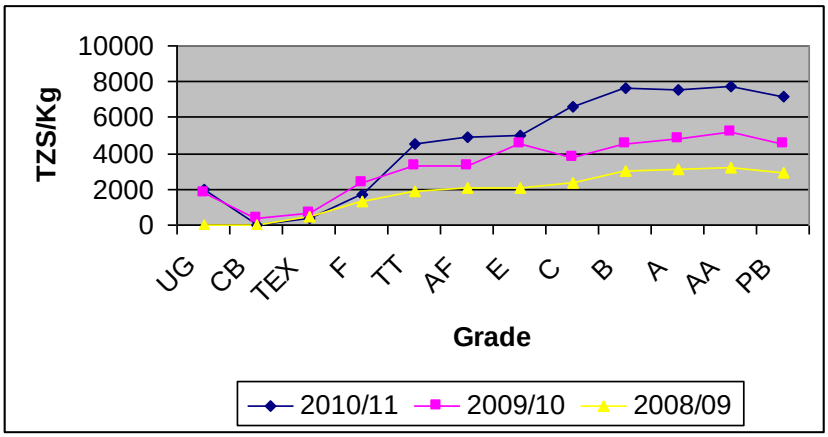

Figure 3. The average trend of coffee quality.

Implementation of CFC/ICO/45 Project in Tanzania

The main focus of the project CFC/ICO/45 is to build coffee certification capacity for selected farmers so as to produce a differentiated product that would attract sustained market recognition. The target group were farmers with existing expertise in practicing good management practices (GMP) and good agricultural practices (GAP). The approach used by this project to attain its goal includes training of Master Trainers (MT), trainers of trainers

(ToTs), verifiers/certifiers and farmers. These categories of trainers were drawn from the different institutions and/or other coffee organisations. Also baseline survey was conducted to identify eligible famers from primary cooperative to be trained.

Progress of the CFC/ICO/45 project in Tanzania

Significant progress has been noted in the implementation progress of the project in Tanzania. Key deliverable in the period under review is the training of four (4) Master Trainers, 19 trainer of trainers (TOTs) and 1000 lead farmers. Also the project trains four (4) auditors on certification and verification standards so as to have our own auditors who will then help the trained cooperatives during auditing about the standard if they aim to be certified. Majority of trained farmers were impressed with project and show interest to be certified by fair trade and organic certification standards. About 49 primary coffee co-operatives (17 under VUASU and 32 under G32) were linked with fair trade certification and verification standard. Meanwhile farmers demonstrated understanding of the important for certification in line with environmental protection, and social protection through implementation of good agricultural practices.

Challenges toward coffee certification in Tanzania

During the implementation of the project it was noted that, majority of farmers were not aware about certification and verification schemes and this is due to the fact that, there is no local institution that promote certification and the current schemes are dominated by foreign institution. Presences of many certification schemes confuse farmers to opt the suitable schemes. Meanwhile respondents from certified co-operative members claimed that, they are not benefiting from the certification due to low coffee price in the market. Hella (2005) documented that low price of coffee is among the major constrains towards increase coffee productivity and quality. Given the fact that, there was no significant difference in price paid to certified farmers against non-certified farmer, there is a need to provide more training to farmers in order to increase competence in terms of quality and volume and complying with the standard. Likewise old coffee trees which are highly affected by coffee berry disease and coffee leaf rust that lead to low yield and poor quality (Teri *et al* 2004) are the reason that also course farmers not to meet the volume required for certification. Therefore farmers need to rehabilitate their farm by planting improved coffee varieties which are resistance to the mentioned coffee diseases and they are high yielding. Aged coffee producer in the study area was the other factor that limits adoption of improved technologies, so there is a need to encourage youth to invest in coffee farming so as to increase production and quality.

