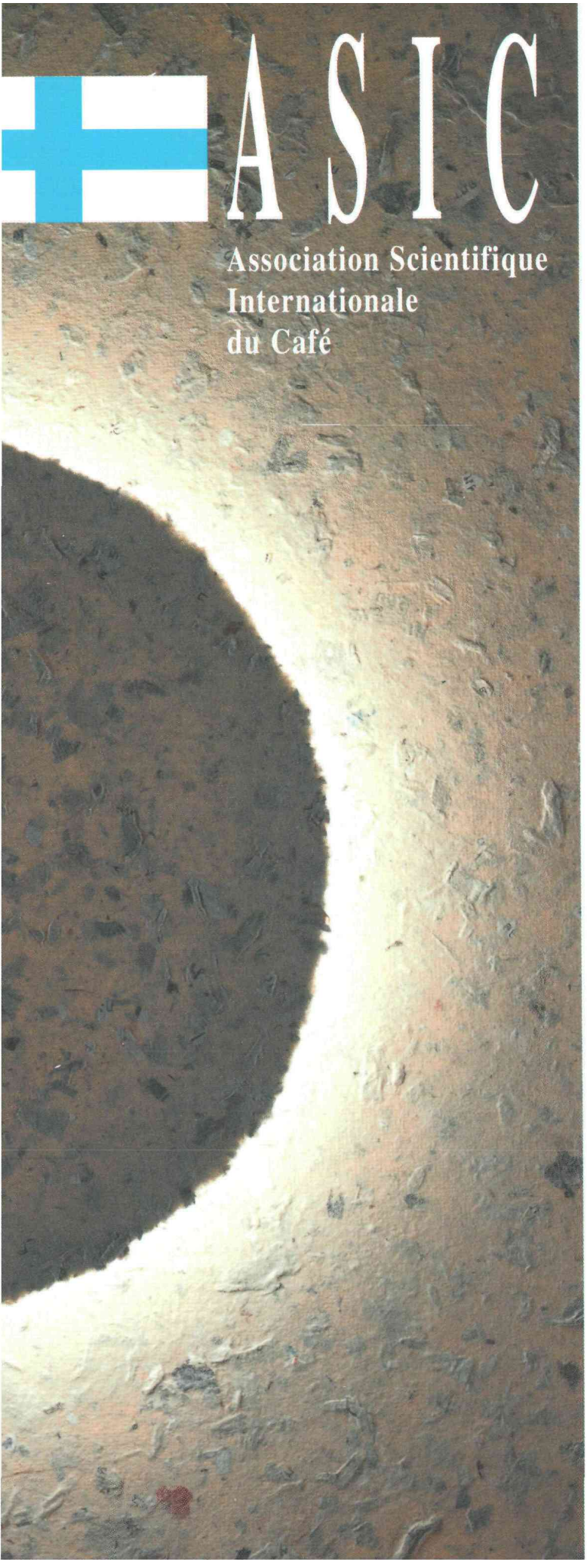

DIX-HUITIÈME COLLOQUE SCIENTIFIQUE INTERNATIONAL SUR LE CAFÉ
Helsinki, 2-6 août 1999

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Introduction

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UNIVERSITIES AND COMPANIES IN COLLABORATION

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When in the course of evolution, the human race developed the ability to think, it also developed a thirst for knowledge. Soon people discovered that knowledge could be harnessed to improve the quality of live, to facilitate moving from one place to another, but also, alas, to further the effectiveness of warfare. The tree of the knowledge of good and evil had been found. Today, the applicability of knowledge is essential for the success of companies growth of economies, a high standard and quality of human life, and preservation of nature and the environment. The amount of available information grows not only rapidly, but exponentially. Not very new bit of knowledge can instantly be applied to practical use, in fact, the applicability of new knowledge for the use of society and industry is by no means self-evident. The so-called chain of innovation, moving from the producer of knowledge through its applications to the end-user, does not always work as desired. For example, here in Finland we often feel that the system of innovation leaves room for improvement. In order to speed up the practical application of innovation, various forms of cooperation have been launched betwwen the producers of knowledge, meaning universities and research institutes, and companies that put theorretical knowledge to practical use. However, this cooperation has been hampered by problems arising from the attitudes and actions of scientists, companies and political decision-makers.

The academic world has feared that interference by outside parties may disrupt the ideal of the freedom of research. Science seeks the truth and aims to expand our knowledge. Certain essential criteria must be met before the mentioned objectives of science can be achieved. First of all, research must be objective. Research results may not be dependent on the subjective wishes and ideas of a researcher or company, let alone a politician. For this reason, other scientists must be able to repeat the study and test the reliability of the results. Second, science must remain critical throughout the process, from the presentation of hypotheses to the testing of results. In principle, all previous hypotheses and statements can become nullified based on new scientific knowledge. This aspect is closely related to the third criterion of research, that of self-correction. New theories, methods and equipment lead to results that are more accurate and thus closer to the truth than previous research results. According to the fourth criterion, science must be autonomous. Only the academic community itself may correct research that have been achieved under its auspices. Thus, political, economic or religious interests and expectations may not correct scientific knowledge.

Upholding the mentiond criteria continues to be a primary responsibility within the academic community. By observing them the academic community has managed to obtain the freedom of research, according to which researchers must be able to create scientific knowledge free from external pressure, guidance or interference. The famous ancient philosopher, Arsitotle, came to the conclusion that thinking is most productive in idleness. Since Aristotle, many scholars from Galileo to Darwin have been subjected to outside pressures. Indeed, there have been many attempts in the course of history to yoke science to serve various ideologies. In the Middle Ages science – if we can talk of science as we understand it today – was controlled by the church. Ever since, secular rules have tried to achieve the same control by such dubious methods as distorting history.

Science has also been used to achieve morally and ethically desirable results ; think of the methods of the notorious Russian geneticists Trofim Lysenko. The machinery of war has exploited scientific achievements as

the superpowers have invested massive sums of money into arms development. Today's governmental research policies manipulate scholarship in a kind of an enlightened way as funding steers research into areas which are, or are believed to be , beneficial to social development. Free and independant research has tried to seek a balance in the midst of these kinds of pressures. In the Western countries this has been possible thanks to the fairly autonomous status of universities. For example, for more than 350 years the autonomy of the University of Helsinki secured not only freedom of research, but also freedom of speech for the Finnish academic community during the country's periods of foreign rule before 1917.

The business sector has also shown an interest in the advancements of science, although this concern is rather recent. Only the great discoveries of the 18th and 19th centuries – the steam engine, electricity and the combustion engine – awoke the interest of companies in the results of scientific research. At first, interaction between companies and researchers was rather one-sided, with companies trying to find practical applications for research results to the best of their abilities. For instance, note that over 150 years ago a representative of the British Ministry of Finance paid a visit to the physicist and chemist Michael Faraday, to inquire what use Mr. Faraday's electromagnetic experiments could possibly have. He obviously could not foresee that electricity would later turn into a lucrative source of taxation. It is only during this century that the economy and companies have actively tried to influence the direction of research.

The relationship between research and the business sector is marked by a duality : on the one hand, the academic community strives to maintain the freedom of research, on the other hand, the connection between research and economic growth is generally accepted. Companies and the economy as a whole want to benefit from the research they have funded. The benefits to research, in turn, come in the form of increased opportunities for funding. In fact, some fields of science have worked for the business sector in a rather straightforward way. Such applied sciences as technology and economics, and the agricultural, food and forestry sciences have in their time been developed to serve the needs of the economy. Even such fields as law and, to some extent, medecine, have their repercussions on the economy through their close connections with the surrounding society.

One of the most important factors for the success of modern companies is effective product development, which is, of course, dependent on applied research. As the amount of applied research has been considered insufficient, large companies especially have increasingly been starting their own programmes in applied research, some even in basic research. During the past two decades, Finnish industry has invested in research at a significantly faster rate than the public sector has. Today, over two-thirds of all research funding in Finland comes from the private sector. This has greatly contributed to the fact that the share of research and development accounts for more than three per cent of th Gross National Product, which is among the highest ratios in the world.

Despite the increasing interest of companies in product development and even applied research, it has become clear that a sensible division of labour requires that new knowledge is produced in universities and the practical applications are carried out by companies. This entails that universities and companies seek functional and efficient forms of cooperation. Companies in the fast-developing high tech fields are locating themselves in the immediate vicinity of universities. This has led to the development of technology and science parks, where the producers of knowledge and companies applying the knowledge together form an adequately large and functional critical mass. The first science park was established at the University of Stanford in the 1950's. By the 1980's there were only ten science parks in the entire world. By 1990 the figure had risen to over 300, and, at the moment, depending on how the term « science park » is defined, they number well over 1 000. The most famous science park is probably California's Silicon Valley, which has had a tremendous impact on the development of electronics. The next field with great promises for a major breakthrough is biotechnology. Since biotechnology has close connections with food production, we can expect that it will have some kind of impact on the cultivation and breeding of coffee as well. Science parks concentrating on biotechnology are an example of modern co-operation that is mutually beneficial to universities and companies.

Cooperation between scientists and companies is one of the projects spearheaded by the University of Helsinki ; it is presently in full motion at the Viikki Campus. The Viikki Campus , which lies about ten kilometres northeast of the centre of Helsinki, has for the last 30 years accomodated a number of departments of the Faculty of Agriculture and Forestry, including the agricultural, food, household and environmental sciences. Some 13 years ago, at the 20th anniversary celebrations of academic teaching in the food sciences, I proposed the idea of establishing a science park on the campus. The original idea was to provide favourable circumstances for finding a solution to the problems of Finnish food production in relation to the international markets. The core of these problems was high production costs due to our northerly location.

The science park project, which got under way as late as 1993, currently has objectives differing from those of the original plan : rapid advances in the field of biotechnology and the University's desire to be at the forefront of these advances have directed the plans and objectives in a new direction. The Viikki Campus will be developed to form the largest concentration of biosciences in whole Europe at the beginning of the next millenium. There, companies that make practical use of research results will work in close cooperation with university departments. The Viikki Science Park will be made up of three research sections. One of these sections will be the Biocenter, consisting of three departmental buildings and accomodating all the basic biosciences, including biochemistry, genetics, plant and animal physiology, ecology, hydrobiology, pharmacy and environmental research. The building complex will also provide facilities for the Institute of Biotechnology, an independent institute of the University of Helsinki, which currently employs over 200 researchers. The second research section will consist of such applied sciences as agricultural and food sciences, home economics and environmental and forestry sciences, which will be moving from the centre of Helsinki to the Viikki Campus. The third research section, slightly smaller than the two others, will comprise the Faculty of Veterinary Medicine, which also will transfer from its premises in Helsinki to new facilities in the science park in the near future.

The Viikki Science Park will thus accomodate the basic sciences in biology, responsible for producing new knowledge in the field, as well as all the applied sciences, which are responsible for the practical application of this knowledge in agriculture, food production, veterinary practice and environmental control. A third element, product development, will join this chain of basic applied sciences to ensure that innovations are transformed into marketable high tech products. This will take place in two ways :

First, existing companies in the fields of biotechnology, pharmaceuticals, food productions and forestry may relocate their research and product development units in th Viikki Science Park. Negotiations are underway with major international companies.

Second, the science park will include business incubators, which provide facilities for small business starting their operations based on research findings done at the university. The first business incubator building was completed in June, and now accomodates 30 newly established bio- and food- production companies. University employees and recent graduates are encouraged to start their own companies. In Finland we lack the tradition of self-employment among academics, and for this reason the threshold for starting a company is high. In order to lower this threshold, a small consultancy, the Helsinki Science Park Ltd, operates within the science park and provides services, including financing, for companies, especially small companies wishing to locate in the science park. There are also plans to establish a « school of biobusiness », which would provide tailored business training for graduates in the biological, food and forestry sciences.

Science parks offer excellent circumstances for efficient co-operation between basic research, applied research and commercial product development. Let me give you an example. Two years ago a programme on the biotechnology of forest trees was launched in the Institute of Biotechnology. With the help of solid basic research, the aim of the programme is to discover the genetic map of the birch tree, as well as the effects of various genes on the properties of birch. The forestry sciences, such as silviculture and forest engineering are also involved in the project. The overall aim is to find out whether gene transplants affect the stress tolerance of birch against disease, drought, frost during the growing season and pollution. It is possible that the expansion of the greenhouse effect and the resulting global warming will increase such stress factors. As forests are a crucial resource for the Finnish economy and constitute a central element fo the Finnish landscape, maintaining the health of Finnish forests is a vital question from the point of view of future generations. This research project will also study the possibilities of manipulating the fibre structure of birch through genetechnology in order to produce high-quality material for industrial processes.

I can imagine that similar projects could be built around coffee. The genetic map of the coffee plant could be worked out through basic biotechnological research. Perhaps a gene from a plant with higher resistance against frost could be transferred into the coffee plant. This would salvage the Brazilian coffee harvests from frost. As many other tropical plants, coffee is known to be chilling sensitive, which means that the plant dies even before ice crystals are formed. I am not aware wether this kind of research has been done. However, I did find on the Internet some information about molecular research on the resistence of the coffee plant against insects and diseases. And could perhaps the flavour of coffee be altered through gene transplants? As far as I know, coffee produced in different countries has distinct aromas depending on the climate, soil and probably also production techniques. Consumers in different countries also prefer different kinds of flavours. Finnish coffee is different from, say, Italian or American, which every tourist has surely noticed. Finns are among the most avid coffee drinkers in the world. It is difficult to estimate whether there is any room for an increase in the consumption of

coffee in Finland. However, in countries with lower consumption figures, new flavours might attract new coffee drinkers. Coffee producers should, and I am sure they will, seize every opportunity offered by biotechnology. Co-operation between universities and companies is another opportunity offering countless possibilities for coffee producers, too.

Biotechnology certainly holds great promises for the future. The faster the knowledge generated in universities can be relayed to benefit the economy and us, the ordinary people, the better. The companies applying biotechnological knowhow and operating in connection with universities are often small or middle-sized, but they may be significant employers in their field, and thus contribute to the positive employment prospects of academically educated people.

If the greenhouse effect expands according to prognoses, producing food for the ever-increasing world population will be a major challenge in the future. The expansion of the greenhouse effect would mean that the climate of the most important agricultural areas in the world would dry up as the subtropical anticyclones would move further away from th equator. You can imagine the dramatic consequences of this for food production. In coffee-producing area, coffee would have to give way to essential food plants. The field of science that could open up new opportunities for food production and even solve the world's food problem is biotechnology. For coffee, this means that land would still be available for growing coffee, and that new flavours developed with the help of biotechnology would perhaps increase th number of coffee drinkers and thus the number of people finding pleasure in a good cup of coffee.

NEW CHALLENGES FOR THE COFFEE SECTOR

Bertel Paulig
Chief Executive, Paulig Group
President of ASIC

Ladies and Gentlemen,
It is my pleasure and honour to welcome you to Helsinki and to Finland – the country with the highest consumption of coffee per capita in the world.

You have all by now, at the latest this morning over breakfast, tasted our Finnish coffee and thus know that the secret behind the high consumption is :

- high quality of green coffee,
- light roast,
- giving a high acidity and
- always served fresh.

Thus we bring to the consumers an absolutely « clean cup » of coffee.

ASIC is unique in so far that a truly independent organisation, devoted to the secrets of coffee it brings together this week :
- scientific,
- technical and
- applied **knowledge**.

ASIC covers a wide range of coffee research :
- agronomy,
- chemistry,
- technology,
- physiological effects and
- packaging and storage.

Let me now try to link together the work of science and research with the commercial realities of trade and industry :

Responsible business

- Relevant components of responsible business are :
  - environmental issues in European countries (specifically packaging and packaging waste),
  - sustainable production,
  - organic production,
  - genetically modified products,
  - fair trade,
  - ethical sourcing /labour conditions /child labour /human rights.

In general terms, a distinction can be made between « technical » issues (environmental, sustainable production, organic production, genetically modified products) and « ethical » issues (fair trade, ethical sourcing, labour conditions, child labour, human rights).

I will this morning, obviously focus on the « technical » issues :

Environmental issues

Respect for the environment is not only a legal obligation, but also an important element in consumers' appreciation of food industries. Perceived non-respect of environmental matters can be very damaging. For coffee roasting industries the distinction must be made between environmental issues in origin (soil depletion, erosion, water usage, pesticides) and in consuming countries (emissions into air, water and soil, energy efficiency, packaging waste).

Within the overall context of the European food and drink industry, we are committed to sustainable development. It is essential to preserve the environment in which raw materials are grown and ensure the long-term sustainable food availability.

The food and drink industry is continually making efforts to reduce as much as possible its limited impact on the three environmental media (air, soil and water) while continuing to provide safe, wholesome and convenient products which meet consumers needs. This is basically implemented through :

- the integration of environmental considerations into day-to-day activities in the framework of existing quality and environmental management systems,
- waste minimisation and efficient waste management (reduction at source, recovery, recycling),
- energy and water savings,
- conservation of resources by efficient use of raw materials,
- contribution to consumer awareness by providing relevant environmental information.

Packaging and packaging waste

Although packaging waste generated by the food and drink industry is only a small part of the total waste stream, it receives a large share of the political attention, probably because it is a clearly visible part of the waste stream. On the political level there is a strong pressure to promote reusable forms of packaging and to reduce the single-use forms of packaging. This is relevant to the coffee sector since the quasi-totality of the packaging material used is « one-way », be it the industrial packaging waste (jute bags, liner for bulk containers) or the household waste (roasted coffee packaging material).

Packaging fulfils three essential roles in ensuring that products are moved from source of production to their destination in the best possible condition :

Protection – packaging is needed to protect consumers products from damage and deterioration and tampering. The amount and type of packaging needed will vary according to the nature of the product.
Containment – packaging contains the product to eliminate spillage and waste and to aid handling and distribution.
Preservation – packaging provides safety and freshness for food products, ensuring that a maximum is consumed and not wasted. The preservation role is of utmost importance to coffee. The very specific characteristics of roasted and ground coffee make it an absolute necessity that very high standards are kept in the design and manufacturing of the « primary » packaging which is directly in contact with the foodstuff in order to ensure that the organoleptic qualities of the product are fully preserved. Any change in the technical characteristics of the packaging leading to a lowering or these high standards is not acceptable, as this would jeopardise the product as a whole.

Sustainable production

It could be argued that environmental policy in producing countries is a matter for national legislation and that its implementation is the responsibility of the coffee growers, not the green coffee buyers. However, the European food industry - including the coffee sector – cannot avoid becoming increasingly involved in the first stages of production in producing countries. Concepts such as product liability, HACCP and Integrated Product

Policy in one way or another require closer control by the manufacturer of the final product over the total chain, and this includes – directly or indirectly – environmental issues. Proper use of fertilisers and pesticides for instance is not only a food safety, but also an environmental issue. Although green coffee buyers cannot interfere directly in national environmental legislation in producing countries, they can encourage their suppliers to adopt best practices, possibly going beyond legal requirements. In some instances this has already been realised, taking the form of projects, financially supported by roasting industries.

Sustainable agriculture

Sustainable agriculture refers to an agricultural production and distribution system that :

- achieves the integration of natural biological cycles and controls,
- protects and renews soil fertility and the natural resource base,
- optimises the management and use of on-farm resources,
- reduces the use of non-renewable resources an pruchased production inputs,
- provides an adequate and dependable farm income,
- promotes opportunity in family farming and farm communities, and
- minimises adverse impact on health, safety, wildlife, water quality and the environment.

Applied to coffee growing, there is no single uniform concept on what constitutes sustainable development. Elements mentioned frequently include :

- use of shade tree,
- density of coffee plants,
- pruning,
- soil erosion measures,
- use of agrochemicals,
- water management.

Organic coffee

There is a certain overlap with sustainable development in producing countries, but organic production has a connotation that goes beyond the environmental issues : consumer perception is that the product is more wholesome and healthier. Contrary to sustainable agriculture, organic production is identified on tne final product. It is therefore much more a marketing issue than sustainable agriculture which is PR related. Until recently, the sale of organic products was almost exclusively through specialised shops. This limited market expansion because these shops reached only a relatively small group of dedicated customers. A shift in distribution channels can be noted . Large supermarkets are introducing ranges of organic products, making them more accessible to a much larger group of consumers.

Genetically modified products (GMO)

After the introduction of products containing GMO maize and soya on the EU market, the question is largely one of consumer acceptance and – related to that – the marketing of GMO-free products. The food industry's current concern is the different application (or even non-application) of the EU legislation in the various EU member countries, with the associated problems for the free movement of goods.

As far as coffee is concerned, there is on-going research into genetic modifications that wpuld result in increased pest or frost resistance. in uniform ripening of the cherries or in caffeine –free coffee. So far this research has not reached the commercial production phase.

The essential requirements that need to be satisfied before any genetically modified coffee is introduced on the European market are :

- assured safety for the consumer and the environment,
- real benefits for the producer (for instance : increased yields), the environment (for instance : reduced use of pesticides) and the consumer (for instance : better quality),
- clear and unequivocal approval for admission by the relevant EU authorities.

Prevention of mould formation

A wide range of raw materials, including most grains and beans, are susceptible to naturally occuring mould growth. Moisture in the raw product during harvesting, drying, storage and distribution is the most important factor for mould growth.

The coffee industry has for many years been aware of the possibility of mould growth on coffee beans, and knows that such growth produces spoiled beans. By implementing stringent quality procedures, spoiled beans with any sign of mould growth are automatically rejected.

The European Commission is currently considering proposals to implement measures to prevent mould-affected products, including coffee, from entering the European market, with a view to ensure that consumer is provided by sound and wholesome foodstuffs. The European Union should develop a policy that takes the specific situation of each commodity into account. A measure that is often proposed for other products, namely checks of the raw material at the point of entry into the European Union, creates major problems for coffee :

- there are as yet no agreed sampling plans,
- checks on green coffee do not prevent the entry of potentially contaminated processed coffee coming from third countries.

In the case of coffee, maximum limits on the raw material are unnecessary (because coffee is only a very minor contributor), impractical (because sampling and analysis of the huge volume of coffee imports would be excessively burdensome), disruptive to international trade (negatively affecting the exports of developing countries), ineffective (because finished coffee products manufactured outside the EU and subsequently imported would not be covered) and premature (because preventive measures throughout the chain are being developed).

The coffee industry takes seriously any possibility, however small and remote, that any of its products might prove unwholesome to its consumers, but responses other than maximum limits for green coffee are more practical and effective. The European coffee industry has already taken a number of actions in addressing the issue. ISIC (Institute for Scientific Information on Coffee) has funded a large screening programme of EU market samples of both roast and ground and soluble coffees for occurrence of mould residues. From this it has been concluded that coffee is only a minor contributor among several other food items. For such contributors preventive actions are generally considered to be the appropriate control instrument.

As a first step to develop a prevention program for mould formation in coffee, an ISIC-funded fact finding pilot study (with a.o. field sampling in a number of producing countries) has been completed. As a next step the ICO and FAO, supported by the European coffee sector, have drafted a *Project Proposal for the enhancement of coffee quality through the prevention of mould formation* which has received co-financing from the Common Fund for Commodities.

The European coffee trade and scientific organisations have set up the *European coffee co-operation Task Force on OTA* to discuss with all relevant Directorates General of the European Commission the negative implications of maximum limits and preferred alternative of preventive measures.

It is regrettable that several EU member countries are applying checks on green coffee on an individual basis. This is unwarranted give the stringent quality controls already applied by the industry, unnecessarily anticipates the discussion in the European Union and the evaluation of the Scientific Committee on Food on the need to take measures regarding coffee, and provides an unjustified obstacle to the free movement of goods.

So, Ladies and Gentlemen, with these reflections on challenges we are facing together, I have tried to bring together on one hand science and research with, on the other hand, trade and commerce.

Clearly, the way forward is with **co-operation and communication.** Open and factual communication is needed not only between all of us attending this ASIC Conference, but also in relation to relevant authorities and to the final consumers of coffee.

Please do enjoy this week together in summery Helsinki !

Key Odorants of Roasted Coffee: Evaluation, Release, Formation

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1 Introduction

Identification of the compounds causing the pleasant aroma of roasted coffee is a challenge for flavor research. In my lecture I would like to demonstrate the progress which was reached in recent studies. The following points will be discussed:

- Key odorants of raw Arabica coffee. Their changes during roasting.
- Evaluation of the key odorants of ground roasted coffee by sensory studies.
- Determination of the release of odorants.
- Formation of 2-furfurylthiol.

2 Odorants of raw coffee

Vitzthum et al. (1976) were the first to analyze the volatiles of raw coffee by combining instrumental with sensory methods. They concluded that 3-isopropyl-2-methoxypyrazine and the corresponding isobutyl-derivate contribute to the characteristic „peasy-like" smell of raw coffee. We differentiated by dilution experiments the potent odorants occurring in raw Arabica coffee from the great number of odorless volatiles. These odorants were quantified in the provenance Colombia (Col) before and after roasting using stable isotope dilution assays. An extract of the results is shown in Table 1. To get an insight into the key odorants of raw coffee, odor activity values (OAV, ratio of concentration to odor threshold) were calculated on the basis of the odor threshold values of the compounds using cellulose as base. The highest OAVs were found for the methoxypyrazines **1** and **2** (Table 1). This result confirmed the statement of Vitzthum et al. (1976) mentioned above. In addition, their suggestion that the methoxypyrazines occurring in raw coffee are not degraded by the roasting process was corroborated by the quantitative data in Table 1.

Table 1. Concentrations, and odor activity values of potent odorants in raw Arabica coffee and their changes during roasting

No.	Odorant	Concentration (µg/kg)	
		raw	roasted
1	3-Isobutyl-2-methoxypyrazine	97 (490)[a]	97
2	2-Methoxy-3,5-dimethylpyrazine	0.5 (83)	1.1
3	Ethyl 3-methylbutyrate	22 (37)	14
4	3-Isopropyl-2-methoxypyrazine	2.3 (23)	2.4

[a] The odor activity values given in brackets were calculated by dividing the concentration by the odor threshold value in cellulose.

Source: Czerny and Grosch (in preparation).