CONCLUSION AND RECOMMENDATIONS

In general, farmers are willing to go for certification and produce enough volume of quality coffee that will fetch good price in the market. In order to achieve this, famers need to be financed and receive more training on certification and verification. Also encouraging farmers to adopt implementation of good agricultural practices, planting hybrid coffee varieties that are resistant to coffee berry disease (CBD) and coffee leaf rust (CLR) and rehabilitating their coffee farms. There is also a need to strengthen partnership between farmers and other actors in the chain by providing market information.

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Spent Coffee Grounds Obtained from Ready-to-Drink Coffee-Making Factories for Use in the Fabrication of Negative Electrode Materials of Lithium-ion Batteries

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SUMMARY

Spent coffee grounds are by-products of the ready-to-drink coffee industry, and are discharged in large amounts in Japan. Carbonaceous materials obtained from spent coffee grounds have been proposed as potential anode material for lithium-ion batteries. In this work, we examined whether spent coffee grounds could be stored for a long period of time as a crude material. The viable microbial counts of spent coffee grounds stored at 25 °C for 70 days reached 2.8×10^{12} cfu/g on standard plate-count agar and 1.9×10^{12} cfu/g on potato dextrose agar. When we used these samples to fabricate negative electrode materials, the COD value in demineralization was found to be significantly increased at 25 °C and 35 °C compared to that observed at 10 °C. However, there was no significant difference in the mineral behavior (K, Ca) and carbonization yield. Moreover, samples using these negative electrodes did not suffer any quality loss. We conclude that even if the spent coffee grounds are subjected to microbial contamination, the partially corrupt grounds are removed by demineralization, and the grounds can therefore be used as a nongraphitizable carbon material.

INTRODUCTION

Recently, lithium-ion secondary batteries, used in small electronic devices such as personal computers, mobile phones, and digital cameras, have become essential for our daily lives. The electrode materials used in lithium-ion batteries are made of various components. In general, lithium chalcogen compounds are used as the positive electrode, and graphite or nongraphitizable carbon is used as the negative electrode. In particular, it is expected that the market of nongraphitizable carbon, as the major anode material of lithium-ion batteries used in environmentally friendly vehicles such as electric and hybrid vehicles, will further expand because the durability of nongraphitizable carbon is much higher than that of graphite. Until now, solid petroleum pitch has been used as a nongraphitizable carbon material; however, various natural materials are currently being considered as alternative nongraphitizable carbon sources. The structure of nongraphitizable carbon is stable and shows little change even after repeated input and output of lithium ions because it has a random layout of the carbon hexagonal plane, which creates a nano-scale sheaf space (Figure 1). Kalyani et al. and Sonobe et al. reported that spent coffee grounds could serve as a useful anode material. We have confirmed that nongraphitizable carbon can be obtained from spent coffee grounds by a process of demineralization, drying, pre-carbonization, grinding, classification, and carbonization for use as an anode material (Figure 2). Furthermore, the performance (in terms of charge capacity, discharge capacity, and irreversible capacity) of the products obtained using such nongraphitizable carbon is equivalent to that of the products fabricated using

carbon obtained from solid petroleum pitch. We have estimated that at least more than 140,000 tons of spent coffee grounds are discharged per year in Japan. Therefore, it is necessary to elucidate the microbiological behavior of spent coffee grounds in long-term storage to obtain nongraphitizable carbon material from the coffee grounds because the summer temperature and humidity levels are particularly high in Japan. Therefore, we investigated the number of bacteria, external structure, and extraction behavior of minerals (demineralizing process), and the COD (drainage) of spent coffee grounds discharged from a beverage factory for up to 98 days at 10 °C, 25 °C, and 35 °C.

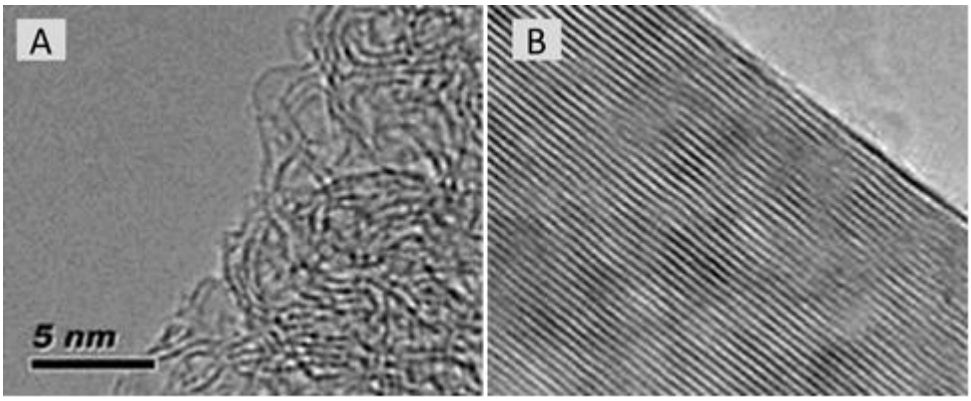

Figure 1. The structure of nongraphitizable carbon (A) and graphite (B).

Figure 2. Manufacturing flow chart of producing anode material from spent coffee grounds.

MATERIALS AND METHODS

Sample collection and storage

Spent coffee grounds were obtained from the outlet of the storage hopper in a ready-to-drink coffee factory on October 14, 2013 (Hyogo beverage factory, UCC Ueshima Coffee Co. Ltd., Tatsuno City, Japan). These samples (1 kg) were divided into seven 45-L polyethylene bags and stored at 10 °C, 25 °C, and 35 °C for 98 days. The samples were collected before the test (day 0) and on day 28, 42, 56, 70, 84, and 98. Initial analysis was carried out less than 24 h after the sample collection. The humidity condition ranged from 30% to 90% over the course of the testing period.

Sample analysis

Viable microbial counts for all samples were determined by the standard plate method from duplicate tests on standard plate-count agar (SPC, for viable bacterial counts) and potato dextrose agar (PDA, for determination of yeast and fungi) (Nissui Pharmaceutical Co.; Tokyo, Japan).

Potassium (K) and Calcium (Ca) contents were determined by fluorescent X-ray analysis. The COD of wastewater after the washing process was measured. The demineralized spent coffee grounds (by acid demineralization) were dissolved in three volumes of water, followed by

vigorous stirring for 5 min at room temperature. The COD of the supernatant was measured according to the method of JIS (Japanese Industrial Standards) K 0102-20. Carbon yield was calculated using the following formula:

Carbon yield (%) = (dry weight after carbonization sample) / (dry weight after demineralization sample) × 100

K, Ca, COD, and carbon yield analyses were conducted on the samples before storage (day 0) and on the test samples at day 42, 56, 70, and 98.

RESULTS AND DISCUSSION

A substantial number of viable microbial cells (SPC: 1.8×10^8cfu/g; PDA: 1.6×10^6cfu/g) was observed in the samples at day 0 despite the fact that the spent coffee grounds were not kept for more than one week in the storage hopper in the factory (Figure 3). Furthermore, the average temperature of the location where the factory is located is 17.3°C in October.

These results suggest that the spent coffee grounds are rich in nutrient sources for microbial growth. The viable counts of spent coffee grounds stored at 25 °C for 70 days reached 2.8×10^{12} cfu/g (SPC) and 1.9×10^{12} cfu/g (PDA). Viable cell counts of the 35 °C storage group were lower than those of the 10 °C and 25°C storage groups, respectively. However, the 35 °C storage group showed the most significant microbial contamination by visual observation. We did not evaluate the species of microorganisms and microflora present in the samples; however, these results suggest no relationship between the corruption level and the viable counts (Table 1). In a preliminary experiment, we observed a tendency of increased fungal growth in environments with air present in the vessel (oxygen-rich condition in the head-space volume). In fact, although no fungi were observed inside of the polyethylene bags, fungal growth was observed on the surface layer of the coffee residue.