3 Evaluation of the key odorants of roasted coffee

In several studies the potent odorants of roasted coffee were screened by dilution experiments, e.g. aroma extract dilution analysis (AEDA). Altogether, 27 odorants were identified showing higher OAVs (review by Grosch, 1998). In AEDA the odor impact is evaluated after separation of the volatiles by gas chromatography. Interactions of the odorants are abolished. Therefore, the question, which compound among the potent odorants actually contributes to the flavor, remains open. To answer this question we performed the following experiments:

- Preparation of a synthetic blend of the odorants on the basis of quantitative data.
- Critical comparison of the synthetic blend, denoted aroma model, with the view of trying to duplicate the odor profile of the original sample.
- Comparison of the overall odor of the aroma model with that of models in which one or more components are omitted. In other words, in triangle tests the assessors have to find out by sniffing the sample what is different in the overall aroma (omission experiments).

As reported by Czerny et al. (1999), this procedure was applied on a sample of medium-roasted ground coffee which originated from Colombia (Col). As it is very difficult to deodorize ground coffee, we had to look for a suitable base for the aroma model. A comparison between cellulose and water and a mixture of sunflower oil and water in a volume ration of 1:20 indicated that this latter base was the more reliable.

The flavor profile of the model containing the odorants dissolved in the mixture of sunflower oil and water (MI) was close to that of the original (Table 2). In particular, the very characteristic roasty/sulfurous odor note of coffee was as intense as in the real coffee sample. The results of the omission tests are summarized in Table 3. Experiment (exp.) no.1 indicates that the model in which acetaldehyde, propanal and the three *Strecker* aldehydes were lacking, was sensorially discriminated from the complete model. The assessors reported that the reduced model smelled less malty. Exp. 2, without acetaldehyde and propanal, confirmed this evaluation. Due to the presence of the *Strecker* aldehydes, the malty odor was perceptible. In exp. 3 the model without butanedione and pentanedione was not clearly differentiated from the complete model. This result indicated that they did not play a significant role in the coffee flavor. The omission of the pyrazines in exp. 4 affected the flavor. Exp. 5 indicates that the overall flavor of the model was already changed when only the three alkylpyrazines were lacking. The assessors did not perceive the presence of the isobutylmethoxypyrazine in exp. 5.

Table 2. Aroma profile of roasted ground coffee and the corresponding aroma model M I

Attribute	Col	MI[a]
	Intensity[b]	
Sweetish/caramel	1.0	1.4
Earthy	1.6	1.3
Roasty/sulfurous	2.3	2.1
Smoky	1.7	1.4
Similarity[c]		2.3

[a] Altogether 27 odorants were dissolved in sunflower oil-water (1:20, v/v).
[b] The intensity of the attributes was scored by 10 assessors on a scale of 0 (absent) to 3 (strong).
[c] Similarity rating scale: 0 (no similarity) to 3 (identical with the coffee sample).
Source: Czerny et al. (1999).

This result is of special interest, because, as reported before, this methoxypyrazine is the most odor-active constituent of raw coffee (Table 1). On the basis of the sensory experiments listed in Table 3, we conclude that roasting of the raw material does not only produce the pleasant aroma which is characteristic for coffee, but, in addition generates odorants which mask the peasy odor note caused by the 3-isobutyl-2-methoxypyrazine. The absence of furanones I and II in exp. 6 as well as of furanones III and IV in exp. 7 was not recognized by a significant number of panel members (Table 3). However, when the four furanones were lacking, as can be seen in exp. 8, the odor of the aroma model was significantly different. In contrast, the absence of ß-damascenone was not missed (exp. 9). The impact of the phenolic odorants on the coffee flavor was evaluated in exp. nos. 10 to 12. The absence of the four phenols in exp. 10 was clearly recognized. Exp. nos. 11 and 12 in which either guaiacol or 4-vinylguaiacol were omitted showed that guaiacol did not reach the confidence limit for significance by one answer whereas vinylguaiacol reached this limit. A comparison of exp. nos. 13 and 14 indicates that 2-furfurylthiol is the outstanding odorant in the group of sulfur compounds (Table 3). Its absence in exp. 14 changed so clearly the odor of the model that 15 out of 20 answers were correct. The assessors reported that the intensity of the sulfurous/roasty note in the odor profile had decreased. Exp. no. 13 indicates that the remaining six sulfur compounds evaluated by AEDA did not belong to the key odorants of the coffee sample studied here. In exp. nos. 15 to 17 methylfuranthiol, mercaptomethylbutyl formate and methylbutenthiol were singly omitted. In agreement with exp. no. 13, in none of these experiments the confidence limit for significance was reached. However, one or the other sulfur compound might reach or even exceed this limit in other provenances in which it may occur in a higher concentration than in the provenance Colombia here discussed.

4 Release of odorants

The flavor of freshly roasted and ground coffee is not stable (Table 4). The intensity of the sweetish/caramel note in the aroma profile decreases within 15 min from 1.5 to 0.8 points. Further changes refer to the earthy and smoky notes, which increased during the storage period of 3 hours. To get an insight into the losses caused by evaporation of the odorants from coffee and other foods, an apparatus (Figure 1) was designed for the quantification of the more potent odorants in the headspace (Zehentbauer and Grosch, 1997).

Table 3. Aroma of the model for roasted ground coffee as affected by the absence of compounds

Exp. no.	Compound(s) omitted	Number[a]
1	Acetaldehyde, propanal, methylpropanal, 2- and 3-methylbutanal	17*
2	Acetaldehyde, propanal	12*
3	2,3-Butanedione, 2,3-pentanedione	6
4	2-Ethyl-3,5-dimethylpyrazine(I), 2-ethenyl-3,5-dimethyl-pyrazine(II), 2,3-diethyl-5-methylpyrazine(III), 3-isobutyl-2-methoxypyrazine	* 13
5	Alkylpyrazines I to III as in exp. 4	12*
6	4-Hydroxy-2,5-dimethyl-3(2H)-furanone (I), 2-ethyl-4-hydroxy-5-methyl-3(2H)-furanone (II)	8
7	4-Hydroxy-4,5-dimethyl-2(5H)-furanone (III), 5-ethyl-3-hydroxy-4-methyl-2(5H)-furanone (IV)	9
8	Furanones I to IV	11*
9	ß-Damascenone	6
10	Guaiacol, 4-ethylguaiacol, 4-vinylguaiacol, vanillin	13*
11	Guaiacol	10
12	4-Vinylguaiacol	11*
13	2-Methyl-3-furanthiol, dimethyl trisulfide, methional, 3-mercapto-3-methylbutyl formate, 3-methyl-2-buten-1-thiol, methanethiol	10
14	2-Furfurylthiol	15*
15	2-Methyl-3-furanthiol	10
16	3-Mercapto-3-methylbutyl formate	10
17	3-Methyl-2-buten-1-thiol	10

[a] Number of assessors who find out by sniffing that sample what was different in the overall aroma (maximum 20).

* Significant result ($p < 0.05$). Source: Czerny et al. (1999).

Table 4. Changes of the aroma profile of freshly ground coffee

Attribute	Time (h)			
	0	0.25	1	3
Sweetish/caramel	1.5	0.8	0.6	0.3
Earthy	1.5	1.8	1.8	2.1
Roasty/sufurous	2.5	2.5	2.5	2.5
Smoky	1.5	1.5	1.8	2.1

Intensity rating scale: 0 (absent) to 3 (strong).

A sample (2.5 g) of roasted Arabica coffee (Col), was placed into tube no. 8 which then was sealed. Valves nos. 3 and 4 were opened and a solution containing definite amounts of the odorants in diethyl ether was injected on the glass finger no. 10 which was heated up to 80°C. These odorants were used as internal standards. They were labeled with stable isotopes to differentiate them from the analytes. The release of odorants was determined for a period of 15 min or 30 min during which the coffee sample remained in the apparatus. To equilibrate the odorants and the internal standards in the apparatus, the piston (no. 5) was pushed and pulled during this period. Subsequently, the valves nos. 3 and 4 were closed, and nos. 1 and 2 were opened. After opening valve no. 1, the gaseous nitrogen pressed the headspace collected in tube no. 6 into the Tenax trap no. 7 by moving the piston. After headspace sampling, the Tenax

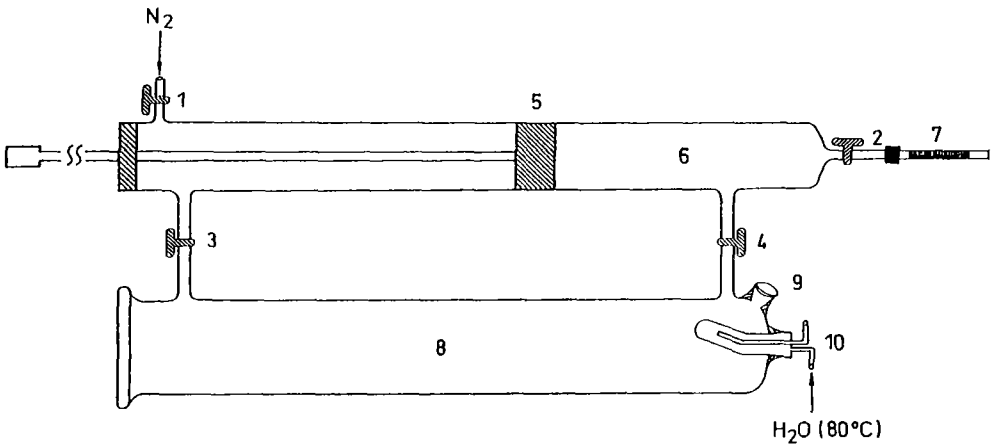

Figure 1. Glass apparatus (volume 6.84 L) for quantitative headspace analysis
1-4 Valves, *5* piston, *6* tube, *7* Tenax trap, *8* tube for the sample, *9* septum, *10* glass finger

trap no. 7 was put in the desorption heating block of a gas chromatograph. The odorants and their labeled internal standards were desorbed and then analyzed by mass chromatography.

To investigate the changes in the composition of the odorants, the coffee sample was stored for different periods of time at room temperature. Then it was placed in the apparatus and the release of some odorants during 15 min was measured. The results in Table 5 indicate that, in particular in the case of the malty/sweet smelling *Strecker* aldehydes methylpropanal, 2- and 3-methylbutanal, the amount which evaporated during 15 min decreased to one third when the coffee sample was stored for 15 min in air. Under these storage conditions, the release of butanedione and pentanedione decreased by 50 % whereas the rate of methanethiol did not change and the relatively high rate of acetaldehyde was scarcely affected. The release of some odorants was measured up to a storage time of 3 h (Table 5). The results indicate that the amounts liberated decreased further, although the differences become much smaller with respect to the amounts found after the first 15 min of storage. In the next experiment, the release of the highly volatile odorants from whole beans as well as from freshly ground beans during a period of 15 min was compared. In Table 6 the percentage of odorant losses in relation to the total concentration in the coffee sample is indicated in brackets. As expected, the increase of surface caused by the grinding process strongly enhanced the amount of odorants which evaporated from the coffee sample. The largest increase was found for 2,3-butanedione. The amount released in the headspace was 8-times larger in ground beans than in whole beans. The smallest difference was found for acetaldehyde, as the amount was only 3-times higher than in ground beans. In relation to the total concentration of the odorants in the coffee sample, 43 % of methanethiol and 39 % of acetaldehyde were lost from the ground material within 15 min (Table 6). An experiment, not shown in the Table indicated that a prolongation of the release period to 30 min led to an evaporation of 66 % of the methanethiol occurring in the coffee sample. The remaining odorants listed in Table 6 are not so volatile. The losses within a period of 15 min lay in the range of 15 to 27 %.

The concentrations of some sulfur and phenolic compounds were compared in the coffee powder and its headspace (Table 7). In these experiments the release period amounted to 30 min. The number in brackets indicates that 23 % of 2-furfurylthiol and 29 % of methional evaporated. Also the losses of guaiacol (18 %) and vanillin (20 %) are comparable with that of the thiol. In the cases of ethyl- and vinylguaiacol only 8 % and 5 %, respectively, were lost in 30 min. The lowest evaporation rates were found for the furanones (Table 7). Only one percent of the amount present in coffee was lost in 30 min.

Table 5. Release of highly volatile odorants after storage of coffee at room temperature[a]

Odorant	Storage time after grinding[b]			
	0	0.25	1	3
Methanethiol	1.9	1.9	n.d.	n.d.
Acetaldehyde	45.7	40.0	n.d.	n.d.
2,3-Butanedione	7.5	3.5	2.6	2.1
2,3-Pentanedione	7.4	3.7	3.7	3.3
Methylpropanal	4.9	1.7	1.2	1.0
2-Methylbutanal	7.0	2.3	1.8	1.6
3-Methylbutanal	3.6	1.4	1.1	1.0

[a] Concentration in µg/g.
[b] After storage the sample of roasted Col was equilibrated in the apparatus for 15 min.
n.d.: not determined.
Source: Mayer and Grosch, in preparation.

Table 6. Release of the highly volatile odorants from roasted coffee. Comparison of whole with ground beans

Odorant	Whole beans[a,b]	Ground
Methanethiol	0.5 (11)[c]	1.9 (43)[c]
Acetaldehyde	14.1 (12)	45.7 (39)
2,3-Butanedione	0.9 (1.8)	7.5 (15)
2,3-Pentanedione	1.1 (3.1)	7.4 (21)
Methylpropanal	0.9 (3.7)	4.9 (20)
2-Methylbutanal	1.1 (4.3)	7.0 (27)
3-Methylbutanal	0.6 (3.6)	3.6 (22)

[a] Concentration in µg/g.
[b] The sample was equilibrated for 15 min in the apparatus.
[c] Loss of the odorant (%).
Source: Mayer and Grosch, in preparation.

Table 7. Concentrations of odorants in ground roasted coffee and in its headspace: Sulfur compounds phenols and furanones

Odorant	Powder[a]	Headspace[a,b]
2-Furfurylthiol	1.65	0.38 (23)[c]
Methional	0.245	0.07 (29)
Guaiacol	3.42	0.6 (18)
4-Ethylguaiacol	1.78	0.15 (8)
4-Vinylguaiacol	45.1	2.2 (5)
Vanillin	4.05	0.8 (20)
4-Hydroxy-2,5-dimethyl-3(2H)-furanone	144	2.0 (1.4)
2-Ethyl-4-hydroxy-5-methyl-3(2H)-furanone	15.9	0.2 (1.3)
3-Hydroxy-4,5-dimethyl-2(5H)-furanone	1.85	0.02 (1.1)

[a] Concentration inµg/g.
[b] The coffee sample Col was ground and equlibrated for 30 min in the apparatus.
[c] Loss of the odorant (%).
Source: Mayer and Grosch, in preparation.

Altogether, 22 potent odorants were quantified in the headspace of the coffee sample. A sensory experiment was started to examine whether it was possible to imitate the aroma of roasted coffee on the basis of the headspace data. Therefore, the odorants, in the concentrations equal to those in the headspace after a release period of 30 min, were dissolved in pentane (aroma model MII). This solution was injected into the apparatus (Figure 1) which after removal of the Tenax trap was used as an olfactometer (Zehentbauer and Grosch, 1997). Altogether, six assessors compared the aroma of the synthetic mixture of the odorants with that of the original coffee sample. The result in Table 8 indicates that the aroma profile of MII matched very well the aroma profile of the coffee sample. The highest difference in intensity which amounted only to 0.2 points for the sweetish/caramel and the smoky notes, were not significant. The good agreement in the aromas is underlined by the high score of 2.6 given by the assessors for the similarity of the overall odor of the imitate with that of the coffee sample. Therefore, the similarity score of the headspace model MII was higher than that of the mixture of odorants MI dissolved in the oil/water emulsion (Table 2). This lower score of MI is mainly caused by the base which differs from the solid coffee matrix in the binding of the odorants. The high similarity of the headspace model MII with the aroma of ground roasted coffee (Table 8) was firstly caused by the elimination of the matrix. Furthermore, the concentrations of the odorants in the headspace is much lower than in the matrix. Hence, chemical reactions which have been detected in the coffee brew of the odorants (unpublished results) are very unlikely.

Table 8. Odor profile of freshly ground coffee and the corresponding headspace aroma model M II

Attribute	Coffee[a]	M II[b]
	Intensity[c]	
Sweetish/caramel	2.2	2.4
Earthy	1.4	1.4
Roasty/sulfurous	2.5	2.4
Smoky	1.2	1.4
Similarity[d]		2.6

[a] The coffee sample Col was equilibrated for 30 min in the apparatus and then smelled.
[b] Mixture of the 21 odorants which were found in the headspace.
[c] Intensity rating scale: 0 (absent) to 3 (strong).
[c] Similarity rating scale: 0 (no similarity) to 3 (identical).

5 Formation of 2-furfurylthiol (FFT) (Moors and Grosch, in preparation)

The important role established in the sensory experiments for FFT confirmed the assumption of Reichstein and Staudinger in 1930 that this thiol is a key component of the flavor of ground roasted coffee. Therefore, we were interested to identify the precursor of FFT in raw coffee and to get an insight into the formation of FFT during roasting.

Ground raw coffee was roasted in an autoclave. The formed FFT was determined by a stable isotope dilution assay. A comparison of exp. nos. 1 and 2 in Table 9 indicates that the amount of FFT increased 7fold when air was replaced by argon in the autoclave used for the roast process. An increase of the pressure by a factor of 19 did not affect the production of FFT in exp. no. 3. Due to these results roasting was performed in the following experiments after replacement of air by 0.1 MPa argon. Shortage of the sulfur source had limited the production of FFT in exp. nos. 2 and 3. Consequently, addition of cysteine in exp. no. 4 enhanced the amount of FFT strongly.

Table 9. Formation of 2-furfurylthiol (FFT) during roasting of ground raw Arabica coffee[a]

No.	Roast conditions	FFT (µg/kg)
1	Air	118
2	Argon (0.1 MPa)	870
3	Argon (1.9 MPa)	820
4	Addition of cysteine (100 mg)/ argon (0.1 MPa)	9220

[a] The material (5 g) was heated in an autoclave for 2 h at 200°C.

Table 10. Liberation of bound thiols from roasted coffee

Compound	with DTE[a]	without DTE[b]	Increase
		Amount (µg/kg)	
2-Furfurylthiol	1328	414	914
3-Methyl-2-butenthiol	16	4	12
3-Mercapto-3-methylbutanol	1343	543	800
3-Mercapto-3-methylbutyl formate	39	26	13
Methanethiol	1920	610	1310

[a] After roasting and extraction with CH_2Cl_2 the sample was treated at pH 7.5 with dithiothreitol (DTE).
[b] Control experiment.

The experiments summarized in Table 10 indicate that after roasting a portion of FFT and of other thiols are bound to coffee components. After roasting, the coffee sample was extracted with methylenechloride to remove the volatiles. Then the coffee sample was divided. One half was treated with dithiothreitol (DTE) dissolved in a phosphate buffer (pH 7.5), and the other half only with the buffer. Quantification indicated that higher amounts of the thiols were found after treatment of the roasted coffee sample with DTE. In the case of FFT the increase against control amounted to more than 200 %. As the reagent DTE cleaves disulfide bonds we suggest that FFT and the other thiols listed in the table are linked via these bonds to cysteine and cysteine containing peptides and proteins occurring in roasted coffee.

Now to the experiments showing the precursors of FFT. Raw coffee was fractionated and after hydrolysis the monosaccharide composition of each fraction was gas chromatographically determined. As shown in Table 11 under no. 2 for the defatted material, arabinose was the only pentose of which considerable amounts occur in raw coffee. This result is in agreement with those reported by Thaler (1979). Galactose, mannose and glucose are found in the hexose fraction given as sum in Table 11. In addition, the concentration of free cysteine was determined in some fractions. A comparison of exp. nos. 1 and 2 shows that the yield of FFT was lowered when the raw material was defatted before roasting. The defatted raw coffee was separated in a water-soluble and in an insoluble fraction. Roasting of the water-soluble fraction (exp. no. 3) yielded 7 times more FFT than roasting of the water insoluble one (exp. no. 4). However, the sum of FFT resulting from the two fractions (93 µg/kg) was much lower than the amount obtained from raw coffee (exp. no. 2). The main reason for this decrease in the generation of FFT was the separation of cysteine which was completely extracted with water from the major portion of arabinose which remained in the water-insoluble fraction. This suggestion was confirmed in exp. no. 5, as the amount of FFT increased strongly when the water-insoluble fraction was roasted after addition of cysteine.

The importance of arabinose as precursor of FFT was shown after separation of the water-soluble fraction by ultrafiltration since roasting of the permeate (exp. no. 6), in contrast to the retentate, yielded FFT due to the lack of arabinose. The finding that only the pentose but not the hexoses occurring in raw coffee was active as precursor of FFT was in agreement with the results of Hofmann and Schieberle (1997).

Table 11. Formation of 2-furfurylthiol (FFT) during roasting of raw coffee and its fractions

No.	Sample/fraction (yield)	Cys[a]	Arab[b]	Hex[b,c]	FFT
		Amount (g/kg)			(µg/kg)
1	Ground raw coffee (100 %)	n.a.	n.a.	n.a.	689
2	No. 1, defatted (88 %)	n.a.	40	327	477
3	Water-solubles of no. 2 (29 %)	0.6	11	174	81
4	Water-insolubles of no. 2 (59 %)	<0.01	61	401	12
5	Water-insolubles of no. 2 plus cysteine[d]				581
6	Permeate (M $<10^3$) of no. 3 (8 %)	0.41	<2	169	0
7	Retentate (M $>10^3$) of no. 3 (21 %)	0.46	25	138	121

[a] Free cysteine (Cys).
[b] Monosaccharides arabinose (Arab) and hexoses (Hex) after hydrolysis.
[c] Sum of galactose, glucose and mannose.
[d] Cysteine (0.1 g) was mixed with no. 4 (5 g) and then roasted. n.a., not analyzed.

Table 12. Effect of water and acid pH on the formation of FFT

Exp.	Reaction system[a]	FFT (µg)
1	Arabinogalactan (from coffee, 2.5 g)	9.6
2	As exp. 1 plus 10 % water	11.2
3	As exp. 1 plus 10% phosphate buffer (0.5 mol/L, pH 5.3)	16.1

[a] After addition of cysteine (2.5 g) the reaction system was roasted at 200°C for 1 h.

The major portion of arabinose occurring in raw coffee has been identified by Bradbury and Halliday (1987) as building block of polysaccharides. To study bound arabinose as precursor of FFT an arabinogalactan was isolated from raw coffee (Bradbury et al., 1990) and then roasted in the presence of an excess of cysteine. Exp. no. 1 in Table 12 indicates bound arabinose as precursor of FFT. In exp. no. 1 a dry reaction system was roasted. However, raw coffee contains approximately 10% water and, due to the presence of acids, the pH lies in the range of 5. In comparison to the dry reaction system addition of 10% water to the arabinogalactan from coffee increased the production of FFT by 17% (exp. no. 2 in Table 12). A greater effect was caused when the pH was lowered to 5.3 by the addition of a phosphate buffer. The increase amounted to 68 % (exp. no. 3). A partial hydrolysis of the polysaccharide with formation of free arabinose might be the cause for this effect.

6 Conclusions

The key odorants of roasted coffee were verified by sensory experiments which based on the results obtained by a combination of instrumental with sensorial, analytical methods. These compounds could be useful as indicators for the assessment of the coffee quality in dependence, e.g. of the provenance, roast process and storage conditions.

The roasty-sulfurous smelling 2-furfurylthiol (FFT) is undoubtedly confirmed as the outstanding odorant of coffee. During roasting it is formed by reactions of cysteine with arabinose which is released from the polysaccharides in coffee. Substantial amounts of 2-furfurylthiol and of other thiols are linked by disulfide bonds to components of roasted coffee.

7 References

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Summary

An aroma model very close to the aroma profile of a sample of medium roasted Arabica coffee from Colombia was developed by using 27 odorants which had been both screened in preceding dilution experiments and quantified. Omission experiments indicated 2-furfurylthiol (FFT) as the outstanding odorant of roasted coffee. In addition, 4-vinylguaiacol, several alkylpyrazines, furanones, acetaldehyde, propanal and the malty smelling *Strecker* aldehydes are all responsible for the unique flavor of roasted coffee. FFT and the above mentioned odorants mask the peasy odor of 2-isobutyl-3-methoxypyrazine which originates from the raw material and is stable during roasting. The release of important odorants during open storage of ground coffee was also determined, e.g. the losses of methanethiol and acetaldehyde within 15 min amount to 43% and 39%, respectively. In contrast, only 1% of the low volatile furanones evaporate within 30 min. Model experiments indicated that in the roasting process FFT is produced by reactions of cysteine with arabinose which originates from polysaccharides. Bound thiols including FFT were liberated from roasted coffee by a treatment with dithiothreitol. This suggests that they are linked by disulfide bonds to cysteine and cysteine containing peptides and proteins.

Evolution of Coffee Aroma Characteristics during Roasting

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Introduction

Roast coffee aroma is a subtle mixture of more than 800 components, from which a restricted number of components (< 10%) are perceivable using GC-Olfaction (GC-O) on typical aroma extracts. These compounds cover a broad range of odours. Some of them have an odour reminiscent of roasty and coffee character, but do not deliver plain coffee aroma only by themselves.

The knowledge of major coffee aroma impact compounds contributing to coffee odour has significantly progressed during the last decade. The use of GC-O combined with dilution techniques brought to the fore strong odorous compounds, and their concentrations in coffee were measured precisely using Isotope Dilutions Assay. O.Vitzthum et al.[1] and W.Grosch et al.[2-10] have thoroughly described roast coffee key aromas. Reformulation studies were published by W.Grosch et al. for brews[7] (23 compounds) and more recently for roasted coffee powders[9-10] (27 compounds).

Only sparse information is available on the evolution of odorous volatiles during roasting and their impact on the overall aroma characteristics. Compounds having the most impact on green coffee aroma were assessed by H.Steinhart et al.[11], and the levels of some volatile compounds or classes of compounds were measured for various roasting levels by R.Silwar et al.[12], and respectively Y.Kawakami et al.[13] and L.Hashim et al.[14].

The purpose of this study was to correlate global aroma quality of ground coffee with the relative composition of its odorous components, for various roast levels ranging from green coffee to dark roast.