When we used these samples to fabricate negative electrode materials, the COD values in demineralization of the 35 °C and 25 °C storage groups were found to be significantly increased compared to that of the 10 °C storage group. However, there was no significant difference in the mineral behavior (K, Ca) and carbonization yield among the groups (Table 2). Moreover, samples using these negative electrodes did not suffer any quality loss (data not shown).

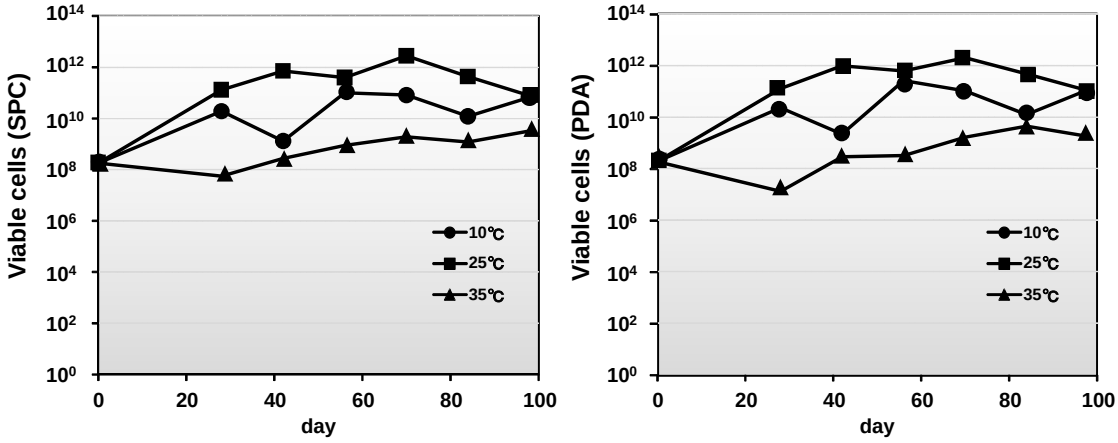

Figure 3. Changes in viable cell counts (A: standard method agar [SPC], B: potato dextrose agar [PDA]) during storage of spent coffee grounds.

Table 1. Changes in sensuous properties of spent coffee grounds.

Temp.	Analytical items	day0	day28	day42	day56	day70	day84	day98
10℃	Picture							
	Visual observation	(−)	(−)	(−)	Visible mold at the surface	In a state of progressive putrefaction(upper layer)	In a state of progressive putrefaction(upper layer)	In a state of progressive putrefaction(upper layer)
	Odor	(−)	(−)	(−)	(+)	(+)	(+)	(+)
	Viable cell counts(SPC agar)	1.8×10^{8}	1.9×10^{10}	1.3×10^{9}	1.0×10^{11}	8.2×10^{10}	1.2×10^{10}	6.8×10^{10}
	Viable cell counts(PDA agar)	1.6×10^{8}	2.2×10^{10}	2.1×10^{9}	2.5×10^{11}	1.0×10^{11}	1.2×10^{10}	1.1×10^{11}
25℃	Picture							
	Visual observation	(−)	Visible mold at the surface	Visible mold at the surface	Mold covering the surface	In a state of progressive putrefaction(upper layer)	In a state of progressive putrefaction(upper layer)	In a state of progressive putrefaction(upper layer)
	Odor	(−)	(+)	(+)	(+)	(+)	(+)	(+)
	Viable cell counts(SPC agar)	1.8×10^{8}	1.3×10^{11}	7.1×10^{11}	3.9×10^{11}	2.8×10^{12}	4.3×10^{11}	7.7×10^{10}
	Viable cell counts(PDA agar)	1.6×10^{8}	1.2×10^{11}	9.3×10^{11}	5.9×10^{11}	1.9×10^{12}	4.5×10^{11}	1.0×10^{11}
35℃	Picture							
	Visual observation	(−)	Mold covering the surface	Mold covering the surface	Mold covering the surface	In a state of progressive putrefaction(upper layer)	In a state of progressive putrefaction(upper layer)	In a state of progressive putrefaction(upper layer)
	Odor	(−)	(+)	(+)	(+)	(+)	(+)	(+)
	Viable cell counts(SPC agar)	1.8×10^{8}	5.5×10^{7}	2.7×10^{8}	8.7×10^{8}	1.9×10^{9}	1.2×10^{9}	3.2×10^{9}
	Viable cell counts(PDA agar)	1.6×10^{8}	1.3×10^{7}	2.8×10^{8}	3.1×10^{8}	1.6×10^{9}	4.3×10^{9}	1.7×10^{9}