Materials and Methods

Coffee: Coffee beans (500 g, Colombia) were roasted with a Neotec roaster at a constant temperature (230°C). Different roast levels were produced according to the length of roasting time (1, 2, 4, 6 and 9 minutes). Roasted coffee samples were characterised by L-values between 52 and 14. The coffee beans were stored in evacuated sachets (100 g) at –80°C, and ground just before use.

Sensory evaluation: An internal panel composed of 15 people was trained to memorise the forty-five "Champ des Odeurs®"[15] references individually. Assessors then worked on mixed solutions of the reference molecules in order to improve their accuracy for olfactive evaluation and finally on complex food products as a test of the method. A further training specific for coffee revealed that certain notes present in coffee could not be described by the basic set of references. Therefore nine further references (to cover amine, sulphur, pyrogenic and woody notes) were added. At the same time, performance tests were done regularly on the learnt molecules. Training took a total of 40 hours.

From the 54 references, assessors eliminated those which did not correspond to notes present in coffee. 26 references were retained. The Colombia samples were evaluated three times. At each session, assessors were presented with 2 brown flasks containing 20g of freshly ground coffee, in a monadic presentation. The assessors rated the 26 references according to their intensity on an unstructured scale of 10 cm with 0 (no value of the reference) at the left and 10 (maximum value of the reference) at the right. The data acquisition and statistical treatments were performed with FIZZ software (Biosystemes, Couternon, France).

Aroma isolation: Volatile aroma from coffee samples was recovered by vacuum steam stripping with water. 50g of ground coffee were placed in a 250 ml round bottom flask. 35 g of sodium chloride were dissolved in 100 ml of water, added to the coffee and mixed. The flask containing the mixture was frozen with liquid nitrogen for 20 minutes and then connected to the vacuum stripping apparatus (0.5×10^5 Pa). The stripping was run for four hours at room temperature followed by two hours at 50°C. The distillate was condensed in two cold traps cooled with liquid nitrogen. Then it was successively extracted with three portions of methylene chloride (3× 33 ml). The aromatic extracts were pooled, dried over anhydrous sodium sulphate and then concentrated to 1 ml in a Kuderna-Danish apparatus. The representativeness of coffee aroma extracts was verified for different roast levels. The aromas from extracts and corresponding R&G were compared using similarity tests.

Aroma analysis: Odorous compounds were located by the CHARM method, then identified and quantified by mass-spectrometry. In both cases, compounds were eluted on a polar column (FFAP from Machery-Nagel).
CHARM analyses (GC-O) were performed with a chromatograph HRCG MEGA CE equipped with a Combi PAL autosampler (CTC) and 3 detectors in parallel: FID, FPD and GC-O. Each extract was evaluated at each dilution by 3 trained assessors, until no odour was perceived (dilution step =3).
Mass spectrometry analyses (GC-MS) were performed with a quadrupole Fisons MD800 coupled with a GC 8000 equipped with an A200S autosampler (CTC). Quantification was done according to an internal standard added to the concentrated extract.

Results

The ***sensory profiles*** of the different coffees were analysed statistically. Results of the PCA are shown in Figure 1. The first factorial plan of the principal component analysis represents 78% of the initial variability. The Columbia coffee samples were coded as A. Similarly, roasting levels

were coded as: 0 for the green coffee, 1 and 2 for the light roast, 4 and 6 for medium roast and 9 for dark roast (these codes refer to minutes of roasting time).
A good agreement between the assessors was found for describing the odour of ground coffees ranging from green to over-roast levels. Out of the 26 references (see Table 1), 16 were characterised as relevant to describing the coffee samples (variance analysis).
The following evolution was observed during roasting. The green coffee was described by fatty, green, lactone and terpenic descriptors. During roasting, sweet notes, pyrogenic sulphurous and finally amino odours were developed. The green coffee (A_0) was characterised by *methional (CO 12)*, *β-caryophyllen (CO 17)*, *ethyl isobutyrate (CO 20)*, *cis-3-hexenol (CO 23)* and *2,6-(E,Z)-nonadienal (CO 47.* The light roast coffee (A_1 and A_2) samples were described by *coumarine (CO 2)* followed by *acetylpyrazine (CO 26)* and *ethylmaltol (CO 30)*. The contribution of the green coffee descriptors was still significant.

CO No.	Molecule	CO No.	Molecule
1	*nonanal*	29	*vanillin*
2	*coumarine*	30	*ethylmaltol*
3	*1-octen-3-ol*	31	*isobutylquinoleine*
9	*butyric acid*	32	*diacetyl*
12	*methional*	43	*isobutylamine*
13	*isoborneol*	46	*acetic acid*
14	*phenol*	47	*2,4(E,Z)-nonadienal*
17	*βcaryophyllen*	48	*furaneol*
18	*dimethyldisulphide*	49	*2-isopropyl-3-methoxypyrazine*
20	*ethyl isobutyrate*	51	*2-ethoxythiazole*
23	*cis-3-hexenol*	52	*ethyl-3-furfurylthiopropionate*
24	*evernyl*	53	*4-methyl-4-mercaptopentan-3-one*
26	*acetylpyrazine*	54	*2-acetythiazole*

Table 1

List of sensory descriptors used for the study. Descriptors 1 to 43 belong to the "Champ des Odeurs©" from J-N. Jaubert.

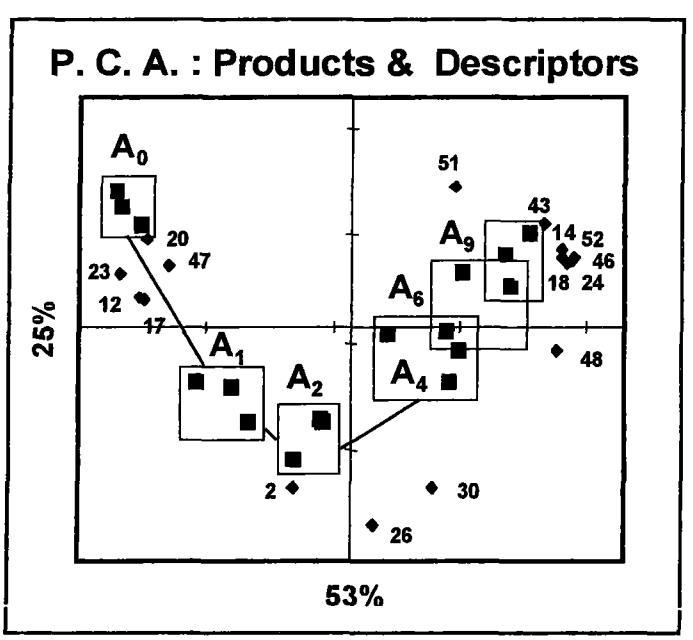

Figure 1

Principal component analysis showing the position of the ground Columbia coffees (A) at different roast levels (green, 1 to 9 minutes roasting), and the associated significant sensory descriptors (axes 1 and 2).

Medium roast sample (A_4) was mainly characterised by *furaneol (CO 48)*. For darker roast (A_6 and A_9), the references used to profile the coffee samples were *phenol (CO 14)*, *dimethylsulphide (CO 18)*, *evernyl (CO 24)*, *isobutylamine (CO 43)*, *acetic acid (CO 46)*, *2-ethoxythiazole (CO 51)* and *ethyl-3-furfurylthiopropionate (CO 52)*.

The **aroma recovered in the organic extracts** of the 6 Colombia coffees was **characterised qualitatively and quantitatively**. Fifty odorous zones were located by GC-O at various dilutions and further characterised by a CHARM value. In addition, 176 compounds were identified and quantified by GC-MS. The evolution of odorous compounds during roasting was compared with that of sensory descriptors presenting similar notes (green, earthy/mouldy, fruity, sweet/buttery, roasty/nutty, sulphurous, smoky/spicy and acidic/pungent). Some examples are presented in the Figures 2 to 4 and discussed below.

Green and earthy sensory notes are important descriptors of green coffee. The intensity of some of them decreases during roasting *(CO 12, 17, 23)*, or remains constant such as *CO 49*. Associated aroma compounds are either not affected by the roasting, as for example 2-isobutyl-3-methoxypyrazine and methional, or in some cases even increase during roasting e.g. 2,3-diethyl-5-methylpyrazine (see Figure 2). The masking effects from other potent aromas generated during roasting probably decreases the sensory perception of some of the green and earthy notes in overall aroma of roasted coffees.

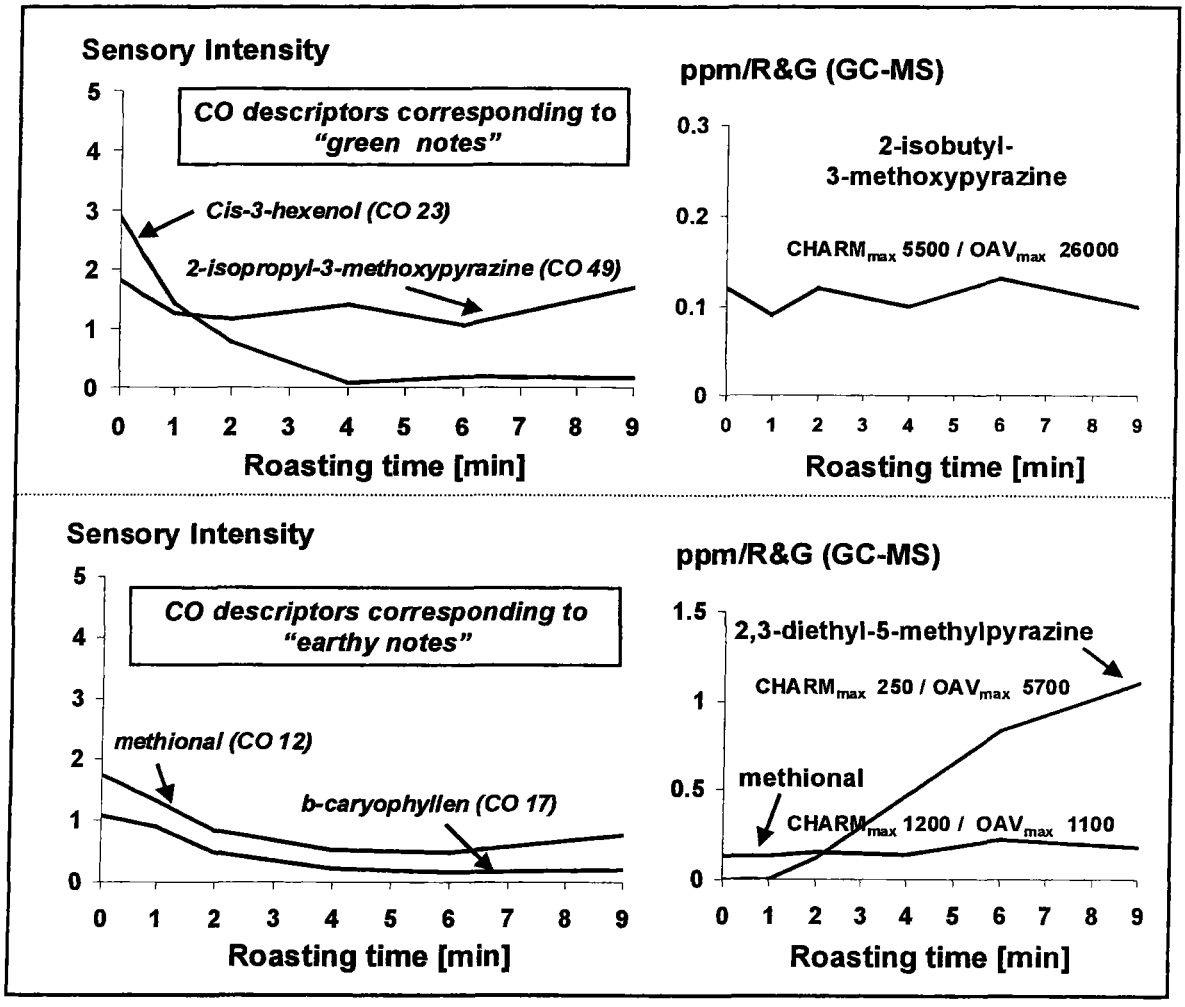

Figure 2 *Evolution of sensory descriptors and volatile compounds presenting similar odours. Top: green notes, bottom: earthy notes*

Sweet notes of overall aroma present a maximum of sensory intensity at the early stage of roasting (light roast 1-2 minutes). Then their contribution decreases in darker roasts (4,6 and 9 minutes), as shown in Figure 3 *(CO 2)*. Most of the volatile compounds characterised by sweet odours present also a maximum of concentration, but this maximum is shifted towards medium roast levels (4-6 minutes). Some other compounds e.g. damascenone increase slightly during roasting. The shift in the maximum between sensory intensity and concentration curves suggests that sweet notes are partly masked by other potent odours in roasted samples.

Figure 3 also shows the patterns observed for "roasty" notes. Some sensory descriptors present a maximum of intensity at early stages of roasting (2 minutes, *CO 26*), others increase in perception and then level off in higher roasted samples *(CO 48)*. The concentration of some compounds characterised by roasty odours increases during roasting e.g. trimethylpyrazine and 2-ethyl-3,5-dimethylpyrazine, or others level off after 6 minutes (2,5 and 2,6-dimethylpyrazine). None of them present a maximum of concentration at early stages of roasting. These results suggest that the evolution of roasty notes is partly correlated with that of roasty-smelling compounds.

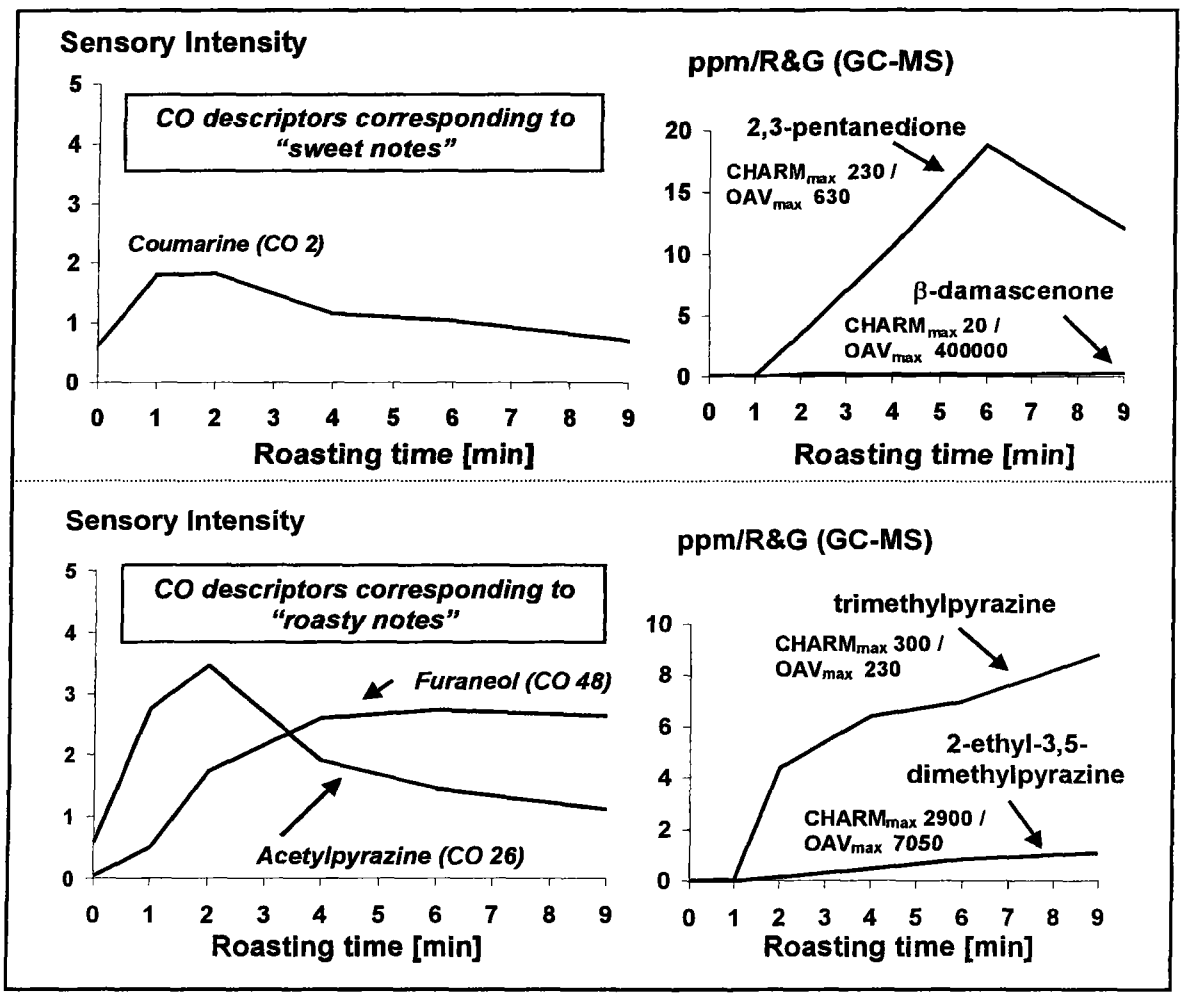

Figure 3 *Evolution of sensory descriptors and volatile compounds presenting similar odours. Top: sweet notes, bottom: roasty notes*

The perception of sulphurous notes increases significantly between 1 and 9 minute's roasting as shown in Figure 4 *(CO 18, CO 52)*. The concentration of associated compounds also shows a steep increase up to 9 minutes (furfurylmercaptan), or up to 6 minutes (dimethyltrisulfide).

The perception of smoky notes increases regularly during roasting (Figure 4, *CO 14, CO 24*) and is well correlated with the increasing concentration of associated compounds such as guaiacol.

Sulphurous and smoky compounds are well correlated with sensory perception, and certainly contribute to the decreased perception of sweet, green and earthy notes in roasted samples.

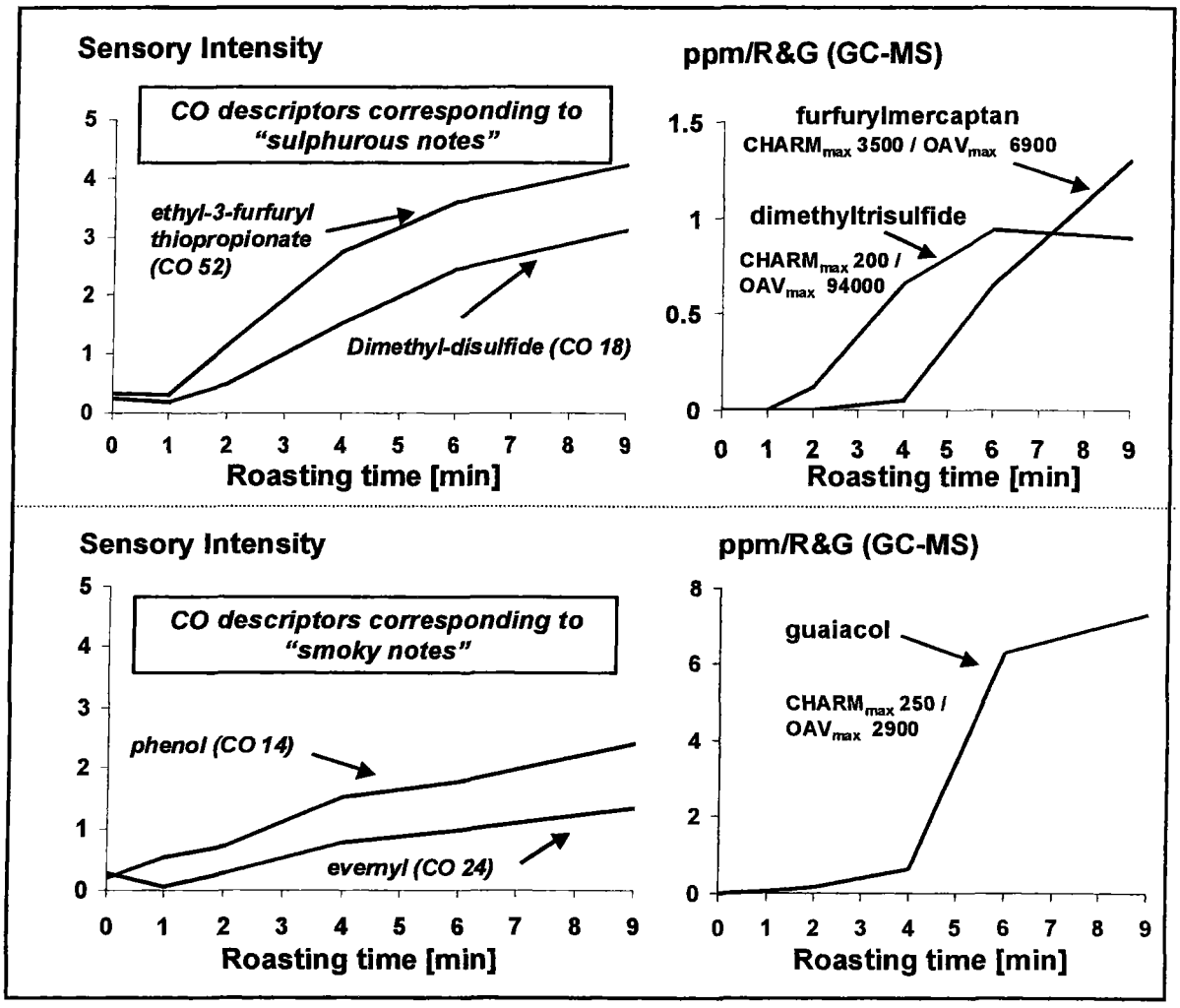

Figure 4: Evolution of sensory descriptors and volatile compounds presenting similar odours. Top: sulphurous notes, bottom: smoky notes

Conclusion

The evolution of odorous aroma compounds during roasting was compared with the evolution of associated sensory descriptors. A correlation between concentration and sensory note intensities was not observed for all compounds. Strong odorants formed in the later stage of roasting tend to mask the sweet, green, earthy and even some roasty notes, despite the presence of associated compounds in roasted coffees. Such interactions need to be taken into account when trying to assess coffee aroma quality by analytical techniques.

Summary

The purpose of this study was to correlate the global aroma of ground coffee (Columbia) with the relative composition of its odorous components at various roast levels, ranging from green to over-roasted coffee. Roast levels were chosen to produce distinct aromas (roasting at constant temperature, from 1 to 9 minutes). The aroma of the various coffees was characterised by sensory profiling using the "Champ des Odeurs" approach. Volatile compounds were recovered by steam vacuum distillation, followed by solvent extraction and concentration. The odour representativeness of the extracts was checked by sensory similitude tests. Odorous compounds were characterised by GC-O (CHARM technique) and identified and quantified by mass spectrometry.

A good agreement between the assessors was found for describing the odour of various ground coffees. Out of the 26 descriptors, 16 were characterised as relevant to differentiate the coffee samples. Fatty, green, lactone and terpenic notes were representative of the green coffee. During roasting, sweet notes, pyrogenic sulphurous and finally amino odours were developed. The evolution of the sensory intensities was compared to the generation of volatile compounds presenting similar odorous characteristics. Green and earthy notes in the overall aroma of roasted coffees were equal or lower in intensity as compared to their level in green coffee while the concentration of associated volatile compounds remained unchanged or increased during roasting. Most of the sweet notes presented a maximum of intensity for both the sensory assessment and concentrations of associated compounds. However the concentration maximum occurred at higher roast levels (4-6 minutes for concentration maximum, 1-2 minutes for sensory maximum). The evolution of roasty notes was found to be partly correlated with that of roasty-smelling compounds. Sulphurous and smoky notes were better correlated with associated compounds, and are certainly at least partially responsible for the masking effect in the perception of sweet, green and earthy notes of roasted samples.

Résumé

Le but de cette étude était de corréler la perception sensorielle de l'arôme de café moulu pur Colombie, avec la composition relative des composés odorants de l'arôme, et ceci pour différents degrés de rôtissage, partant du café vert jusqu'au café très rôti. Les degrés de torréfaction ont été sélectionnés de façon à produire des arômes distincts (rôtissage à température constante, de 1 à 9 minutes). L'arôme des cafés a été décrit selon une approche adaptée du Champ des Odeurs©. Les composés volatils ont été isolés par distillation à la vapeur sous vide, extraits et concentrés. La représentativité odorante des extraits a été vérifiée par des test de similitude. Les composés odorants ont été localisés par GC-O, caractérisés par des valeurs CHARM, identifiés et quantifiés par spectrométrie de masse.

Un très bon accord a été observé entre les membres du jury pour la description sensorielle des cafés. Parmi les 26 descripteurs retenus pour l'étude, 16 étaient significatifs pour différencier les cafés. Le café vert est décrit par des notes grasses, vertes, lactones et terpéniques. Au cours du rôtissage, apparaissent successivement des notes douces, pyrogénées, sulfurées et finalement aminées. L'évolutions de l'intensité des notes sensorielles a été comparée à celle de la concentration des composés volatils de l'arôme présentant des odeurs similaires. Par rapport au café vert, les notes sensorielles vertes et terreuses ont une intensité constante ou plus faible dans les cafés rôtis. Si certains composés volatils présentant les mêmes notes ne subissent pas de variation de concentration

au cours du rôtissage, d'autres voient leur concentration augmenter dans les cafés rôtis. La plupart des notes douces présentent un maximum d'intensité sensorielle et de concentration. Cependant le maximum de concentration est déplacé vers les degrés de rôtissage plus foncés. Il apparaît entre 4 et 6 minutes, alors que le maximum sensoriel est situé entre 1 et 2 minutes. L'évolution des notes rôties est partiellement corrélée avec celle des composés odorant associés. Les notes soufrées et fumées sont mieux corrélées avec les l'évolutions des composés volatils correspondants, et sont certainement responsables d'effets de masquage quant à la perception des notes vertes, terreuse et douces dans les cafés rôtis.

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Differences in Chemical Composition of electronically sorted Green Coffee Beans

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Introduction:

The quality of the coffee beverage is detemined to a very high extent by its aroma and flavor. These in turn can be influenced already at the green coffee level by a variety of factors like botanical variety, soil, green coffee processing, defects caused by insects and microorganisms and also the conditioning of green coffee. Some important parameters in the coffee producing country are the roasting process, packaging, beverage preparation and also staling of the finished product.