Table 2. Changes in K, Ca, COD, and carbon yield during storage of spent coffee grounds.

Storage temp.	Day	K [ppm]	Ca [ppm]	COD [mg/L]	Carbon yield (%)
initial	0	3951	1110	2300	25.4
10℃	42	3758	1001	2300	25.1
	56	3921	949	2100	26.7
	70	3830	955	1900	24.3
	98	4211	1024	1600	24.6
25℃	42	3975	1048	2700	24.6
	56	3629	1182	2900	23.3
	70	3177	902	2200	24.2
	98	4127	1167	1900	25.8
35℃	42	4158	1106	3300	24.0
	56	4276	1126	3500	22.6
	70	4377	1026	3100	24.6
	98	4342	1032	3600	23.4

Therefore, we conclude that the microorganisms in the spent coffee grounds metabolize the components (such as sugar, caffeine, chlorogenic acids, and lipids) that do not affect the quality of the negative electrode fabricated from the coffee grounds. Thus, even though the spent coffee grounds were subjected to microbial contamination, the partially corrupted grounds are removed by demineralization, and the grounds can therefore be used as a nongraphitizable carbon material. Investigations regarding reductions in the transportation and manufacturing costs are required in order to further develop anode material of lithium-ion batteries using spent coffee grounds. These preliminary tests involving the preservation of perishable materials such as spent coffee grounds contribute knowledge toward understanding the yield and quality of carbon biomass for use in lithium-ion batteries.

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A Probabilistic Study Applied to Sensory Analysis of Specialty Coffees Undertaken among Consumers

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SUMMARY

Some methods of descriptive sensory analysis have been adopted for coffee evaluation. Among these methods, that of the Specialty Coffee Association of America (SCAA) stands out, a method performed by trained coffee cuppers qualified as Certified Judges of Specialty Coffees. However, most consumers of (specialty) coffee do not receive any type of specific training in relation to its sensory characteristics. Given this situation, the aim of this study was to carry out a probabilistic study considering diverse distributions belonging to the generalized class of extreme values based on the results of sensory analysis of specialty coffees with distinct profiles. The coffees evaluated were grown at different altitudes and underwent wet processing or dry processing. The sensory panel composed of untrained consumers was considered for this analysis. It was observed that the distribution of extreme values was that which best fitted to the final score. In addition, the probability of a consumer providing a maximum score of 9.0 points was low. Therefore, there is evidence to conclude that the consumer lacks training to differentiate specialty coffees in an effective manner.

INTRODUCTION

The sensory analysis of coffee requires that it goes through a certain process: roasting, standard concentration, cupping preparation and pouring. Then, the sample in question is tested and evaluated. The results of sensory evaluation are established by the Specialty Coffee Association of America (SCAA Protocols - Cupping Specialty Coffee) based on a 0-10 quality scale which represents increasing levels of quality coffee.According to this protocol, the results vary according to a quality scale where scores 6, 7, 8, 9 correspond respectively to: good, very good, excellent and outstanding.However, for the final score, coffees scoring below 6 are not specialty quality. An appropriate approach for this type of study is necessary given that different tasters may judge differently, randomly assigning maximum sensory scores. First espoused by Fisher and Tippett (1928), Extreme Value Theory (TEV) considers three types of probability distributions: Gumbel, Fréchet and Weibull. In 1955, Jenkinson proposed that these distributions were represented in a single parametric form, called a generalized extreme value distribution, GEV. Considering these aspects, this paper analyzed the sensory ratings given to four different types high quality coffees: Acaiá and Yellow Bourbon, being that each of them went through two different processes and environments. The extreme value theory was used, aiming to find the most appropriate distribution to the data. Since high sensory scores are rare events, this study also sought to determine the probability of high scores for the four types of coffees analyzed.