Since the aroma of coffee plays such a big role, much effort has been put into identifying the compounds that are responsible for the good flavor of this beverage. While the quest for the key flavor compounds in coffee could not yet be answered in a satisfactory way in complete detail, many of the less desirable compounds in coffee could be clearly identified. And the first criterium for quality of course is the absence of those compounds.

Examples for these types of "off-flavor" compounds are methoxyisopropylpyrazine (Becker et al., 1987) which was found to be the culprit for a "peasy" note in certain coffees from Ruanda, 2,4,6-trichloroanisol (Spadone et al., 1987), responsible for the medicinal, musty note in socalled "rio" coffee. Methylisoborneol (Vitzthum et al., 1990) has been found to be the main contributor to the typical "earthy" note of Robusta coffee. Certain ethylesters were found to be the culprits of the typical taste and smell of overfermented and/or stinker beans (Bade-Wegner et al., 1997).

Usually these "off-flavor" compounds are present in very small amounts but due to their extremely low odor threshold, they do negatively influence the overall flavor. In addition, many of these compounds are thermally stable and survive roasting, giving rise to off-flavors also in the beverage. Especially in the making of espresso the presence or absence of these "off-flavors" influences very much the quality of the final beverage since in the "espresso

type" coffee preparation the aroma carrying lipids are extracted to a higher extend than in other common types of coffee preparation.

Defects that are present in green coffee play a big role in classifying the product for trade purposes. For example in the New York classification system the grading of green coffee is based on the quantity of imperfections found in a one pound sample.

Wet processed - or washed - coffee usually shows less defects, because it is already "pre"sorted depending on the floating or sinking properties of the green beans in water. This "preselection" is missing in socalled "Natural coffees", the coffees that are prepared in the sundried process. They usually exhibit more types and quantity of defects than the washed coffees.

Electronic sorting of green coffee beans by means of color mapping is a technique that has been studied and developed extensively since the 1980's (Maughan et al., 1980; Milo, 1980; Illy et al., 1982; Suggi-Liverani et al., 1987; Illy and Maughan, 1989; Suggi-Liverani, 1991;). The optical principle of this type of sorting machines is based on measuring the reflectance ratio of two different wavelenghts. Beans are illuminated with white light. The resulting reflected light is then filtered by two different filters at visible wavelength (usually red and green) and detected. The ratio of these two wavelenghts exhibit characteristic values for various defects as well as for "normal" beans. The sortable defects range from immature, waxy, chipped, insect damaged or broken beans to black or whitish beans. In addtition also the size of the beans can be measured. For the industrial sorting process the intensity and wavelength of the beans are compared to a previously established map.

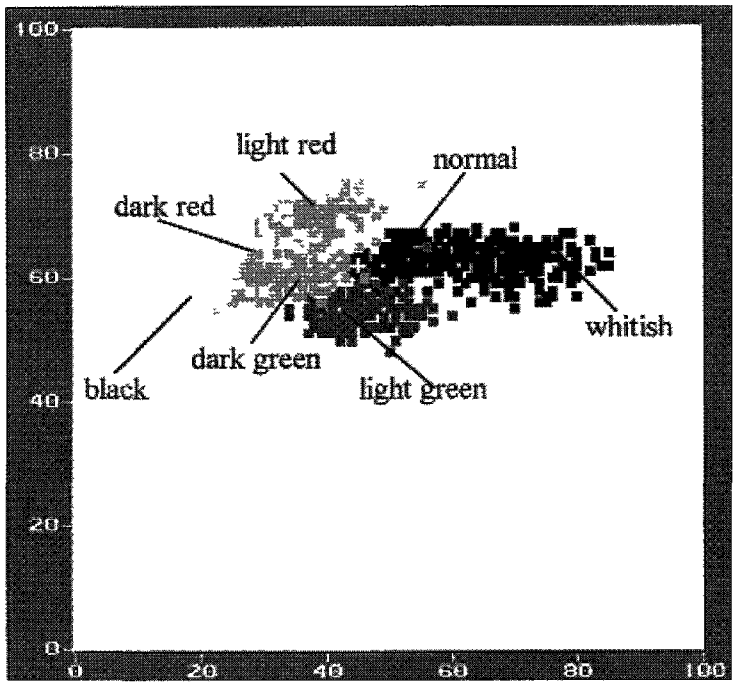


Fig. 1: Classes of defective beans that can be distinguished by the sorting machine

The machines used for this type of sorting generally consist of a delivery system for the green beans (A), an optical box (B), which is responsible for the sorting process, a control and acquisition system (C), processing software (D) and a mechanism for removing the defective beans, usually by a stream of air.

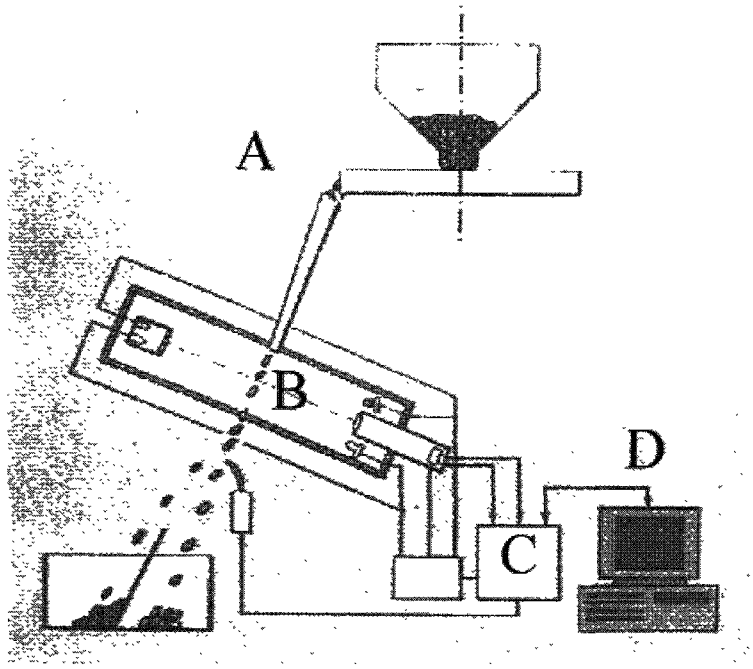


Fig. 2: Scheme of the sorting machine's setup.

Experimental

Extraction procedure: 100 g of green coffee (whole beans) were put into a glass flask along with 1 liter of demineralized water and 50 µg of internal standard (used standards: Undecane and/or Dimethoxytoluene). Boiling chips were added and the flask subjected to simultaneous distillation/extraction (SDE, Schulz et al, 1977) procedure. Organic solvent used for the SDE was 50 ml of pentane/ether (1:1). Before use the ether has been distilled to remove the stabilizer (BHT). Cooling of the SDE apparatus was held at – 4.5°C. SDE was conducted for 2 hours. The obtained extracts were concentrated to 0,5 – 1 ml using a Vigreux column and subsequent blowing down with nitrogen gas.

Gas Chromatography:

GC-Sniff: The analysis by gas chromatography was performed on an HP 6890 GC with a Gerstel cooled injection system. Capillary column was a DB-Wax (J & W, DB-Wax, 60 m x 0,32 mm, df = 0,25 µm). The system was held in splitless mode for 1.2 minutes. Then a purge flow of 15 ml/min was applied. The column oven was held at 35°C for 3 minutes, then programmed at 4°/minute to 220°C, held for two minutes then programmed at 20°C/min to

240°C and held for 15 minutes. Flow was held constant at 39 cm/s. Carrier gas was helium, detector gases 300 ml/min synthetic air and 30 ml/min hydrogen. Make up gas for the detector was Nitrogen at 30 ml/min.

GC-MS: Analysis were performed on a Finnigan GC-Q Ion trap Mass Spectrometer coupled to a Finnigan GC. Capillary column was a DB-Wax (J & W, DB-Wax, 60 m x 0,25 mm, df = 0,25 µm). Split flow was 3,5 ml/min. The column oven was held at 35°C for 3 minutes, then programmed at 4°/minute to 220°C, held for two minutes then programmed at 20°C/min to 240°C and held for 15 minutes. Ionization was done in Electron Impact mode at 70eV. Full Scan Spectra were recorded from m/z 39-250.

Identification of compounds was done by comparison of chromatographic behaviour with mass spectral and organoleptical data with those of authentic reference materials. Quantification was performed by GC-MS using internal standards and 5 point calibration curves for each compound.

Results and discussion

In this paper we'd like to show how certain off-flavor containing green coffee beans in Arabica coffees from Brazil can be eliminated by electronic sorting.

Using the technology of sorting machines it is possible to sort out various defects simultaneously. Since we were interested in the flavor compounds for specific defects only, the sorting was adjusted accordingly.

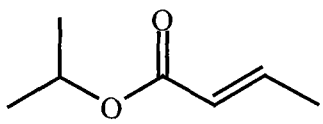
To analyze the flavor compounds, extracts of the green coffee were obtained by simultaneous distillation-extraction. These extracts were subsequently analyzed by gaschromatography with masspectrometrical detection and also by gaschromatography with Sniff-Detection.

The most abundant defects that could be electronically sorted out were two types of immature beans: light green and dark green immature beans. Cuptasting of the dark green immature beans is described as bitter, astringent and reminiscent of rotten fish (Illy and Viani, 1995). Usually these beans also exhibit the "overfermented" flavor. The light green immature beans are described as astringent and fishy, metallic.

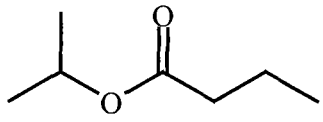
In the GC-Sniffing the main differences between the "accepted" and the defective "rejected" green coffee were some fruity notes. In fact, by GC-MS analysis we found relatively large amounts of some esters that have been published earlier by Bade-Wegner and coworkers to be responsible for the flavor of overfermented and stinker beans (Bade-Wegner et al, 1997).

Ethylisovalerate **1** and ethyl-2-methylbutanoate **2**, are compounds with a very low odor threshold and a fruity, silage like note. This explains their contribution to the "fermented" flavor, even when present in relatively low amounts. Since immature beans are known to ferment very easily, this result is expectable.

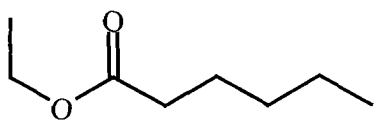
In addition to this two compounds we could also find the presence of two more esters that possess similar flavor properties but have a higher threshold. The chemical structures of these compounds are shown in the figure 5. Ethyltiglate **3** and ethylhexanoate **4** are reported for the first time in green coffee. While ethyltiglate is present in amounts comparable to ethylisovalerate, ethylhexanoate is only present in small amounts. These two compounds also impart the fermented flavor when added to a cup of espresso, however due to their relatively high odor threshold, their presence may be seen more as an indicator.



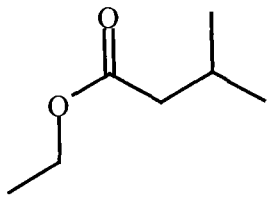
Ethyl-2-methylbutenoate (Ethyltiglate) **3**
Threshold: 65 ppb fruity, caramel note



Ethyl-2-methylbutanoate **1**
Threshold: 0,006 ppb (S-enantiomer), fruity, apple



Ethylhexanoate **4**
Threshold: 1 ppb, pineapple, banana, fruity



Ethyl isovalerate **2**
Threshold: 0,01 ppb, fruity, apple

Figure 3: Structure of various esters found to be present in elevated levels in immature beans. Odor thresholds in water according to literature (Takeoka et al., 1995, 1998).

Ethylisovalerate and Ethyltiglate were present roughly in a relation of 1:10:50 going from normal beans to light and dark green immature ones.

Figure 4 shows the chemical analysis with the GC-MS system. The ion traces are selective for ethyltiglate (m/z 100, 113 and 128) and ethylhexanoate (m/z 88 and 99). The upper chromatogram (A) shows the accepted beans; the two esters are not visible. The middle trace (B) corresponds to the light green immature beans where ethyltiglate is easily detected while the quantity of ethylhexanoate is somewhat less. The chromatogram on the bottom (C) represents the dark green immature beans, where both compounds can easily be detected.

The quantitative results obtained by chemical analysis are shown in figure 5. In this radar plot each axis of the "star" represents the amount of a particular flavor compound. The thin straight line represents the normal coffee, whereas the dotted and the thick straight line represent the light green and the dark green immature beans.

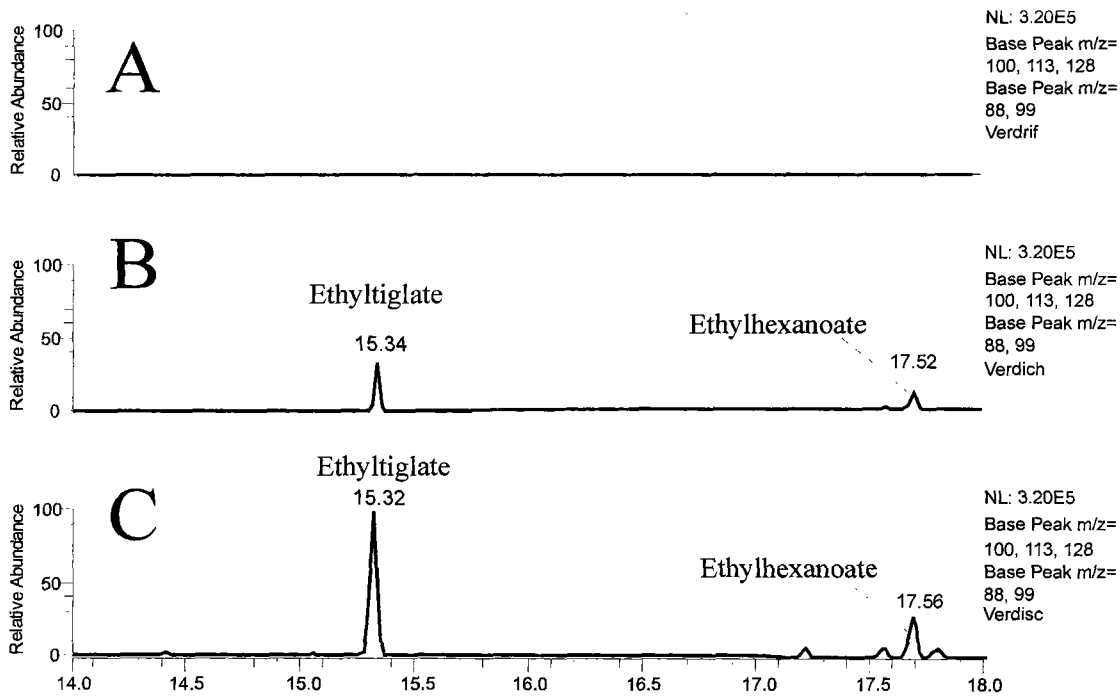


Fig 4: GC-MS analysis of of "accepted" and "rejected" beans. (A) Normal, (B) light green immature and (C) dark green immature beans.

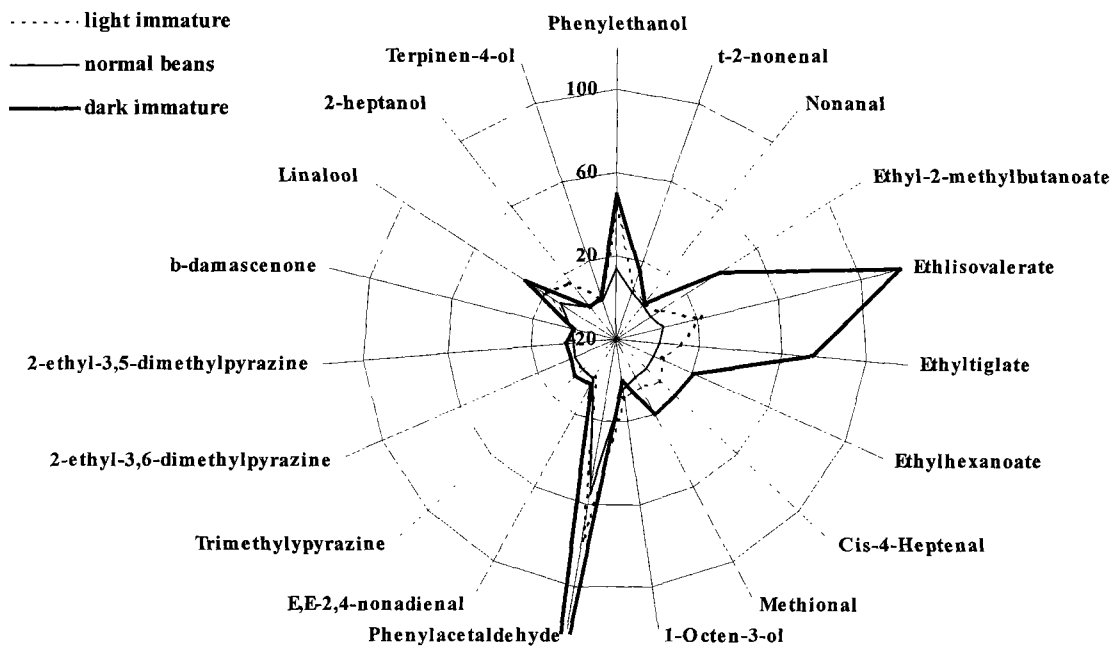
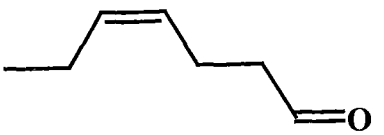


Figure 5: Radarplot of the chemical composition of normal, light green immature and dark green immature beans.

The rotten fish flavor and astringency that goes along with the immature beans could be attributed to elevated levels of cis-4-heptenal **5**.



cis-4-heptenal (5)

Figure 6: Structure of cis-4-heptenal **5**.

This compound has a low odor threshold of 0.4 ppb and it's odor is described as fishy, sardine like (Buttery, 1999). An addition of 1 ng to a cup of espresso rendered the typical "immature" sensation of astringency and the taste of rotten fish. It is a product of the autoxidation of linoleic acid (Grosch, 1998). Linoleic acid is known to be present in higher levels in immature beans (Illy and Viani, 1995).

Cis-4-heptenal could be found in about 10 fold higher concentrations in the immature beans than in the normal beans.

Summary:

The purpose of this paper was to demonstrate the effectiveness of electronic sorting to remove certain defects. This was done by chemical analysis of the flavor compound of "accepted" and "rejected" beans, respectively. The coffee batches available to us contained only two types of defective beans in sufficient amounts for chemical analysis. Light green and dark green immature beans. The chemical differences that could be found when comparing "accepted" and "rejected" beans were quite substantial, especially when looking at the low odor threshold esters that are responsible for the overfermented flavor. As compound responsible for the typical sensation of "immature beans" cis-4-heptenal could be identified.

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Characterisation of Mouldy/Earthy Defect in Green Mexican Coffee

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Introduction

The sporadic appearances of off-notes and taints in green coffee that persist all the way to the cup, are of major concern to cultivators and processors alike.[1-3] This is mainly due to two reasons. *First*, flavour is a key quality criterion of coffee and one of the major drivers for consumer preferences. Whatever affects the flavour of coffee in an objectionable way will also affect the sales figures of cultivators and processors. *Second*, the price of the raw material is a major cost factor of finished coffee products. Both these reasons have lead to the elaboration of strict quality evaluation procedures of green beans.

Here we investigate the mouldy-earthy note, which has appeared in green Mexican coffees (Arabica) and has not yet been characterised in coffee. However, musty, mouldy, earthy notes were already reported in foodstuffs others than coffee and associated with the presence of 2,3,4,6-tetrachloroanisole,[4] 2,4,6-trichloroanisole (2,4,6-TCA),[1] geosmin,[5-7] 2-Methyl isoborneol (MIB),[8,9] 2-methoxy 3-isopropyl pyrazine (MIP),[10] or alkyl methoxy pyrazines. Recently, a review of Maga cited geosmin, MIB and MIP as the main responsibles for mouldy-earthy notes in foodstuffs and water.[11] The aim of the present study is to identify the chemicals responsible for the mouldy-earthy off-flavour in Mexican green coffee.

Experimental

Material: Green coffee beans (500 g) from the Chiapas area in Mexico, obtained by dry post-harvest treatment,[12] and that were judged by a Mexican expert panel as mouldy-earthy, was compared to a coffee of the same origin but without noticeable sensory defect.

Sensory evaluations: Absolute sensory profiling on a 6-point scale and ten attributes was performed at the Nestlé Research Center in Lausanne, with a panel of 12 trained tasters. Blind tastings of the defect and reference samples were performed in two repetitions, on very lightly roasted coffee (120 CTn) and served at 55°C.

Extraction of volatiles: Green coffee beans were frozen in liquid nitrogen, finely ground, and extracted by vacuum hydrodistillation at ambient temperature (Θ < 25-30°C). 100 g of green ground coffee were mixed with 350 ml demineralised and degassed water. 100 ml water was added every two hours. Volatiles were condensed in three cold traps (-196°C). The total extraction time was 6 hours. This allowed to recover 250-300 ml of aqueous extract. The procedure was repeated five times. A total extract of 1.2 l was recovered. Distillates were extracted with CH_2Cl_2 in a Mixxor extractor. The organic phases were collected, dried over Na_2SO_4, concentrated to 1 ml on a Widmer distillation column, and then under a nitrogen stream to 500 mg.

Analyses: The extracts were analysed by GC-FID/sniffing, GC-FID/FPD and GC-MS. Two stationary phases were used for each analysis: *polar* - DB-WAX (J&W Scientific) 30 m x 0.25 mm i.d., 0.25 µm film thickness – and *non-polar* - DB-1 (J&W Scientific) 60 m x 0.25 mm i.d., 0.25 µm film thickness. The analysis conditions were identical on both polar and non-polar columns (splitless). Linear retention indices were calculated for each analysis by a separate injection of a series of n-alkanes (C_5-C_{28}), under the same conditions as the analysis.[13] The oven temperature was held for 30s at 20°C, and was then ballistically increased to 60°C, followed by an increase of 4°C/min to 220°C with a 20 min hold. Injector temperature was 250°C, detector was 275°C. MS analyses were performed on a quadrupole mass filter (HP 5973) either in full scan or in SIM mode. The sniffing profiles were recorded in quadruple, by four different trained sniffers and added to integral sniffing profiles.

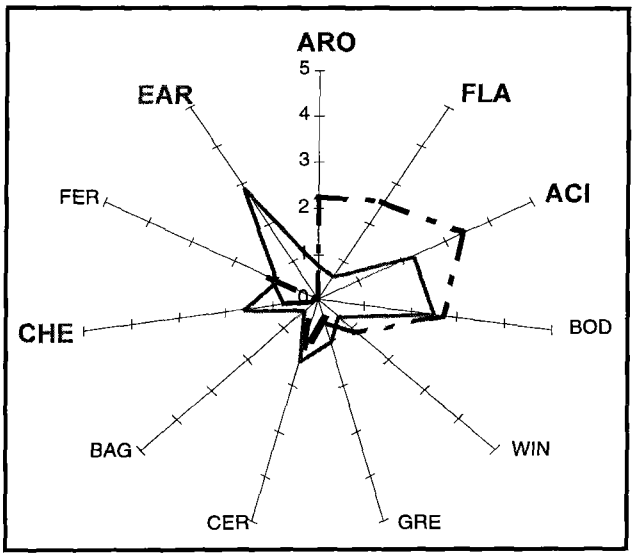
Preparative chromatography: A Hewlett-Packard model 5890 gas chromatograph, modified by Gerstel GmbH (Mühlheim a.d. Ruhr, Germany), was used for the enrichment of earthy/mouldy fractions. Up to seven fractions were pooled in liquid N_2 traps.[14-16] Separation was achieved on two fused silica non-polar capillary columns, a DB-1 (J&W Scientific) 5m x 0.53 mm i.d., 1.05 µm film thickness, and a HP-1 (Hewlett-Packard) 12m x 0.53 mm i.d., 1.05 µm film thickness, connected in series. A temperature programme starting at 60°C up to 220°C (12°C/min) was used. The outlet of the second column was connected to a collector. 40 injections of 5 µl were performed with the defective extract. Seven fractions were collected at -30°C. Starts and ends cuttings were precisely determined after sniffing investigations. Concentrated extracts were rediluted into the minimum amount of solvent (20 µl), analysed and quantified.

Quantification: Quantitative estimation of six substances was realised by external standardisation.[17] A stock solution was made by diluting standards at a concentration of 10 ppm (w/w) in CH_2Cl_2. It contained five different references, e.g. 2-methoxy 3-isopropyl pyrazine, 2-methoxy 3-isobuty pyrazine, 2-methoxy 3-sec-butyl pyrazine, MIB and 2,4,6-TCA. Calibration curves were established within the range of 100 ppt (50, 100, 150 and 200 ppt), 1 ppb (600, 800, 1000 and 1200 ppt) and 20 ppb (15, 20, 25 and 30 ppb).

Results and discussion

Sensory analysis: In Figure 1, the results of the sensory evaluation are shown (star diagram). Comparing the profile of the reference with the mouldy sample, significant differences were observed on the descriptors coffee aroma, coffee flavour, chemical/medicinal, earthy/musty/mouldy flavour and acidity. The reference sample was described as higher on coffee aroma, coffee flavour and acidity. The mouldy sample has lost coffee aroma and flavour intensity and was characterised as earthy/musty/mouldy and slightly chemical/medicinal. The defect seemed to have no influence on body, winey/aromatic, green/grassy, cereal/woody/papery descriptors which were already low in the reference sample.

Figure 1: Sensory profile comparison of reference and mouldy Mexican coffees



ARO: coffee aroma
FLA: coffee flavour
ACI: acidity
BOD: body
WIN: winey/aromatic
GRE: green/grassy
CER: cereal/woody/papery
BAG: baggy
CHE: chemical/medicinal
FER: fermented/rank
EAR: earthy/musty/mouldy

— - — **reference**
——— **mouldy**

GC-FID/sniffing, GC-FID/FPD and GC-MS analysis: GC-profiles obtained from the reference and the mouldy sample were similar (with both polar and non-polar columns). Minor instrumental differences were observed but can not be responsible for the defect. It was not possible to locate the origin of the defect on the basis of GC –FID/FPD/MS profiles.

Sniffing analyses were performed both on polar and non-polar columns. Approximately 40 undertones were detected in each extract. The majority of them are commonly found in coffee. Yet, the sniffing traces also allowed identifying several important differences between the extracts that can be related to the off-note (Figure 1). Six different earthy, green, chemical and mouldy chromatographic zones were located on the two different columns. The agreement in the location and in the descriptor terminology was established by four trained panellists (Table 1).

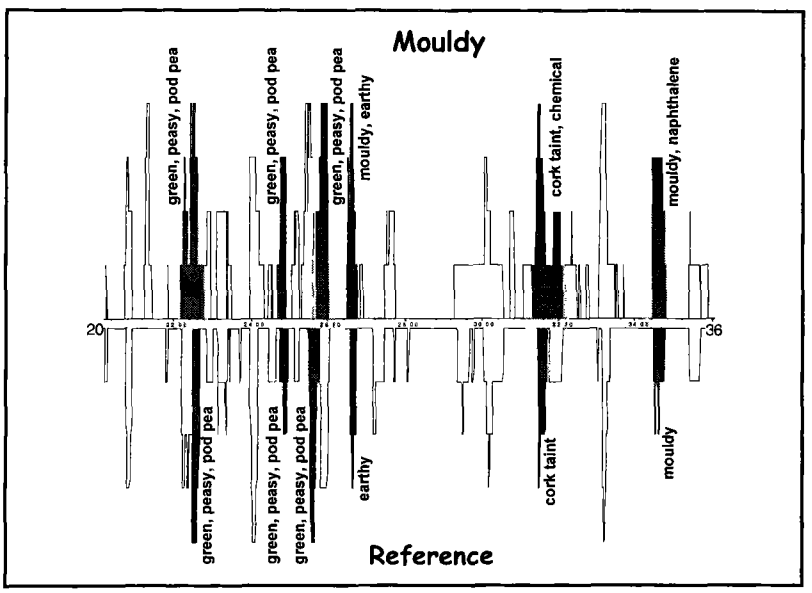
Table 1: GC-sniffing of raw extracts (reference and defective samples).

Attributes	I(x) DB-WAX	I(x) PONA-1	Tentative Assignment
Earthy	1413	1080	2-Methoxy 3-isopropyl pyrazine, (MIP)
Green - earthy - pod broad bean – peasy	1503	1151	2-Methoxy 3-sec-butyl pyrazine, (MsBP)
Green - earthy - pod broad bean – peasy	1529	1170	2-Methoxy 3-isobutyl pyrazine, (MiBP)
Earthy - dry earth	1599	1188	2-Methylisoborneol, (MIB)
Cork taint – chemical	1817	1331	2,4,6-Trichloroanisole, (2,4,6-TCA)
Mouldy	1823	1423	Geosmin

These six important notes are present in both reference and defect samples (Figure 2). Yet, large quantitative differences appeared in term of perception at the end of the chromatographic column. In the reference, the duration of the olfactive sensation for these notes is to 3 to 6 seconds. For the mouldy

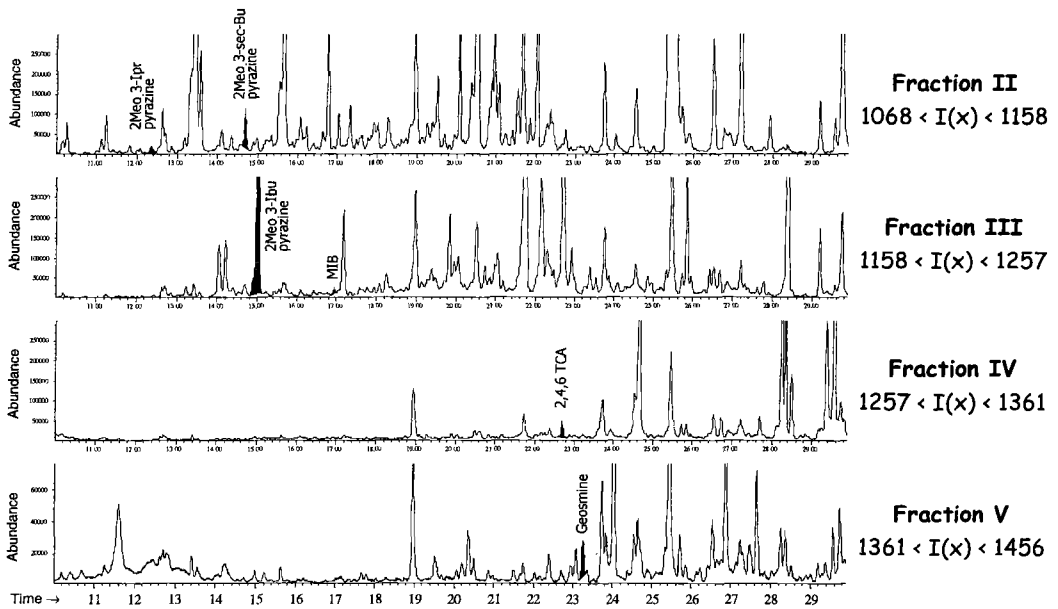
sample the duration of the signals was extended to 25 seconds. Based on both sensory and crossed-chromatographic data, it was possible to make tentative assignments of the culprits, as listed in Table 1. Yet, at this stage, the only compound detected by GC-MS was MiBP. Preparative GC was necessary to obtain GC-MS data on the remaining five compounds and make sound assignments.

Figure 2: *GC-sniffing comparison of mouldy and reference coffee*



Preparative gas-chromatography: Preparative gas-chromatography was performed in order to collect and concentrate aroma fractions, and subsequently to identify and quantify the compounds of interest. After 40 trapping cycles, four fractions were investigated.

Figure 3: Chromatographic profiles of fractions II to V



Fraction II, collected from retention indice 1068 < I(x) < 1158, presents two green, peasy, bell-pepper notes which correspond to MIP and MsBP. The latter compound has never been identified in coffee, but is known to be present in different vegetables such as carrots, lettuce, peas, sweet and bell pepper, pumpkin, beetroot but also in Swiss type cheeses, white wine and ginger.[18] Its odour threshold is very low (approx. 1 ppt in water). The nature of the optical isomer was not determined in this study.

Fraction III, collected from retention indice 1158 < I(x) < 1257, contains MiBP and MIB. MiBP is encountered in approximately twenty different food products.[18] It was quantified as 17 ppb in the mouldy sample, approximately 1000 times its odour threshold. It makes a major contribution to the greenish overall odour of the extract and is probably involved in the masking of the earthy/mouldy notes. MIB was found at 100 ppt in the mouldy sample and approximately 20 ppt in the reference, in which a weak dry earthy, dusty sensory impression was detected by sniffing.

Fraction IV, collected from retention indice 1257 < I(x) < 1361, presented the well known 2,4,6-TCA quantified at a level of 300 ppt in the mouldy sample (six times more than in the refererence sample). Its sensory properties brought a cork-taint odour and taste. The threshold is comprised between 1 to 8 ppt, depending on the media. This is one of the most intense odorants known.

Fraction V, collected from retention indice 1361 < I(x) < 1456, allowed the identification of geosmin at a concentration of 1 ppb (eight times more than in the reference sample).

Quantification: The compounds present in fractions II, III, IV and V were quantified (in SIM and scan mode respectively) with assuming 100 % recovery. Quantification with any of the five standards used gave similar results.

Table 2: Quantification and odour-threshold of chemicals responsible of the mouldy/earthy off-flavour.

Compounds	Conc (ppt) *Reference*	Conc (ppt) *Mouldy*	Threshold (ppt) Determined in water	
2-Methyl isoborneol	***< 20***	***100***	**30,**	ref.{20}
2,4,6-Trichloroanisole	***< 50***	***300***	**1-8,**	ref.{1}
Geosmin	***130***	***1000***	**10-20,**	ref.{5}
2-Methoxy-3-isopropyl pyrazine	200	400	2-10,	ref.{20}
2-Methoxy-3-sec-butyl pyrazine	300	400	1,	ref.{21}
2-Methoxy-3-isobutyl pyrazine	8000	17000	2-20,	ref.{5}

Geosmin and MIB concentrations clearly indicate a contamination leading to the earthy-mouldy defect. Geosmin was strongly correlated with a mouldy note at a concentration of 1 ppb, whereas MIB was described as earthy, dusty at 0.1 ppb. In a former study both geosmin (at a concentration of 17 ppb) and MIB (at a concentration of 1 ppb) were found to give this musty-earthy note in wheat grains.[8] 2,4,6-TCA also contributed to the overall defect in Mexican sample. Geosmin, MIB and 2,4,6-TCA were also present in the reference sample of the same origin. Their concentrations are respectively 8, 5 and 6 times lower than in the defected sample.

The dry post-harvest treatment is believed to be at the origin of the formation of geosmin, MIB and 2,4,6-TCA. These compounds are known to be produced by micro-organisms and can be absorbed during the dying of the beans on the ground. This is also in line with the fact that we were not able to

detect these defaults in subsequent investigations on Kenyan or Colombian coffee obtained by wet processing. Further investigations should confirm these results.

The level of green pyrazines remains of the same order of magnitude in the reference and the defect samples. Nevertheless, the olfactive contribution of MiBP present at a concentration of 17 ppb must be important since its odour threshold is 2-20 ppt (in water).

Conclusions

Chromatographic profiles (GC –FID/MS/FPD) alone do not allow to differenciate mouldy and reference samples. Only with GC-Sniffing were we able to indentify differences relevant to our problem. After locating mouldy zones along the retention indices axis, preparative chromatography was used to obtain enriched fractions of 4 four selected I(x)-zones, containing the chemicals responsible for the off-notes. This allowed us to identify the chemicals by GC-MS and quantify using external standards.

The alkyl methoxy pyrazines are among the main odoriferous constituents of green coffee. Three of them were detected both in the reference and mouldy samples. They evoke strong bell-pepper, green, earthy notes. The concentration of the MiBP has been shown to be very high (10-20 ppb). Additionally, in the present work the MsBP was detected for the first time in coffee.

Three contaminants, MIB, 2,4,6-TCA and geosmin were identified both in the reference and mouldy samples. Their concentrations in mouldy samples are between 0.1-1 ppb (5 to 10 times more than in the reference sample). They contributed to the mouldy–earthy default.

The dry post-harvest treatment is believed to be at the origin of these substances in green coffee beans. Wet post-harvest treatment would most probably avoid the appearance of the mouldy/earthy off-flavour. Further investigations on Arabica and/or Robusta coffee types from different origins and post-harvest treatments have already confirmed these observations. Nevertheless, more thorough investigations are needed to confirm these results.

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Summary

The quality of coffee can be severely affected by off-flavours. A Mexican coffee (Arabica) with a pronounced earthy/mouldy off-flavour is investigated, and compared to a reference from the same origin. The culprits are identified by gas chromatography-olfactometry, isolated by preparative GC, and characterised by GC-MS. Six chemicals participate to this off-flavour. Geosmin, MIB, 2,4,6-TCA were found to be the main culprits, while methoxy pyrazines (2-methoxy 3-isopropyl/ 3-sec-butyl/ 3-isobutyl pyrazine) contributed to a lesser extend to the earthy/green undertone. The occurence of the this off-flavour is believed to be linked to the dry post-harvest treatment.

Résumé

La qualité du café peut-être affectée par la présence de défauts dont l'origine reste souvent mal déterminée. Le but principal de ce travail est de caractériser chimiquement les molécules responsables du défaut moisi-terreux du café vert Arabica d'origine Mexicaine. Les substances responsables ont été localisées par GC-sniffing, isolées et concentrées par chromatographie préparative, enfin identifiées et quantifiées par GC-MS. Six substances participent à ce defaut: géosmine, 2-methylisobornéol et 2,4,6-trichloroanisole contribuent majoritairement à ce défaut alors que trois methoxy pyrazines apportent une note verte–terreuse secondaire. La présence de ce défaut est probablement lié aux conditions de traitement du café vert par voie sèche.

The Detection and Characterization of Free Radicals generated during the Decomposition of Solutions of the Coffee Flavour Compound Furfuryl Mercaptan

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INTRODUCTION

Furfuryl mercaptan (Fur-SH) has been reported to be an important flavour compound of coffee *(1)*, but its concentrations in coffee brews are appreciably lower than that in roast and ground coffees *(2, 3)*. This is thought to be due, at least in part, to the sensitivity of Fur-SH to oxidative processes. Recently we have investigated its stability in solution under Fenton reaction conditions *(4)*, where H_2O_2, which is formed during preparation of coffee brews *(5)*, reacts with an oxidizable metal ion to produce hydroxyl radicals.

$$H_2O_2 + Fe(II) \rightarrow \cdot OH + OH^- + Fe(III)$$

These hydroxyl radicals are highly reactive and readily abstract hydrogen atoms from organic molecules to form water and organic radicals that participate in further chemical reactions.

In our previous work *(4)* we showed that a high proportion of the Fur-SH was lost from solutions containing H_2O_2 and the Fe(II)-generating system (Fe(III) + ascorbic acid) after one hour at 37°C. Volatile compounds, which accounted for about one third of the decomposition products, were mainly of the form Fur-S_n-Fur (n = 0-3) and suggested the generation of the free radical Fur-S· as an initial product and the involvement of radicals of the type Fur-S_n· as intermediates in the reaction. In an attempt to identify free radical intermediates, preliminary measurements were made using electron paramagnetic resonance (EPR) spectroscopy, a technique that is able to detect specifically free radical species. By using chemical spin traps, which are compounds that are able to react with unstable free radicals to generate stable radicals, it was shown that at the end of the incubation period major amounts of adducts of ·C-centred radicals were present along with some adducts of the ·OH radical. There was, however, no evidence for the presence of ·S-centred radicals in the solutions investigated.

The present paper reports the results of further EPR measurements that were undertaken with the objective of resolving some of the puzzling features of our initial experiments and improving our understanding of the oxidative chemistry of these coffee model solutions.

EXPERIMENTAL

Materials: Furfuryl mercaptan or 2-furfurylthiol (Fur-SH), α-(4-pyridyl-1-oxide)-*N*-*t*-butylnitrone (POBN), ethylenediaminetetraacetic acid disodium dihydrate (EDTA $Na_2 2H_2O$), hydrogen peroxide (H_2O_2), ferric chloride hexahydrate ($FeCl_3 6H_2O$), ferrous sulfate heptahydrate ($FeSO_4 7H_2O$), potassium phosphate tribasic (K_3PO_4) and sodium dodecyl sulfate (SDS) were purchased from Sigma Chemical Company Ltd. (Dorset, England, UK). Ascorbic acid was purchased from FSA Laboratory Supplies (Loughborough, UK) and 5-(diethoxyphosphoryl),5-methyl-1-pyrroline-*N*-oxide (DEPMPO) from Calbiochem-Novabiochem (UK) Ltd. (Beeston, Nottinghamshire, UK).

Sample preparation: The preparation of the model coffee solutions for the EPR measurements at room temperature (20±2°C) was similar to that described for model solution 1 in *(4)*. It was prepared fresh immediately before use and contained 1mM ascorbic acid, 0.25mM EDTA, 0.1mM $FeCl_3$, 3.3mM Fur-SH (dissolved in 3.3mg/mL aqueous SDS), 5.3mM DEPMPO or 100mM POBN in 16mM phosphate buffer solution and 0.015% H_2O_2. The H_2O_2 was added last in order that the Fenton reaction was initiated only after all of the other reagents were present. Samples were taken at intervals during the one-hour incubation period at 37°C and transferred to a quartz flat cell for the EPR measurements, which were made at room temperature. Ethanol, which in the former study (*4*), was used to terminate the reaction, was added to the solution at the end of the one-hour incubation. Spectra were also obtained from solutions that were incubated at room temperature in flat cells in the EPR cavity. Further solutions were prepared without Fur-SH with and without addition of ethanol at the end of the incubation period. For low temperature (77K) EPR measurements, a solution was prepared with just $FeSO_4 7H_2O$ (0.4mM), H_2O_2 (38mM) and Fur-SH (87mM). Samples were taken at intervals after mixing the reagents at room temperature, transferred to a 5mm o.d. quartz tube and frozen immediately in liquid nitrogen. For EPR measurements the tubes were transferred to a quartz "Finger Dewar" filled with liquid nitrogen, which was inserted into the microwave cavity.

EPR Spectroscopy: EPR measurements were made at X-band frequencies (~9.5GHz) using a Bruker ESP300E computer-controlled spectrometer incorporating an ER4103TM cylindrical cavity. Microwave generation was by means of a klystron (ER041MR) and the frequency was measured with a built-in frequency counter. All spectra were collected in 1024 data points using a modulation frequency of 100kHz. A microwave power of 10mW and modulation amplitude of 0.1mT were used for fluid solution measurements at room temperature, whereas the respective values for low temperature (77K) spectra were 0.5mW and 0.5mT. As in conventional EPR spectroscopy, spectra were recorded as 1st derivatives of the microwave absorption and displayed as functions of absorption versus magnetic field at a constant microwave frequency.

RESULTS AND DISCUSSION

EPR spectra at room temperature are shown in Fig. 1 of samples of the model coffee solution taken at 15 minute intervals during the one-hour incubation at 37°C in the presence of DEPMPO. These spectra are typical of free radical adducts of DEPMPO (Fig. 2) and consist of a doublet from interaction of the unpaired electron with ^{31}P (I=½), which is split into a doublet from interaction with the ^{1}H (I=½) on the carbon atom adjacent to the nitroxide group and a triplet from interaction with the ^{14}N (I=1) nucleus.

Throughout the incubation period the EPR signal corresponded to that of the ·OH radical, which is characterised by the hyperfine splittings a_N = 1.40 mT, a_H = 1.36 mT, a_P = 4.71 mT *(6, 7)*. This grew in intensity with time of incubation, showing that the Fenton reaction continued to produce ·OH radicals throughout the incubation period. After the addition of ethanol to the solution at the end of the incubation period, the EPR spectrum was transformed immediately to that of a ·C-centred radical adduct, a_N = 1.47 mT, a_H = 2.13 mT, a_P = 4.91 mT *(6, 7)*. The characteristic signal of the ascorbate radical (a_H = 0.18 mT, *ref. 8*) was seen in the samples taken from the early stages of incubation, but had disappeared before the end of the one-hour incubation period.

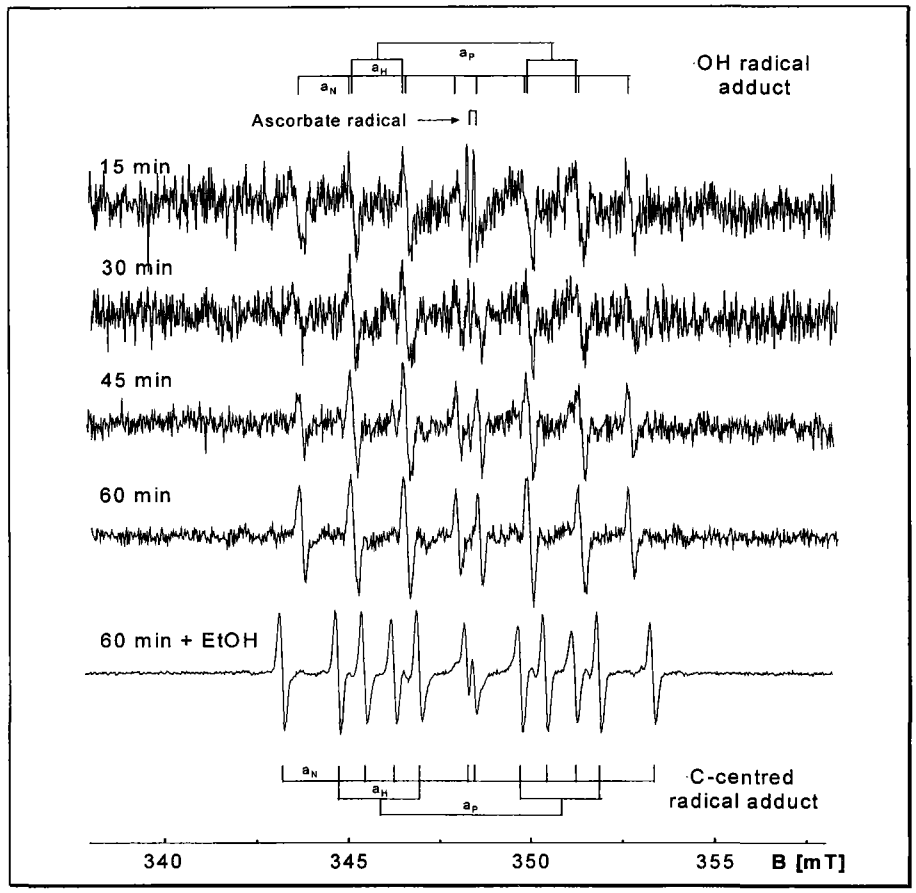

Fig. 1. Room temperature EPR spectra of coffee model solution plus DEPMPO during incubation at 37°C and after addition of ethanol.

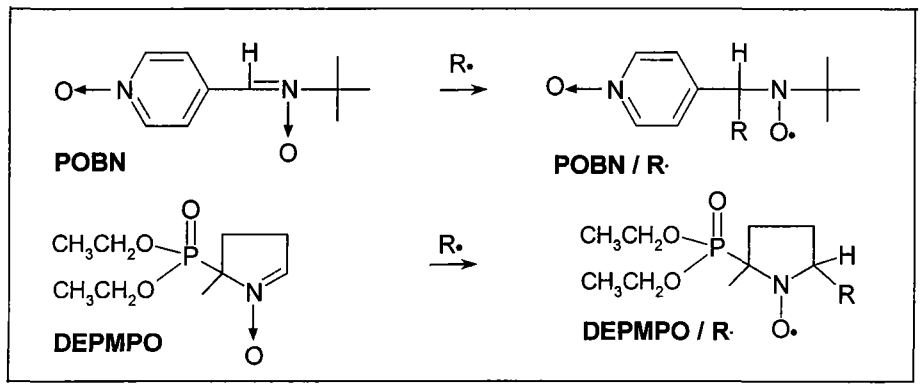

Fig. 2. Chemical formulae of POBN and DEPMPO spin traps and their radical adducts.

The evolution of the spectra was also followed at room temperature over a 20-hour period. The results (not shown) were qualitatively similar to those described above, but the development of the signal was slower. As in the previous measurements, the spectra were all dominated by the ·OH radical adduct. The ascorbate radical, which was seen initially, decreased in intensity with time and was not detectable after about one hour. There were also small amounts of the component from ·C-centred radical adducts in spectra from about six hours, but it was never more than a minor fraction of the total signal.

When POBN was used as spin trap, the spectra (Fig. 3) were also dominated by the signal from the ·OH radical adduct, a_N = 1.50 mT, a_H = 0.160 mT, a_H = 0.033 mT *(9)*. This is characterised by a small doublet from interaction of the unpaired electron with the ^1H of the OH group in addition to the triplet from the ^{14}N and doublet from the ^1H nucleus on the carbon atom adjacent to the nitroxide. As with the DEPMPO solutions, the ascorbate radical signal could be seen in the early measurements, but disappeared completely within one hour. In these solutions, however, some ·C-centred radical adduct was detected during the 1-hour incubation period at 37°C. As with DEPMPO, addition of ethanol to the solution at the end of the incubation resulted in the immediate transformation of the spectrum of the ·OH adduct to that of a ·C-centred radical adduct, with a_N = 1.56 mT, a_H = 0.258 mT *(9)*. In the incubation of a similar solution at room temperature, the ·OH-centred radical adduct was dominant in the early spectra, but the signal from the ·C-centred radical adduct grew progressively with time and dominated the spectra after about 15 hours.

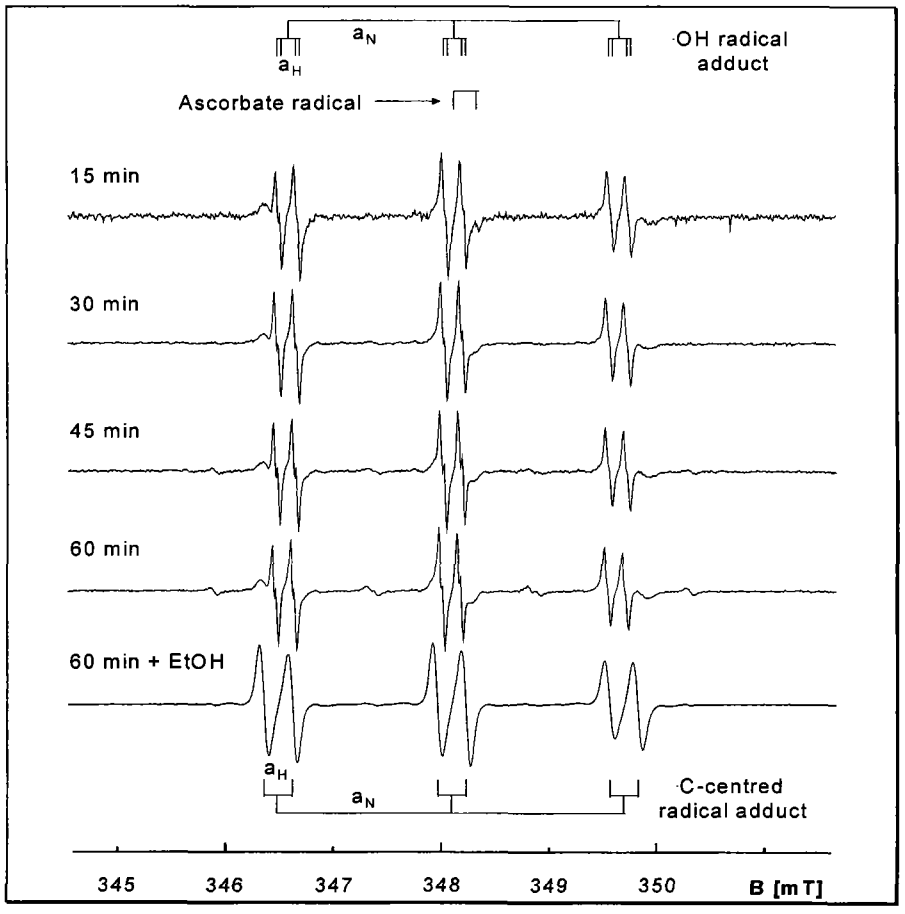

Fig. 3. Room temperature EPR spectra of coffee model solution plus POBN during incubation at 37°C and after addition of ethanol.