MATERIALS AND METHODS

This experiment obtained the data of the scores assigned for each type of coffee in the tests performed at the Federal University of Lavras. Four attributes were evaluated in four different

types of coffee, so that the assessment was made by a particular group of people at different times. The maximum score was determined by a scenario that assessed the highest rating assigned by a taster for each type of coffee, i.e, not considering the attribute of each coffee or when the test was administered.Thus, the database comprises six hundred and ninety six (696) sensory SCORES. Normal probability distributions, GEV and Gumbel were considered. The probability distribution function (PDF) of the Normal is given by:

$$f(x;\mu,\sigma)=\frac{1}{\sqrt{2\pi\sigma^2}}\exp\left\{-\frac{1}{2}\left(\frac{x-\mu}{\sigma}\right)^2\right\}, \tag{1}$$

wherein $-\infty<x<\infty$, μ is the location parameter, σ the scale parameter and $\sigma>0$ .A p.d.f. GEV is given by:

$$f(x;\mu,\sigma,\xi,)=\frac{1}{\sigma}\left\{\left[1+\xi\left(\frac{x-\mu}{\sigma}\right)\right]^{\left(-\frac{1}{\xi}\right)-1}\exp\left\{-\left[1+\xi\left(\frac{x-\mu}{\sigma}\right)^{-\left(\frac{1}{\xi}\right)}\right]\right\}\right\}, \tag{2}$$

wherein $-\infty<x<\mu=\sigma/\xi$ for $\xi<0$, $\mu-\sigma/\xi<x<\infty$ for $\xi>0$ and (μ,σ,ξ) parameters are position, scale and form respectively and $\sigma>0$. Making $\lim_{\xi\to 0} f(x;\mu,\sigma,\xi)$ obtains the p.d.f. Gumbel, which is given by:

$$f(x;\mu,\sigma)=\frac{1}{\sigma}\exp\left\{-\left(\frac{x-\mu}{\sigma}\right)-\exp\left[-\left(\frac{x-\mu}{\sigma}\right)\right]\right\}, \tag{3}$$

The parameters of the distributions (1), (2) and (3) were estimated by the maximum likelihood method. Then, we can proceed with the calculation of probability.

In order to calculate the probability of a sensory score exceed a certain value, we calculate $P[X>x]=1-P[X\le x]=1-F\left(x;\hat{\theta}\right)$,wherein $\hat{\theta}$ is a value or vector of maximum likelihood estimates of c.d.f.Normal, Gumbel or GEV. The c.d.f.Normal is given by:

$$F(x;\mu,\sigma)=\frac{1}{\sigma\sqrt{2\pi}}\int_{-\infty}^{x}\exp\left(-\frac{1}{2}\left[\frac{t-\mu}{\sigma}\right]^2\right)dt=\Phi_{\mu,\sigma}(x) \tag{4}$$

where $\Phi_{\mu,\sigma}(x)$ is the notation for p.d.f Normal, since there is no closed-form expression for the c.d.f., the c.d.f. GEV is given by:

$$F(x;\mu,\sigma,\xi)=\exp\left[-\left(1+\xi\left(\frac{x-\mu}{\sigma}\right)\right)^{\frac{-1}{\xi}}\right]. \tag{5}$$

For the Gumbel distribution we have:

$$F(x;\mu,\sigma)=\exp\left\{-\exp\left[-\left(\frac{x-\mu}{\sigma}\right)\right]\right\}. \tag{6}$$

The Kolmogorov-Smirnov (KS) test was used to check the fitting of the data distribution and to verify the assumption of independence of observations for the method of maximum likelihood of the Ljung-Box test. In both tests, it was considered 5% significance level. The Computational System R was used to make the adjustment distributions, hypothesis testing and calculation of probabilities.