The above result suggests that, although some of the ·C-centred radical adduct is formed during the incubation period, much of it is generated by reaction between ethanol and the ·OH-centred radical adduct.

In order to investigate this reaction further, EPR measurements were made on simplified systems. Firstly, the model solution was prepared without Fur-SH and spectra were compared from solutions with and without addition of ethanol at the end of the incubation period (Fig. 4). As expected, the EPR spectrum from the solution without ethanol showed only the ·OH radical adduct from DEPMPO (and an unknown adduct that has also been seen in a simple Fenton reaction (Fe(II) + H_2O_2) + DEPMPO - *EP and BAG unpublished results*), but addition of ethanol resulted in little change to the spectrum and only a trace of the ·C-centred radical adduct was seen.

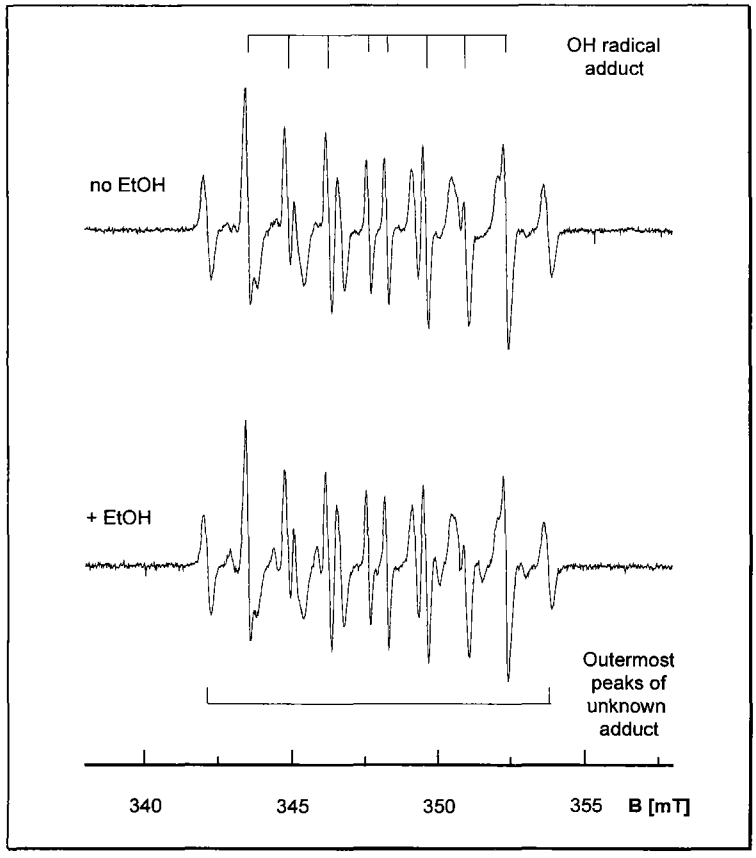

Fig. 4. Room temperature EPR spectra of DEPMPO adducts from model coffee solution minus Fur-SH.

This result demonstrates that the ·OH-centred radical adduct of DEPMPO is stable in the presence of ethanol and that Fur-SH is a precursor of the ·C-centred radical adduct that is seen in the EPR spectra; it is however not the only source in the model coffee solution.

It is also possible that radicals are generated through reaction of Fur-SH with Fe(III), which could produce Fe(II) and Fur-SH-derived radicals. Also the ascorbate radical, which is formed when Fe(III) is reduced to Fe(II), may participate in further reactions, possibly with Fur-SH. We have, therefore, studied radical generation in a 2nd simplified system in order to investigate the extent to which such reactions might participate in the solutions studied above. In a solution, which contained just H_2O_2, Fe(II), Fur-SH and DEPMPO, (thereby eliminating the Fe(III) reduction stage prior to the initiation of the Fenton reaction), only the EPR spectrum of the ·OH adduct was seen (Fig. 5a). When ascorbic acid was included in the reaction mixture, the spectrum remained predominantly

that of the ·OH adduct but some (*ca.* 20%) of the ·C-centred adduct was also present (Fig. 5b). Addition of ethanol to the initial solution immediately after starting the reaction resulted in the generation of a mixture of ·OH and ·C-centred radical adducts (Fig. 5c). Taken with the results discussed above, these measurements show that the behaviour of the original model solution is complex and involves a reaction of Fur-SH or one or more of its decomposition products with ethanol to produce a product that reacts with the ·OH adduct of DEPMPO to form the ·C-centred radical adduct. The ascorbate would also appear to be a precursor of some of the ·C-centred radical adduct.

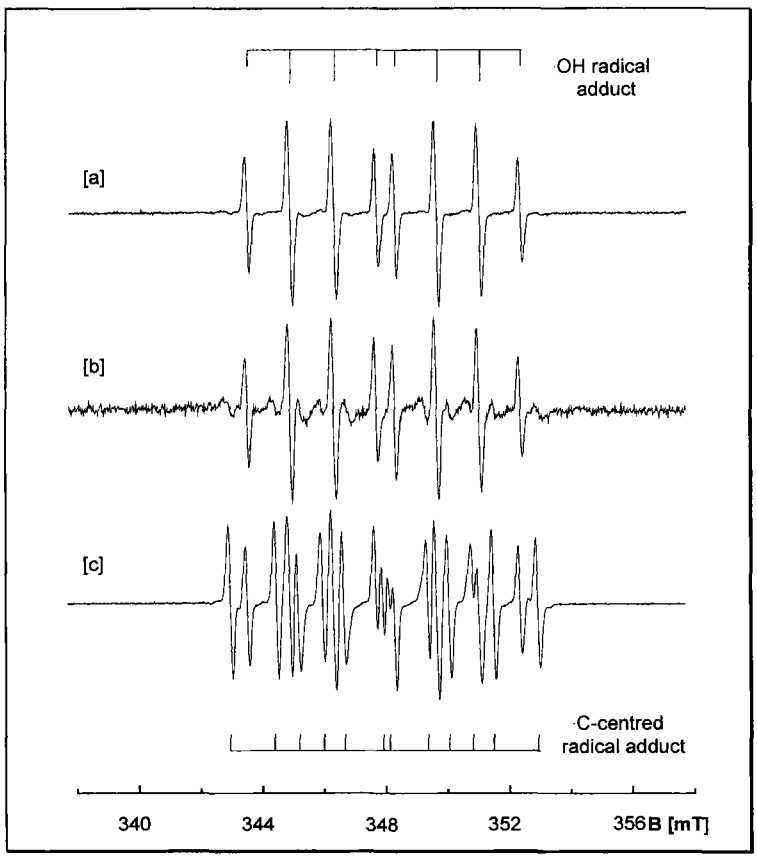

Fig.5. Room temperature EPR spectra of DEPMPO adducts from [a] the simple Fenton system + Fur-SH, [b] as [a] but with ascorbic acid and [c] as [a] but after addition of ethanol.

There was no direct evidence for any ·S-centred radical species in these spin trapping experiments, despite the strong evidence that such radicals are intermediates in the formation of some of the reaction products identified by gas chromatography *(4)* and the EPR evidence for the presence of some undetected Fur-SH-derived species that reacts with ethanol to produce a ·C-centred radical. Another experiment was conducted, this time using higher concentrations of reagents and a freeze quenching technique to stabilise any radical(s) that might be generated. EPR spectra recorded at 77K of solutions that were sampled at intervals after initiating the Fenton reaction and then frozen immediately in liquid nitrogen. An anisotropic radical signal was observed (Fig. 6) with *g*-values of 2.002, 2.011 and 2.023. These are similar to those reported for species of the type of $RS^{\cdot}$-SR^{+} *(10)*. The radical had moderate stability, with a half-life of several minutes and is probably an intermediate in the decomposition of Fur-SH. It does not, however, appear to be reactive with the spin traps used in the present investigation. Also, because of its limited stability, it was probably not the precursor of the ·C-centred radical adduct that was formed when ethanol was added to the model solution.

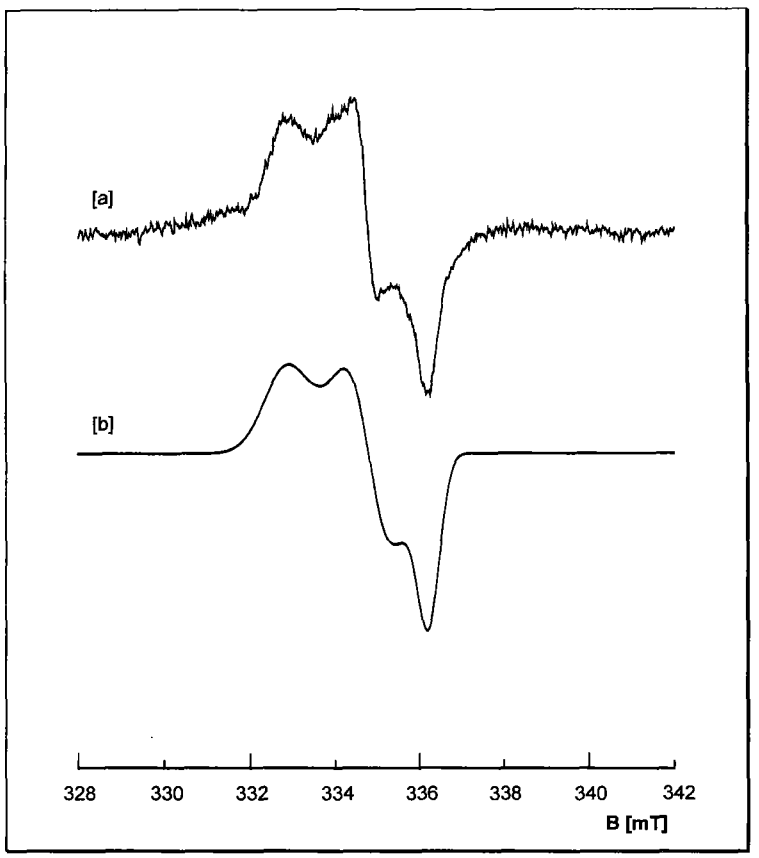

*Fig. 6. [a] EPR spectra at 77K from a simple Fenton system plus Fur-SH and [b] a simulation with **g**-values of 2.002, 2.011 and 2.023 Gaussian lineshape.*

CONCLUSIONS

Free radicals generated during the decomposition of model coffee solutions containing the flavour compound furfuryl mercaptan have been detected by EPR spectroscopy after reaction with chemical spin traps. The spectral parameters are characteristic of adducts of ·OH and ·C-centred radicals. The ·C-centred radical adduct was formed slowly in the solutions, but was generated rapidly in the presence of ethanol, which appears to react with the Fur-SH (or its one or more decomposition products) to form the product that then reacts with the ·OH adduct of the spin trap to give the ·C-centred radical adduct.

In solutions containing spin traps, no spectra from adducts of ·S-centred radicals were seen. Such radicals could be detected in solutions at 77K, however, when high concentrations of Fur-SH were reacted with ·OH radicals from a simple Fenton reaction.

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SUMMARY

The generation of free radicals during the decomposition of a coffee model solution containing the flavour compound furfuryl mercaptan (Fur-SH), has been investigated by EPR spectroscopy. By using the chemical spin traps DEPMPO and POBN, adducts of ·OH and ·C-centred radicals could be detected. The ·OH radical was generated by Fenton chemistry, but the origin of the ·C-centred radical was more complex. No ·S-centred radicals were seen in the spin-trapping experiments, despite a strong indication in previous work that they are intermediates in the formation of volatile compounds following the decomposition of Fur-SH. However, short-lived radicals of the type $RSSR^{\cdot+}$ were detected in model solutions at 77K.

RÉSUMÉ

La formation de radicaux libre a été étudiée par spectroscopie RPE, sur une solution modèle contenant le furfuryl mercaptan (Fur-SH), composé clé de l'ârome de café. En utilisant les "spin trap" DEPMPO et POBN, des produits d'adjonctions du radical ·OH et de radicaux centrés sur le carbone ont été observés. Le radical ·OH a été généré par la réaction Fenton. Par contre l'origine des radicaux centrés sur le carbone est plus complexe. Aucun radical centré sur le soufre n'a été observé. Ceci, malgré une forte présomption de leur présence lors d'une étude précédente. Lors de cette étude, la formation de composés volatiles générés par la décomposition de Fur-SH a été observé, indicant la présence de radicaux centrés sur le soufre comme intermédiaires. Cependant des radicaux à très courtes durée de vie de type $RSSR^{\cdot+}$ ont été observés dans des solutions modèles à 77K.

Retardation of Coffee Beverage Volatiles by different Milk Products

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Introduction

Investigations of the coffee flavour have been confined to the analysis of the aroma substances (1, 2, 3). These investigations showed that about 30 volatile compounds were substantially responsible for the coffee flavour. KIM et al. (4) were the first to investigate the effects of milk additives on the coffee flavour; they used the conventional static headspace technique and only instant coffees. The major objective of this work was to investigate the influence of different milk additives and one coffee whitener on the release of flavour impact compounds from different coffee beverages.
The purposes of adding these products to the coffee beverage are: to develop a desirable colour change, to impart a body to the coffee beverage, to reduce bitter and sour tastes and to reduce the astringency of the coffee. Ingredients of these additives such as lipids, proteins and carbohydrates affect the retention of volatiles (5). Consequently, these aroma interactions affect quality and quantity of the coffee headspace aroma. Direct injection of a headspace sample onto a GC capillary column gives the most accurate composition of flavours. However, working with static headspace (gastight syringe) was not sufficient, because only low amounts of aroma compounds were collected. Therefore a new device had to be developed which could collect a larger volume of headspace above the coffee beverage. In the present study external static headspace was carried out by GC-FID / olfactometry and GC-MS / olfactometry.

Experimental

For the beverages the two economically important coffee species were used: one Arabica coffee (Columbia) and one Robusta coffee (Indonesia), both with an average roasting degree. All samples were supplied by Kraft Jacobs Suchard (Bremen, FRG).
Eight products, purchased from a local market or from Kraft Jacobs Suchard (Munich, FRG), were selected as typical coffee additives.
The different types of dairy and vegetable products were also chosen because of their different lipid, protein and carbohydrate contents. These components have the greatest influence on the retardation of volatiles. The ingredients of the additives are listed in Table I (6).

Table I. *Ingredients of the additives (in %)*

Additive	Lipid	Carbohydrate	Protein
UHT-Milk	0.3	4.8	3.5
UHT-Milk	3.5	4.8	3.3
Condensed milk	0.1	10.6	7.8
Condensed milk	10.0	12.5	8.8
Coffee creamer	10.0	3.1	4.0
Whipping cream	30.0	3.2	2.5
Skimmilk powder	1.0	65.5	24.0
Coffee whitener (vegetable)	34.0	55.0	6.0

Sample Preparations

The beans were stored at -17°C and ground directly prior to use in a coffee grinder of the style that is normally used in coffee shops. The brew was prepared in a household coffeemaker with 12g coffee powder and 225g tap water.

For the external static headspace device 220g of coffee beverage and 45g of additive were used. These liquids were filled in the lower of the two glass vessels (Figure 1).

After an equilibrium time of 15 minutes at room temperature the lower vessel was replaced by an empty one. Nitrogen was flushed with 100mL/min for 30 minutes and the volatiles were collected on a Tenax TA tube. Coefficients of variance of 8 GC-MS replications ranged between 2 and 20% for most of the peak areas of volatile compounds.

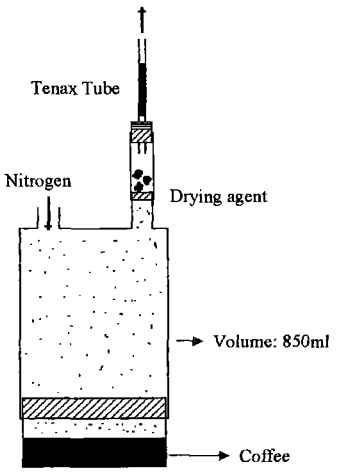

Figure 1. *External Static Headspace device*

GC-FID, GC-MS and GC-Olfactometry

The volatile compounds were analysed on an HP 5890 GC equipped with an FI-Detector and Sniffing-Port or MS-Detector and Sniffing-Port. The volatiles were desorbed by a thermal desorption device and injected onto a DB-5 capillary column (30m x 0.53mm x 1.5µm). In addition to the DB-5, an OV-1701 (60m x 0.32mm x 1.0µm or 60 x 0.25mm x 0.5µm) and an FFAP (60m x 0.25mm x 0.5µm) fused silica capillary column were used.

The identification of the compounds was achieved by comparison of retention data on DB-5, OV-1701 and FFAP and mass spectral data, as well as comparison of sensory properties with those of authentic reference substances. Mass spectra were generated at 70eV in the electron impact mode.

GC/O- analysis

The assessors recorded the aroma substances during the sniff runs (Figure 2): When the compound exceeds its threshold, the sniffer records (chartspeed: 10cm/min) the event; when the concentration drops below the threshold again, the sniffer also records the event. Flavour descriptors were

generated during preliminary GC / sniffing experiments and clustered to descriptors after group sessions of the panel (five trained assessors).

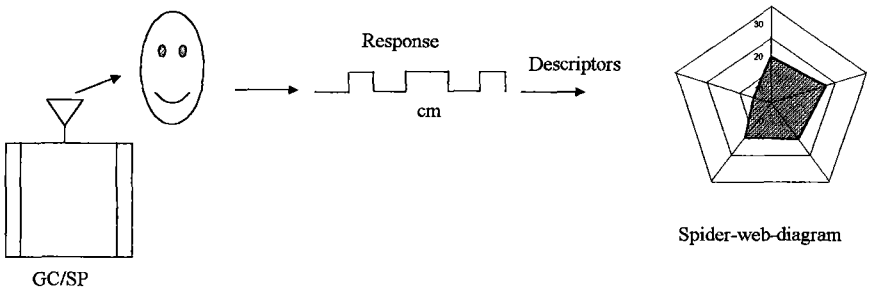

Figure 2. Creation of odour profiles with a GC/O-technique

Results and discussion

With this headspace method over 50 potent odorants (Table 2), resulting in a list of ten descriptors, were recognized at the sniffing port. The identification of these volatiles verified most of the contributors of the coffee aroma described in literature (1, 2, 3).

Table 2. Examples of identified potent odorants

Compound	*Retention index on*		*Aroma quality*
	DB-5	*OV-1701*	
methanthiol	<600	<600	putrid
dimethyl sulfide	<600	<600	putrid
2-methylpropanal	<600	616	cocoa
2,3-butanedione	610	665	buttery
3-methylbutanal	653	720	malty
2-methylbutanal	665	726	fruity / malty
2,3-pentanedione	696	755	buttery
hexanal	797	876	leaf-like
2- & 3-methylbutanoic acid	869	980	sweaty
3-methyl-2-buten-1-thiol	881	728	foxy
methional	903	1027	potato-like
2-furfurylthiol	906	1010	roasty / coffee-like
1-octen-3-one	972	1057	mushroom
2, 3, 5-trimethylpyrazine	1002	1072	roasty
3-mercapto-3-methylbutylformate	1027	1218	foxy
phenylacetaldehyde	1055	1171	sweet / honey-like
2-ethyl-3,5-dimethylpyrazine	1062	1151	earthy / roasty
guaiacol	1096	1219	phenolic / burnt
2-isopropyl-3-methoxypyrazine	1144	1144	earthy / roasty
(E)-2-nonenal	1154	1266	cucumber-like
2-isobutyl-3-methoxypyrazine	1186	1237	sweet pepper - burnt

In general the additives reduced the intensity of the volatiles (Figure 3, 4 and 5). This effect of retardation caused by components of the additives was typical for each additive. Figure 3 represents

the low-fat additives: Only two descriptors, honey and catty, showed a clear decrease for both additives.
As distinct from these products, products with a high fat content, as shown in Figure 4, had significant decrease for most of the descriptors. The roasty descriptor showed only a slight decrease. A likely explanation could be that Robusta coffee has the highest amount of volatiles with a roasty, earthy odour of all coffee species, even a retardation of these volatiles can not change the perception very much.
Because of the manufacturing process - extraction of the soluble constituents from roasted coffee - soluble coffee has fewer flavour compounds.Therefore the odour profile of soluble coffee (Figure 5) showed less intensity for all descriptors with and without additive (here: coffee creamer, fat content 10 %). Nevertheless the difference between the odour profiles was clearly visible. Coffee creamer reduced in general the intensity of all volatiles, especially for the roasty descriptor. Also the descriptors malty and cocoa, fruity and flowery, potato, and honey showed a significant decrease.
The changes in the flavour profile of coffee beverages with an additive may be caused by several effects (7). Interactions (e.g. solution, adsorption, specific binding) of the volatiles with the ingredients of these additives (lipids, proteins and carbohydrates) caused the retardation of coffee volatiles.

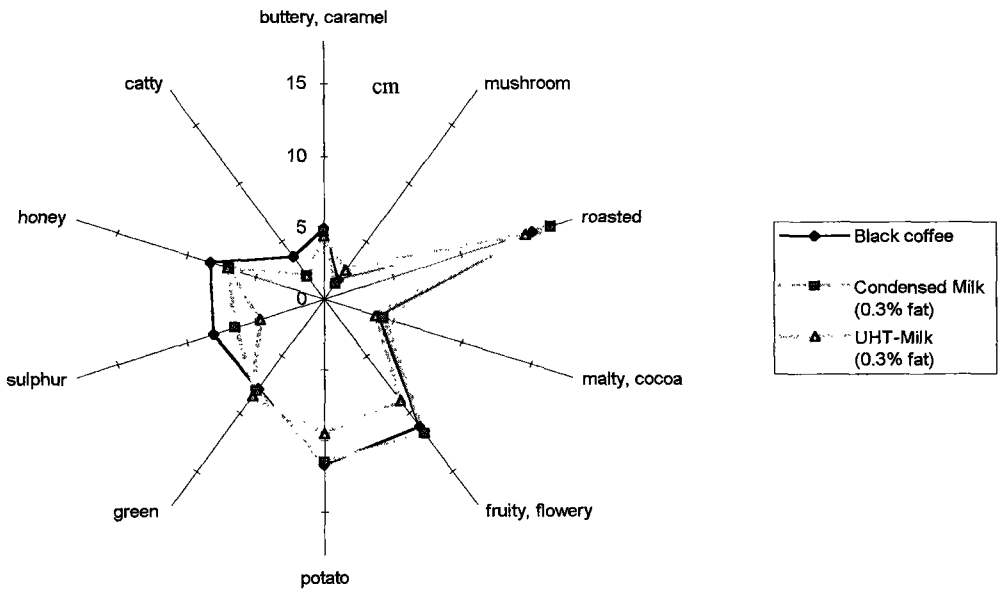

Figure 3. *Influence of condensed milk and UHT-milk on the intensity of GC-O descriptors used for* coffee volatiles (Arabica coffee)

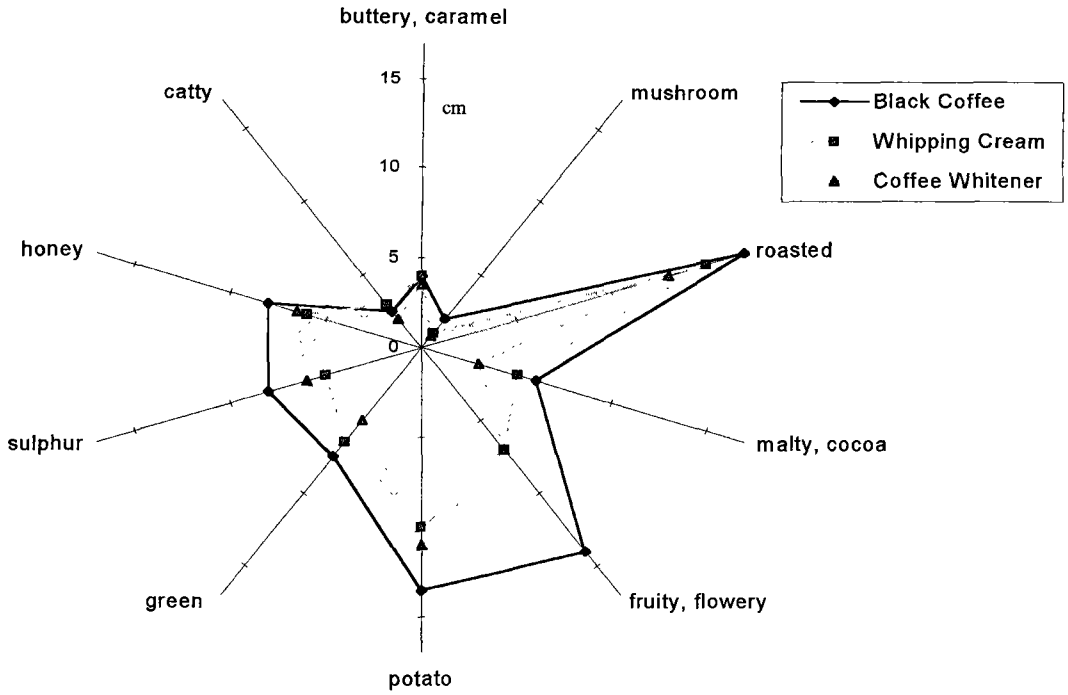

Figure 4. *Influence of whipping cream and coffee whitener on the intensity of GC-O descriptors used for coffee volatiles (Robusta coffee)*

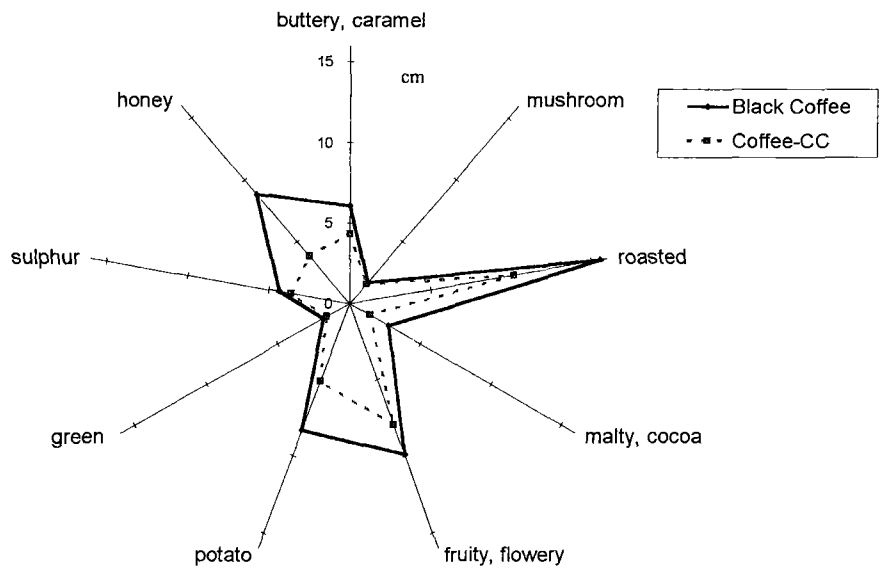

Figure 5. *Influence of coffee creamer (CC, 10 % fat) on the intensity of GC-O descriptors used for coffee volatiles (soluble coffee)*

These results were confirmed by GC/MS-analysis of the beverages. Figure 6 and 7 illustrate the effects of the additives to two potent odorants of the coffee and/or coffee brew: 2-ethyl-3,5-dimethylpyrazine (EDMP), has an earthy and roasty aroma quality, while phenylacetaldehyde has a honey-like, sweetish odour.
The concentration of both substances in the headspace of the coffee brew was in particular influenced by products with a high fat content, like whipping cream and coffee whitener. But also additives with a high protein content such as skimmilk powder may affect the retardation of volatiles. There was no obvious correlation between carbohydrate content and flavour release.

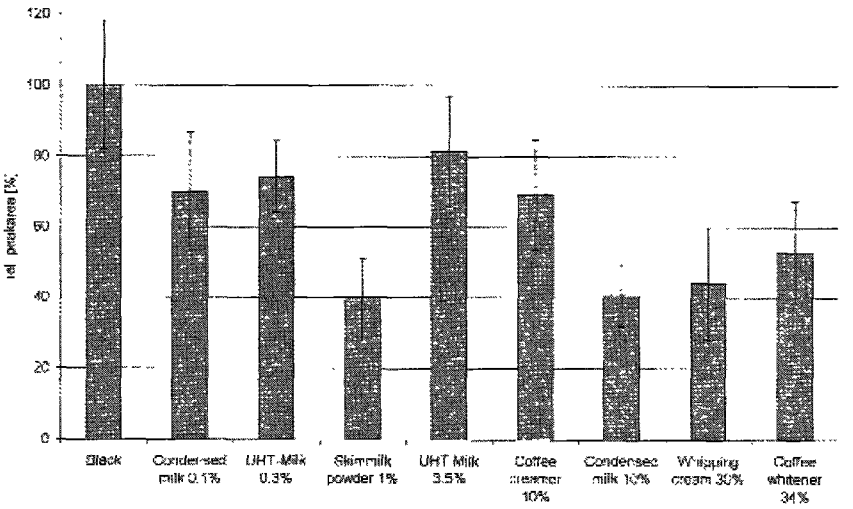

Figure 6. *EDMP: Comparison of the different additives (percentage specifies the fat content of the additive). Results are expressed relative to peak area (targetion GC/MS) of black Robusta coffee*

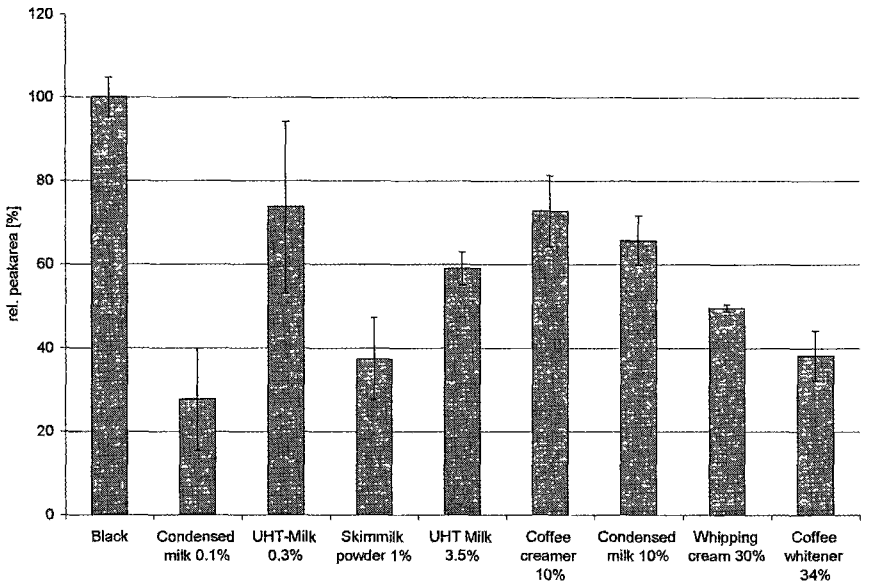

Figure 7. *Phenylacetaldehyde: Comparison of the different additives (percentage specifies the fat content of the additive). Results are expressed relative to peak area (targetion GC/MS) of black Arabia coffee*

Conclusion

With the static headspace method the most potent odorants of coffee beverages were determined. The addition of different milk or vegetable products reduced the amount of volatiles in the headspace of the coffee beverage.
This effect of retardation, caused by components of the additives, was typical for each additive. Further studies will concentrate on structural characteristics that lead to the retention of coffee aroma substances

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Summary

Milk and vegetable products as an additive for coffee beverages have an effect on the release of aroma substances in the brew through their lipid, protein and carbohydrate components. For the investigation of these effects an external static headspace technique was developed. With this technique the most potent odorants of the coffee beverage were determined. Analyses were performed by gas chromatography/olfactometry, FI- and MS-detection. To characterize the odour profiles of the different beverages GC/O analysis was used. All beverages with a milk or vegetable additive showed reduced odour profiles. This effect of retardation, caused by components of the milk and vegetable additives, was typical for each additive.

Zusammenfassung

Milchprodukte bzw. Kaffee-Weißer als Zusatz für Kaffee haben einen Einfluß auf die Freisetzung der flüchtigen Aromastoffe des Kaffeegetränkes. Für die Untersuchung dieser Effekte wurde die Methode der externen statischen Headspace entwickelt. Mit dieser Technik konnten die wichtigsten Aromastoffe bestimmt werden. Die Detektion bei diesen gaschromatographischen Untersuchungen erfolgte mittels FI-Detektor, MS-Detektor und Sniffing-Port (GC/Olfaktometrie). Mittels der olfaktometrischen Untersuchungen konnte gezeigt werden, daß sich das Aromaprofil nach Zusatz von Milchprodukten bzw. Kaffee-Weißer veränderte: Alle Getränke mit einem Zusatz zeigten ein in der Intensität reduziertes Aromaprofil. Diese Retardierung der Aromastoffe wurde durch die Inhaltsstoffe der Zusätze verursacht. Dadurch hatte jedes Getränk mit einem Zusatz ein eigenes, typisches Aromaprofil.

Diterpene Degradation Products in Roasted Coffees

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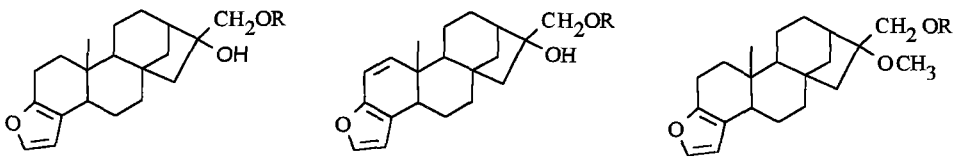
Introduction

Only after roasting of the green coffee beans the coffee beverage appreciated in the whole world could be prepared. The roasting process heating the coffee beans with hot gases changes a lot of coffee ingredients. With temperatures around 230 °C and above in the inner part of the bean, monosaccharides, disaccharides, amino acids, and other compounds are degraded. On the other side new substances are built-up for example the high molecular melanoidines and especially the flavor compounds.
The lipids, however, amounted to 7-17 % due to species and origin remain nearly unchanged in their content as Streuli et al. stated [1]. Therefore, the lipid content is often used as a reference quantity in the analysis of coffee.

However, the lipid fraction does not consist of a unique family. Aside from minor compounds, for example tocopherols, sterols, phosphatides, and carbon acid 5-hydroxytryptamides, the diterpenes and the predominant triglycerides form the main part. The diterpenes -- kahweol, cafestol, and 16-O-methylcafestol (16-OMC) -- are mostly esterified with up to 14 different fatty acids; only small parts, about one to three percent, are present in the free form [2-6] (Figure 1).
It is important to note, though, that the diterpene fraction of roasted coffee differs widely.

Dehydrocafestol and Dehydrokahweol

In 1989, Maier and Nackunstz [7] showed that the diterpenes cafestol and kahweol, determined in the unsaponifiable matter after saponification of coffee oil, were diminished as the roasting temperature increased. Obviously both diterpenes would degrade through the roasting process and due to their chemical structure it was supposed that cafestol and kahweol easily split off a molecule of water. In fact, some years later our research team was able to isolate and identify the two assumed degradation products and named them dehydrocafestol and dehydrokahweol [8].



Cafestol Kahweol 16-O-Methylcafestol

R = H : Free Diterpene
R = Fatty acids : Diterpene Esters

Figure 1. Structural formulae of the three diterpenes

The isolation of dehydrocafestol out of espresso to obtain standard substance turned out to be very toilsome. Therefore, it seemed to be much more effective to get it by directly heating the cafestol. Undoubtably, there are some more elegant and cost-effective methods for isolating and synthesising dehydrocafestol, but we were interested in finding out whether any other components were formed as well. Other components were expected because the contents of cafestol and dehydrocafestol in sum did not correspond to the cafestol content before roasting.

Cafestal

Isolated cafestol was sealed into a thick-walled ampoule under nitrogen atmosphere and heated within defined conditions (time and temperature) in a drying oven. Through this process, we wanted to simulate as closely as possible the normal conditions in a coffee bean. Due to the water proportion contained in green coffee, steam was built during roasting causing an increase of pressure in the bean.

Depending on the chosen conditions, the white cafestol powder became increasingly brown. For sensory analysis the glass tube was opened after it cooled down and a smell reminiscent of freshly baked bread was noticed. The other ampoule was opened under ethyl acetate, an aliquot was evaporated until dry in a gentle steam of nitrogen and after solving it in HPLC eluent, the residue was chromatographed.
As already expected a new compound aside from dehydrocafestol was formed during our model experiments. The structure elucidation was carried out by means of NMR, FTIR, and mass spectrometry and checked by chemical conversion. Following the designation of cafestol, cafestal is proposed as the common name (Figure 2).

Cafestal Kahweal

Figure 2. Structural formulae of cafestal and kahweal

The roasting behavior of cafestol, dehydrocafestol, and cafestal related to heating time at a constant temperature is illustrated in Figure 3. It shows that according to the length of the heating time the amount of cafestol approaches nil.
On the other hand for cafestal and dehydrocafestol, an initial increase is noticed followed by a decrease the longer they were heated.

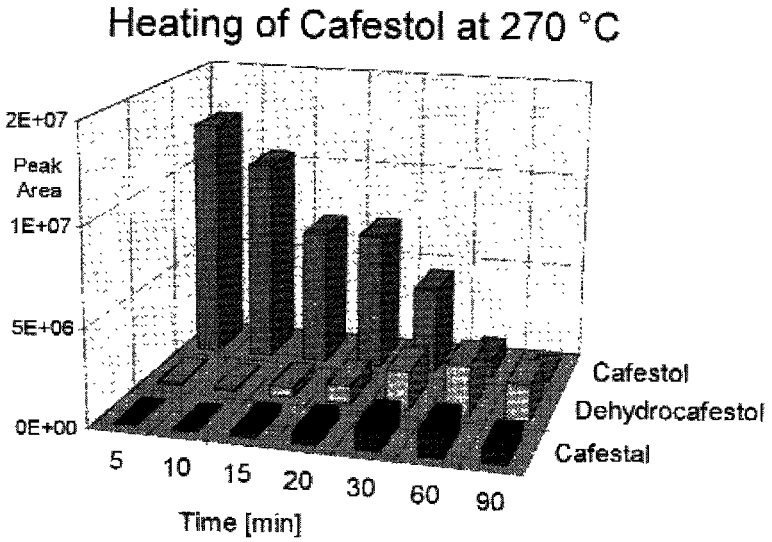


Figure 3. Formation of dehydrocafestol and cafestal during heating of cafestol

Cafestal in Roasted Coffees
We were then interested in detecting and if it was there in determining the new component in roasted commercial coffee. Therefore, a method based on the analysis method which was developed for free diterpenes was validated with a detection limit of 0.1 mg /g coffee lipid.
At first, three commercial coffees and one espresso were investigated, and in addition, in order to better judge of our results, one Arabica and one Robusta coffee were roasted at different roasting degrees. The results are compiled in Figure 4.

In all the examined coffee samples, cafestal was detected and for the three commercial coffees determined at amounts of about 0.3 mg/g lipids, whereas in the espresso twice the amount was analysed. For the model samples with a roasting degree of 6.4, the cafestal contents were 0.25 mg/g lipids for the Robusta and 0.36 for the Arabica and are, therefore, in accordance to the amounts of the commercial coffees.
The contents of the samples with the roasting degree 11.6 ranged from 0.56 mg/g lipids for the Robusta to 0.98 for the Arabica.

Furthermore, in the model samples the contents of free cafestol and dehydrocafestol were analysed. The extremely high dehydrocafestol contents made us suppose that dehydrocafestol esters existed and that dehydrocafestol was released from them (Figure 5).

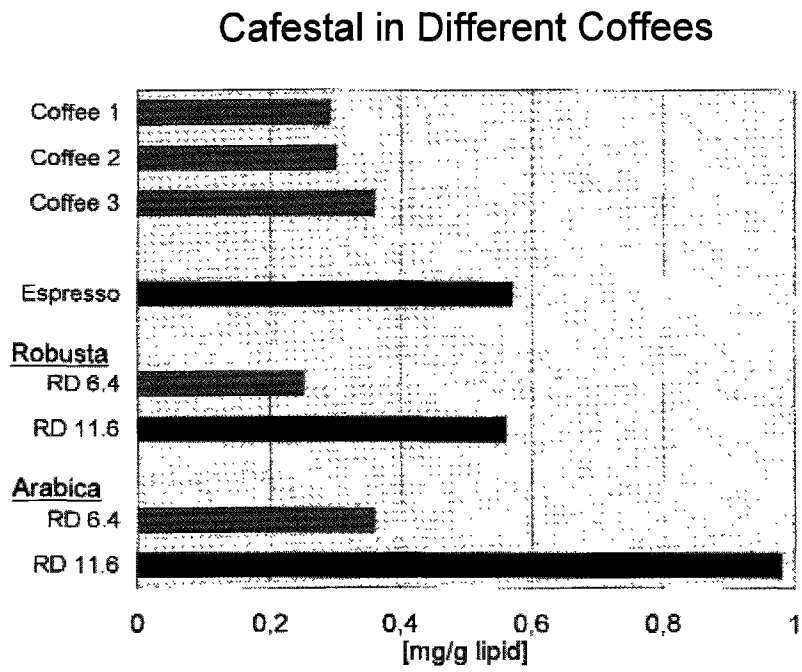


Figure 4. Contents of cafestal in roasted coffees (RD = roasting degree)

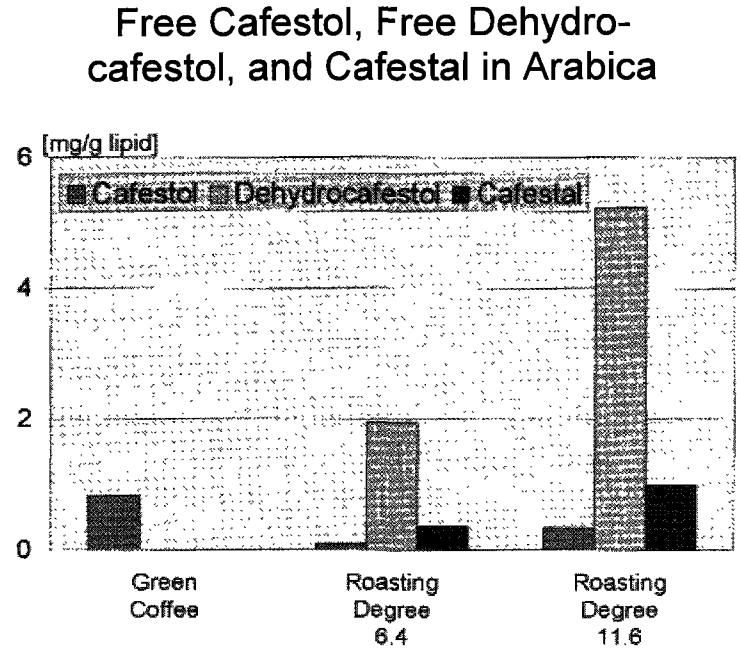


Figure 5. Contents of free cafestol, free dehydrocafestol and cafestal in an Arabica coffee

Dehydro Diterpene Esters

In model experiments with cafestol palmitate and cafestol linoleate it has been confirmed that cafestol fatty acid esters can be dehydrated similar to the free diterpenes kahweol and cafestol. In Figure 6 the mass spectrum of the formed dehydrocafestol palmitate is shown at the top, the mass spectrum of the initial cafestol palmitate is shown at the bottom.

The spectrum of cafestol palmitate has the molecul ion 554, whereas the spectrum of the dehydro component, after the loss of a water molecul, is 536. Further key fragment ions are also shifted by 18 masses.

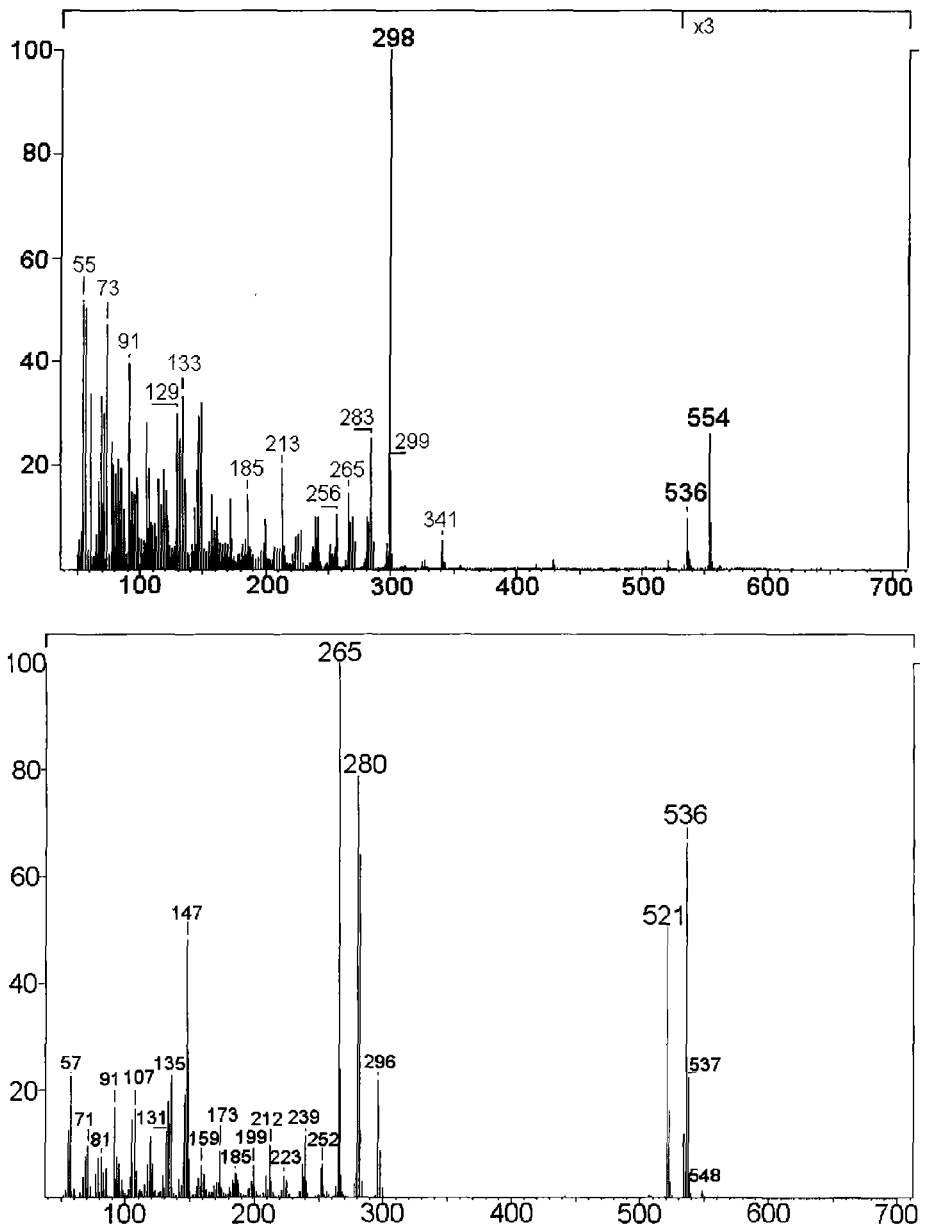


Figure 6. Mass spectra of cafestol palmitate and dehydrocafestol palmitate

The two dehydrated compounds, which were formed in the model experiments, were identified in roasted coffee, too. From this point, it is possible to assume that further fatty acid esters from both dehydrocafestol and dehydrokahweol may be identified. Furthermore, with kahweal, a compound corresponding to kahweol was identified (see Figure 3).

Summary
A brief overview of the composition of the diterpene fraction of roasted coffee was presented. In spite of a very similar lipid content between green coffee and roasted coffee prepared from it, there are several changes in the diterpene fraction due to the roasting process. In comparison to green coffee a large number of additional diterpenes (aside from the diterpenes cafestol, kahweol and 16-O-methylcafestol and their esters) were obtained in roasted coffee. Apart from diterpene derivatives formed by the loss of a water molecule, with cafestal and kahweal two further degradation products were elucidated.

Zusammenfassung
Es wird ein Überblick über die Zusammensetzung der Diterpenfraktion des Röstkaffees gegeben. Trotz eines ähnlichen Fettgehaltes zwischen dem Rohkaffee und dem daraus hergestellten Röstkaffee, ergeben sich mehrere Änderungen in der Diterpenfraktion infolge des Röstprozesses. Im Vergleich zum Rohkaffee enthält Röstkaffee neben den Diterpenen Cafestol, Kahweol und 16-O-Methylcafestol sowie ihrer Ester eine große Anzahl weiterer Diterpenverbindungen. Neben den durch Abspaltung eines Wassermoleküls entstandenen Verbindungen wurden mit dem Cafestal und dem Kahweal zwei weitere Abbauprodukte aufgeklärt.

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Carboxyatractyligenin and Atractyligenin Glycosides in Coffee

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Introduction

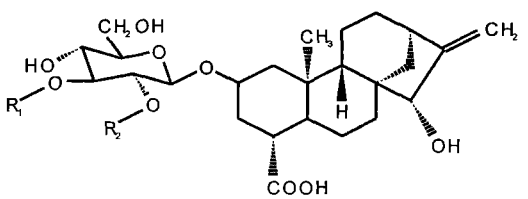
It has been known for some time that atractyloside glycosides (AGs), are present in coffee (1). A summary of detection methods used and their contents in coffee was published by Maier and Mätzel (2). The AGs identified were (3, 4): 2-O-ß-D-glucopyranosyl-atractyligenin **1**, 2'-O-isovaleryl-ß-D-glucopyranosyl-atractyligenin **2**, 3'-O-ß-D-glucopyranosyl-2'-O-isovaleryl-2-O-ß-D-glucopyranosyl-atractyligenin **3** *(Figure 1)*. These compounds have also been termed KA II, KA III and KA I, respectively (3). The aglycon, atractyligenin, a nor-diterpenoid substance of the (-)kaurene series, has also been found in coffee in the free form (5). There have been no reports in coffee of carboxyatractyligenin glycosides (CAGs), which have been observed in other plants and could be precursors for the AGs (6). Reflectance photometry was used by Aeschbach et al. (7) to determine free atractyligenin in coffee using reflectance photometry, and by Maier and Wewetzer (3) to determine glycosides **1-3** following isolation from coffee beans. Since the work of the Maier and Spiteller groups, little attention has been paid towards this class of compounds in coffee. One reason for this could be their lack of analytical sensitivity; they have minimal UV absorbance and are not volatile.
In this publication the use of the electrospray LC-MS technique for the identification and quantification of AGs and, for the first time, of CAGs in coffee is demonstrated.

Identification of AGs in roasted coffees

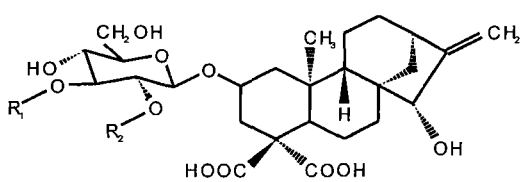
Figure 2 shows the reversed phase liquid chromatography HPLC chromatogram of an aqueous extract (1%, 40°C, ultrasonication) of roasted coffee with UV (second trace) and mass spectrometric (ion trap) detection (upper trace). An electrospray interface (ESI) in negative ionization mode was used to introduce the eluate into the mass spectrometer. The LC eluent consisted of a 1% acetic acid/1% acetic acid in methanol gradient. The chromatogram peaks (UV detection at 220 nm) were mainly attributed to the chlorogenic acid (CGA) fraction. This consisted of isomers of caffeoylquinic

(CQA), feruloylquinic (FQA), dicaffeoylquinic (di-CQA) and caffeoylferuloylquinic acids (CFQA). Assignment of these peaks was by means of their mass spectra together with retention times from literature. Additionally, several non-UV active peaks were eluted at higher retention times. The main peaks in this area showed mass peaks of 481, 565 and 727 (*Figure* 2, lower traces) and could be assigned to the (molecular ion - H^+) peaks from AGs **1**, **2** and **3** (KA II, KA III, KA I), respectively. Identical MS fragmentation patterns indicated that **2** was present in three isomeric forms.