RESULTS AND DISCUSSION

The results of the Box-Ljung and KS (Q) tests are shown in Table 1. Considering a 5% level of significance, it is observed that the distributions Normal and Gumbel did not fit the data of maximum sensory score, once the p value the test for each distribution in each type of coffee was smaller than the nominal level of significance.

Table 1. Estimates of the parameters and results of Kolmogorov-Smirnov (KS) and Ljung-Box (Q) tests for scores of coffee tasters.

Coffee	Distribution	Parameters					KS (p-value)	Q (p-value)
		$\hat{\mu}$	$\hat{\sigma}$	$\hat{\mu}$	$\hat{\sigma}$	$\hat{\xi}$		
	Normal	7,0565	1,8784	-	-	-	0,0181	0,3316
A	GEV	-	-	6,7754	2,0174	0,6827	0,0540	0,3316
	Gumbel	-	-	6,0261	2,3469	-	0,0209	0,3316
	Normal	6,7196	2,2707	-	-	-	0,0104	0,0675
B	GEV	-	-	6,3719	2,5343	0,6776	0,8539	0,0675
	Gumbel	-	-	5,5149	2,5032	-	0,0005	0,0675
	Normal	6,4362	2,3959	-	-	-	0,0007	0,0795
C	GEV	-	-	6,0101	2,6011	0,6284	0,1975	0,0795
	Gumbel	-	-	5,1373	2,7432	-	0,0074	0,0795
	Normal	7,4230	2,04799	-	-	-	0,0019	0,0910
D	GEV	-	-	7,2541	2,2821	0,8202	0,8905	0,0910
	Gumbel	-	-	6,3089	2,3984	-	0,0114	0,0910

On the hand, the same data fit the Generalized Extreme Value (GEV) distribution, indicating that this distribution is most suitable to represent the distribution of the maximum scores of the sensory analysis of the coffees. With respect to the Box-Ljung test, the maximum sensory score may be considered independent, since the null hypothesis test was accepted (p-value> 0.05) for all types of coffee. Thus, once the parameters of a distribution that fits to data were estimated, we calculated the probability. The probability of scores were calculated according to the GEV distribution, the only one adjusted, and the results were greater than 5; 6; 7; 8; 9 and 9.5 points (Table 2).

Table 2. Probability of scores of coffee obtained through GEV distribution.

Coffee	Scoresheet					
	5	6	7	8	9	9,5
A	0,8636	0,7551	0,5896	0,3666	0,1211	0,0236
B	0,7952	0,6834	0,5334	0,3496	0,1537	0,0668
C	0,7572	0,6335	0,4765	0,2970	0,1221	0,0511
D	0,8727	0,7928	0,6712	0,4952	0,2592	0,1257

According to Table 2, it is observed that the probability of occurrence of a high score, or above 9.5 is relatively low compared to the probability of a score greater than 5 for all types of coffee. A practical interpretation of probabilities in Table 2 can be made as follows: the

probability that a taster will give a score higher than 9 points for the coffee A or that it will be considered exceptional by a taster is of 12.11% by the GEV distribution. The same event is more likely to occur for the coffee D, that is, a probability of 25.92%. Therefore, it was possible to verify that among the distributions used, the GEV was the only one adjusted since it was within the significance level at all times. Regarding to the probabilities, the probability that Coffee D receives high scores (above 9.5) is greater between the coffees studied. On the other hand, the likelihood of the coffee A receiving higher scores (above 9.5) is the smallest.

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