Figure 1: Structures of AGs and CAGs in coffee

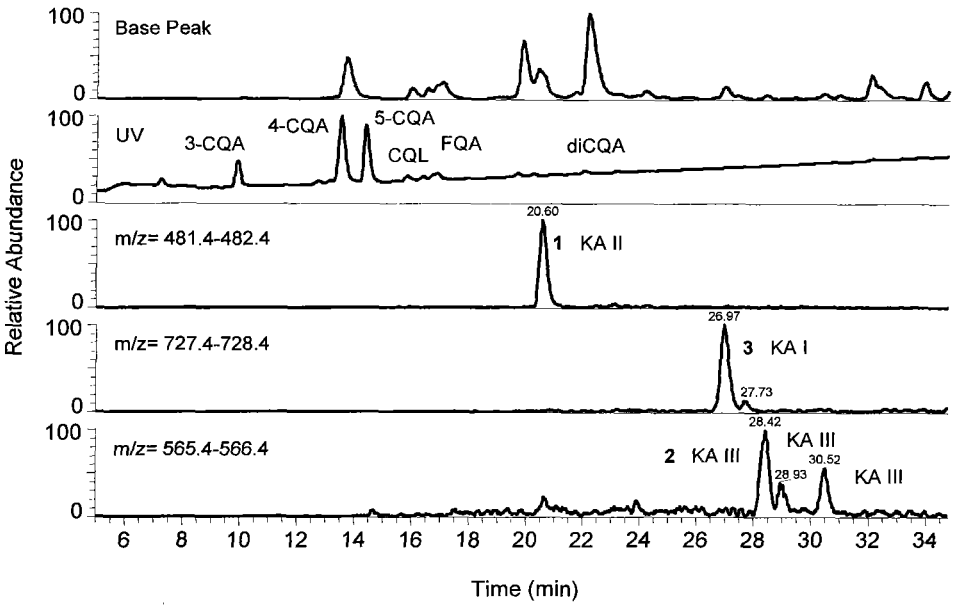


R_1	R_2	Compound		MW
H	H	**1**	KA II	482
H	-C(=O)-CH_2-CH(CH_3)(CH_3)	**2**	KA III	566
ß-D-Gluc$_p$(1→)	-C(=O)-CH_2-CH(CH_3)(CH_3)	**3**	KA I	728



R_1	R_2	Compound		MW
H	H	**4**	CKA II	526
H	-C(=O)-CH_2-CH(CH_3)(CH_3)	**5**	CKA III	610
ß-D-Gluc$_p$(1→)	-C(=O)-CH_2-CH(CH_3)(CH_3)	**6**	CKA I	772

*Figure 2: Reversed phase HPLC/UV/MS chromatogram of roasted coffee extract. Upper trace shows the base peak signal; second trace: UV; lower traces: selected ions that are due to the AGs **1, 3** and **2**, respectively.*



Isolation of AGs

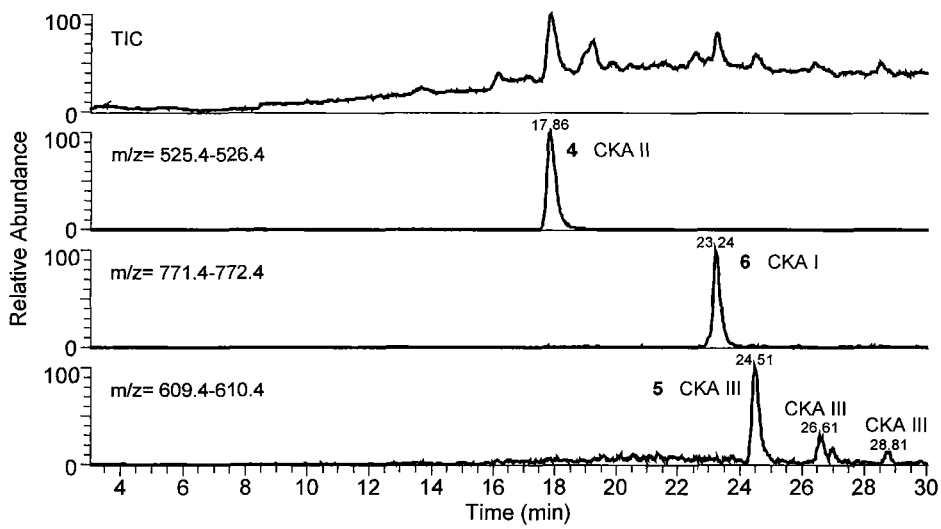
AGs **1** and **3** were isolated from steamed coffee beans according to a procedure based on those of the Spiteller and Maier groups (3, 4). After grinding and defatting, the beans were extracted with methanol in a Soxhlet apparatus. The solvent was evaporated and the residue dissolved in n-butanol and washed with water. n-Butanol was distilled off from the organic phase, the residue dissolved in water and washed several times with CH_2Cl_2 to remove caffeine. The glycosidic fraction was separated from the CGAs by adsorption onto an XAD-2 column. The methanol eluted solids contained the glycosides together with di-CQAs and CFQAs. Final purification of **1** and **3** was by means of preparative HPLC (reverse phase) and subsequent crystallization. Purity was checked by determining the glucose content after hydrolysis with 2N trifluoroacetic acid. The efficiency of each step during the isolation procedure was monitored using reversed phase HPLC/MS analysis.
The structure of the isolated compounds were further confirmed by ^{1}H-NMR and ^{13}C-NMR with final peak assignment by means of two-dimensional H,H-COSY and ^{13}C,H,H-HMQC experiments (8).

Identification of CAGs in green coffees

There was no indication of the presence of AGs in aqueous green coffee extracts (1%, ultrasonication, 40°C) by LC-MS. Instead, other peaks appeared in the LC chromatogram which had different retention times and mass spectra (*Figure 3*). MS base peaks appeared for example at *m/z* 525, 609 and 771, with smaller peaks at *m/z* 481, 565 and 727. Using MS/MS for fragmentation of these ions, the latter peaks became more prominent, attributable to the loss of carbon dioxide (*-m/z* 44). This observation indicated the presence of CAGs corresponding to the AGs **1-3**, each with an additional carboxyl group. The AGs were less polar than the CAGs, causing a shift of the former to higher retention times in the HPLC separation. There have been no reports to date of CAGs in green

coffee beans. Presumably, the lability of the carboxyl group explains why, in earlier work, only the AGs were found in green coffee. The structures of the CAGs (**4, 5** and **6,** termed CKA II, CKA III, CKA I, respectively) are given in *Figure 1.*

Figure 3: *Reversed phase HPLC/MS chromatogram of green coffee extract. The upper trace shows the total ion current, lower traces selected ions that are due to the three glycosides **4, 6** and **5**, respectively.*



Isolation of CAGs

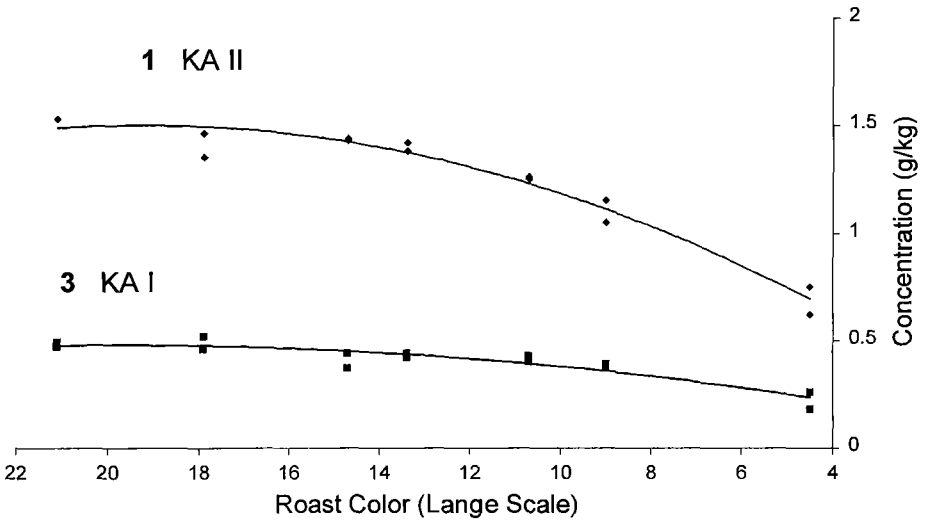
Isolation of CAGs from green coffee was from a methanolic extract as for the AGs above except that i) the n-butanol extraction step was omitted and ii) the more polar **4** was obtained by aqueous elution of the XAD-2 column, **5** and **6** were in the methanol eluate. Isolation of pure **4** and **6** from the mixture was by preparative HPLC using a water/methanol gradient, followed by crystallization. Structural confirmation was by ^{1}H-NMR and ^{13}C-NMR (8).
Glycoside **5** was not isolated due to its low yield. Three isomers of **5** were monitored (*Figure 3*), while **4** and **6** each seem to be present in the single form. It is suggested that isomerisation is due to esterification of the isovaleric group at different hydroxyls on the glucose ring.

Contents of AGs in roast and steamed coffees

Roast coffee
LC/MS offers a fast and convenient means for the analysis of AGs in roast coffee. The analysis can be made directly on a filtered aqueous extract. Best sensitivity was obtained in the negative ionisation mode using the electrospray interface. In order to analyse all the glycosides in one HPLC run, the mass range was set to *m/z* = 450-850. A 20 cm x 2.1 mm RP-column at a flow rate of 0.3 ml/min. gave the highest reproducibility. Data for several coffees at 'medium roast' (Roast color 12.0, Lange scale) are given in *Table 1.* The concentration of **1** for a medium roasted coffee was 1.2-1.4 g/kg in wet processed Arabicas and 0.03 g/kg in a Robusta sample. Contents of **3** were 0.2-0.4 g/kg (wet processed Arabicas), 0.9 g/kg (Brazil) and 0.06 g/kg (Robusta). Contents of **2** were not determined but the three isomeric peaks for this AG were observed in all samples. The effect of

degree of roast on contents of **1** and **3** is also given in *Figure 4*. Degradation of the glycosides was about 30 % at roast colors in the commercial range (ca. 12 roast color, Lange units).
In view of the relatively low level of attention that has been given to these glycosides, the analytical data show unexpectedly high concentrations in roast coffee beans. The levels in roasted coffee determined here were about twice as high as those obtained by Maier's group (3).
Due to their high water solubility, it is apparent that a roast Arabica brew could contain about 0.3% of glycosides (solids basis). Levels in Robusta coffee brews would be lower.

Figure 4: Content of AGs in roast coffee beans as a function of degree of roast.



Steamed coffees
The concentration of **1** in both wet processed (Colombia) and dry processed Arabicas (Brazil) after steaming (148°C, 3.5 bar, 60 min.) was in the range of 1.5-1.9 g/kg. Brazilian coffee contained significantly higher levels of **3** compared to Colombia. In the absence of a standard, **2** was not determined, but estimates were of the order of 0.35 g/kg for the two steamed Colombian samples and 0.5 g/kg for the two steamed Brazilian coffees listed in *Table 1*.

Table 1: Content of AGs in steamed and roasted coffee (roast color 12.0, Lange scale). Each number is given in g/kg and represents an average of duplicates.

		1	**3**
Steamed Coffee	Colombia Excelsa I	1.9	0.8
	Colombia Excelsa II	1.5	0.6
	Brazil Santos I	1.6	1.5
	Brazil Santos II	1.7	1.9
Roast Coffee	Colombia (Excelsa)	1.4	0.4
	El Salvador	1.4	0.2
	Kenya	1.2	0.1
	Brazil	1.2	0.9
	Robusta Indonesia	0.03	0.06

Content of CAGs in green coffee

AGs are formed by decarboxylation of corresponding CAGs. The former compounds were completely absent in untreated green coffee. Using the isolated CAGs as standards, the amounts of **4** and **6** in green coffees were determined for a range of varieties. The concentration of **4** in most wet and dry processed Arabicas was about 2 g/kg. Contents of **6** were more variable (0.2-3.1 g/kg). The estimated level of **5** was 0.5 g/kg for five wet processed Arabicas using the response factor for **4**. The total amount of all CAGs in Arabican green coffee was found to be in the range of 2-4 g/kg.
Long term storage of green coffee beans at ambient temperatures did not lead to generation of **1**, **2** or **3**. For example, Brazil 'Old Crop' beans had similar levels to fresh Brazil beans. Brazil beans contained significantly higher levels of **6** compared to wet processed Arabicas. At accelerated temperatures the decarboxylation of **4**, **5** and **6** in coffee beans occurred rapidly, mild steaming conditions (e.g. 120°C, 30 min) were sufficient to complete the reaction and no CAGs were detected in steamed or roasted coffees. Comparison of glycoside yields in green *(Table 2)* and in the corresponding steamed coffee *(Table 1)*, indicated that decarboxylation was almost quantitative. For example, Colombian Excelsa green beans contained ca. 2.2 g/kg (4.2 mmol/kg) **4** which was reduced to ca. 1.7 g/kg (3.5 mmol/kg) **1** in steamed coffee.

Table 2: Content of CAGs in green coffee (g/kg). Each value represents an average of duplicates.

		4	**6**
Wet Processed Arabicas	Costa Rica I	2.0	0.2
	Costa Rica II	2.0	0.2
	Guatemala	1.9	0.4
	El Salvador	2.0	0.1
	Colombia Excelsa I	2.4	1.0
	Colombia Excelsa II	2.2	0.9
	Colombia Excelsa III	2.2	0.9
	Kenya I	2.2	0.5
	Kenya II	2.0	0.5
	Ethiopia Harar	2.0	1.1
	Ethiopia Djimmah	1.0	3.1
	Papua Neuguinea	1.8	1.0
	Indonesia Sumatra	2.0	1.0
	Jamaika Blue Mountain	1.4	1.1
	Burundi	2.0	0.9
	Zimbabwe	1.0	0.5
Dry Processed Arabicas	Brazil Minas	2.2	1.9
	Brazil Santos	2.2	2.4
	Brazil Old Crop (1 year)	2.1	1.3
	Brazil Old Crop (5 years)	2.1	1.3
Robustas	Vietnam	0.2	0.2
	Indonesia	0.1	0.6
	Decaffinated Indonesia	0.2	0.8

Implication for soluble coffee carbohydrate analysis

Maier and Wewetzer (3) have shown that AGs are present in industrial coffee extract. The data in this present study suggest that AG contents in commercial soluble coffees could be of the order of 0.5%, solids basis. At this level, AGs would contribute ca. 0.2% to the glucose value obtained for the total carbohydrate content obtained by acid hydrolysis.

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8) Balzer, H. H.; Bradbury, A. G. W.; Characterization of Carboxyatractyligenin Glycosides and Actractyligenin Glycosides in Coffee Beans. *In preparation.*

Abstract

HPLC/MS was used to identify and determine three carboxyatractyligenin glycosides, termed CKA I, CKA II, and CKA III, in green coffee beans. CKA III was present as three isomers. Total bean glycoside contents varied between ca. 1 to 5 g/kg and CKA I contents of Arabica beans (1 to 2.5 g/kg) were significantly higher than those of Robusta beans (< 0.2 g/kg). On thermal treatment, decarboxylation to the corresponding atractyligenin glycosides occurred rapidly. The atractyligenin glycosides were relatively stable to roasting, the content being reduced by about half under conditions of dark roast.

Résumé

La chromatographie « HPLC/MS » a été utilisée pour identifier et quantifier trois glycosides carboxyatractyligenin dans les grains de café vert : CKAI, CKA II et CKA III. Le composé CKA III a été trouvé sous la forme de trois isomères. Lors de cette étude, la concentration de "glycoside total" mesurée variait entre 1 et 5 g/kg dans les grains de café. En ce qui concerne le composé CKA I, sa concentration mesurée dans les grains "Arabica" (1 à 2.5 g/kg) était significativement plus elevée que celle mesurée dans les grains "Robusta" (<0.2 g/kg).
Soumis à des traitements thermiques, les glycosides atractyligenin ont été decarboxylès rapidement. Les glycosides atractyligenin se sont montrés relativement stables lors de la torrefaction ; leur concentration n' a été reduite environ que de moitié après une torrefaction « foncée » (Dark roast).

Stable Isotopes and Coffee Quality: Preliminary Report

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ABSTRACT

The aim of this research is to test stable isotopes in coffee beans as tracers for quality. Since the quality of the product is mainly determined by the environmental conditions in which the plant grows and by the genetic origin of the variety, stable isotopes should be a proxi for parameters such as climate, crossing techniques, fertilisation.

A similar approach has been followed in food science for several other products from wine, to honey, milk, fruit juice, in order to identify proxi for climate variability that may indicate the origin of the product and/or the further transformations and sophistications (Martin G.M. et al., 1988).

Relative abundance of stable isotopes in many natural materials have been studied extensively since 1950 to investigate the elemental cycles (C, N, H, O, S) in natural stuff. As a matter of fact, the small variations of the isotope composition of a substance is of interest for the characterisation and identification of food products. The variability is caused by the isotopic fractionations that occur during physical, chemical and physiological processes.

The primary purpose of this study is to asses the feasibility of isotopic techniques to coffee products.

The but de ce projet de recherche est de tester les isotopes stables sur les graines de café entant que traceur. Car la qualitè du produit est presque totalment determiné par les conditions de l'environnement où la plante pousse et par l'origine génétique de la varieté, les isotopes stables pourraient être utilisés pour reconstruire les paramétres comme le climat, les tecniques de croisement et de fertilisation.

Un tel approche à été suivie dans le domain de le science de l'agro-alimentation pour determiner sur de nombreux produits comme le vin, le miel, le lait, le jus de fruit, l'origine et les transformations or sophystications qui ont été apportées.

L'abondance relative des isotopes stables dans de nombreux produits naturels è été étudiée intensément depuis 1950 pour en identifier les cycles élémentaires (C, N, H, O, S). En effet les petites variations de la composition isotopique d'une substance est fondamentale pour la caractérisation et l'identification des produits alimentaires. La variation est produite par le fractionnement isotopique qui se vérifie pendant les transformations physiques, chimiques et physiologiques.

Le premier but de cette étude est la verification des hyp›theses isotopiques sur le café.

RATIONALE BEHIND THE RESEARCH

Coffee is one of the most important products in the stock exchange market with sugar and cocoa.

Many countries in Central and South America and in Africa rely almost entirely on this source that represents nearly the totality of their export budget. The importing countries have on the

other hand, many economic interests mainly due to the investments in the stock market and because of their activity of coffee processing. It is clear how both producers and importers are than concerned about the quality of the product they deal with.

Frauds and adulteration of the product may occur during the whole journey of coffee from the grower to the distribution circuit. According to Zimmering (1998) Brazilian coffee growers are concerned about their rivals adding ground corn and other cheap agricultural products to ground coffee in order to cut costs. Dishonest distributors, on the other hand, label fraudulently coffee to conceal the true origin of the products.

While connoisseurs and coffee experts may expect to easily avoid such tricks, common people represent the true victim of such abuses.

At present EU policy is strongly concerned with the safeguard of consumer. In spite of its efforts for evaluating analytical methods to assess compliance with food legislation, anti-fraud studies and controls on the quality, nothing has been done on coffee products and a well-established analytical method to test its quality is still lacking (Barel and Jacquet, 1994). With the possible exception the new method used by Zimmering (1998). This method is based on measuring gases given off by complex chemical reactions in the heart of the beans or cherries, He observed different release patterns of gases for coffee beans without additives and for coffee beans with additives. Thus the technique was able to determine the level of purity of coffee and could be used as a quality assurance tool. Another method that shows great promise is stable isotopic (B, C, N, O, S) analysis of commercial coffee beans (Wieser, 1997: Wieser et al., 1999).

This research intends to follow the isotopic approach and expand the previous studies analysing a larger number of samples in order to achieve the following goals:

1) Create a databank of isotopic values of coffee according to genetic species and geographic origin.
2) Analysing samples representing several years to evaluate time variability of the yield.
3) Performing the same analysis on roasted coffee to understand whether isotopic signature can be followed even after the product processing.

SCIENTIFIC APPROACH

The research duration will be of 30 months (from January 1999 to June 2001), which will enable us to cope with the three tasks we planned to.

The samples, on the other hand, will cover about three year yields (1998-2000) (taking into consideration that harvesting periods are not synchronous and depend on the local climatic conditions).

In order to collect as many coffee samples as possible (for statistical reasons) we have organised as well a database of climatic and environmental data which will be supported (by means of monthly forms) directly by the plantation owners. This will help us to define external factors, and local farmers to get involved in the project and eventually get to know more about developing techniques such as isotopes.

The analysis that are planned to be performed are:

1) elemental analysis (CNOS) of the whole bean
2) GC-combustion analysis for the chemical characterisation of the bean coupled to a mass spectrometer.
3) NMR analysis
4) SNIFF-NMR analysis on caffeine
5) B analysis using NTIMS (Negative Thermal Ionisation Mass Spectrometer)

METHODOLOGIES

All the data presented in this article as been obtained by a Carlo Erba EA 1110 Elemental Analyser coupled to a Deltaplus mass spectrometer (Finnigan MAT). The samples (about 40 beans or so per sample) have been grounded and subsequently freezed for at least 24h. After that time, the different samples have been insert into a freeze dryer to take out the water from the coffee.

Each data is the mean of 7 analysis, from which the most external two have been taken out. We observed this analytical approach for every sample in order to avoid data scattering due to the isotopic content variations of the different fractions of the beans.

PRELIMINARY RESULTS

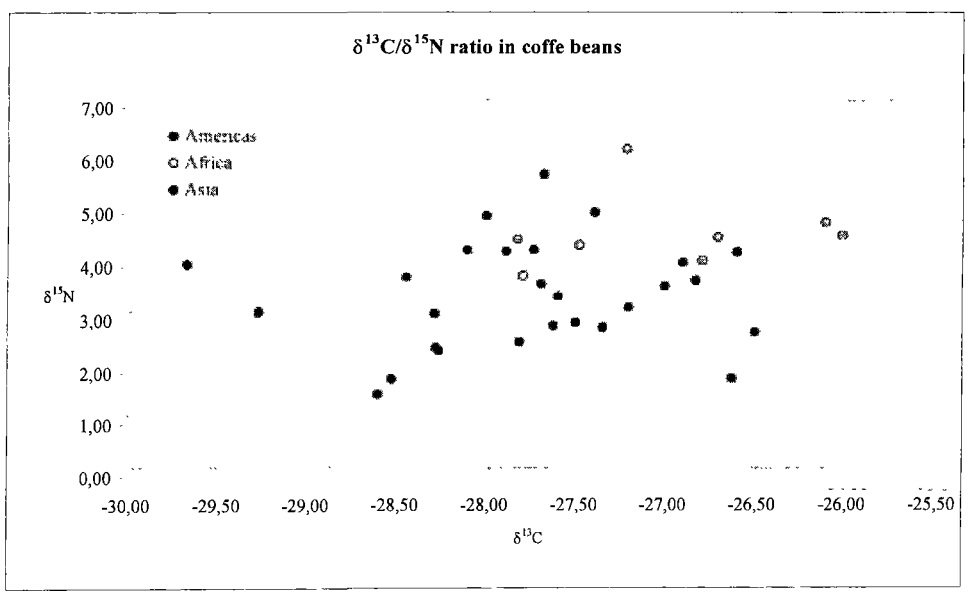
The first analysis we performed have been the isotopic composition of carbon and nitrogen of the whole bean of 35 samples coming from America, Asia and Africa.

The isotopic composition of carbon and nitrogen in plants are influenced respectively by the photosynthetic activity of the species and by the natural content (and isotopic composition) of nitrogen into the soil or into the fertiliser used. Despite the broad literature on the isotopic composition of C3 and C4 plants, little has been done to test how external factors, as precipitation, temperature variations and plant-to-plant interactions, determine isotopic differences among plants of the same variety.

As preliminary test we did not face these particular problems, because of the complexity of the matter and most of all because such an approach would have been much more expensive and time consuming, lacking guaranties of success.

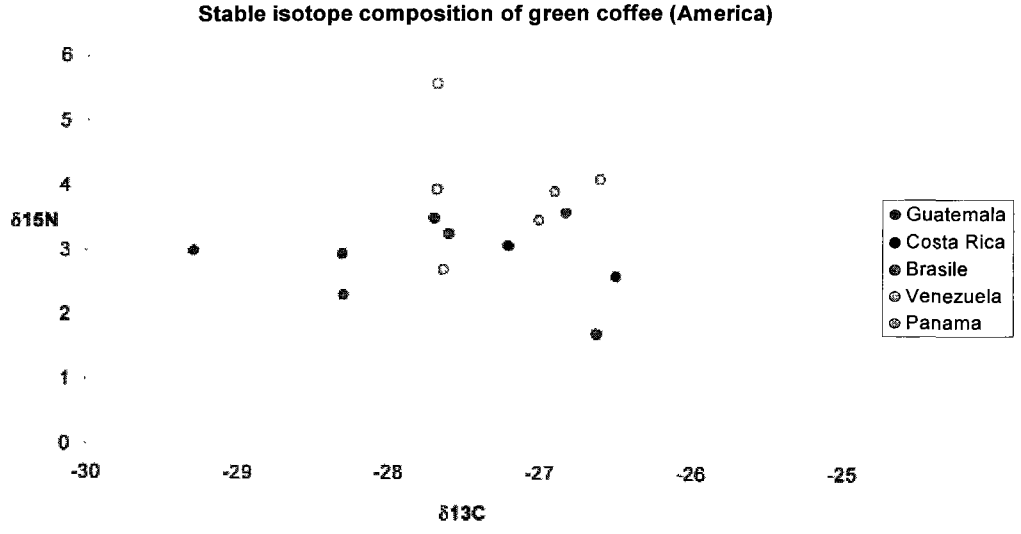
In this optic we preferred to gather as much samples as we could and perform the analysis in order to be able to observe the entire bunch of data from a food-control point of view. Thus, the first question to answer was: "Are the isotopic variations so broad to be detected by an ordinary mass spetrometric analysis?"

From the graph below, the answer that must be given is "yes"! As it is possible to observe the compositional variation of the samples is more than 3 ‰ for carbon and even more for nitrogen, even if the regional origin of the coffee seems not be able to prevent the randomly scattering of the data.



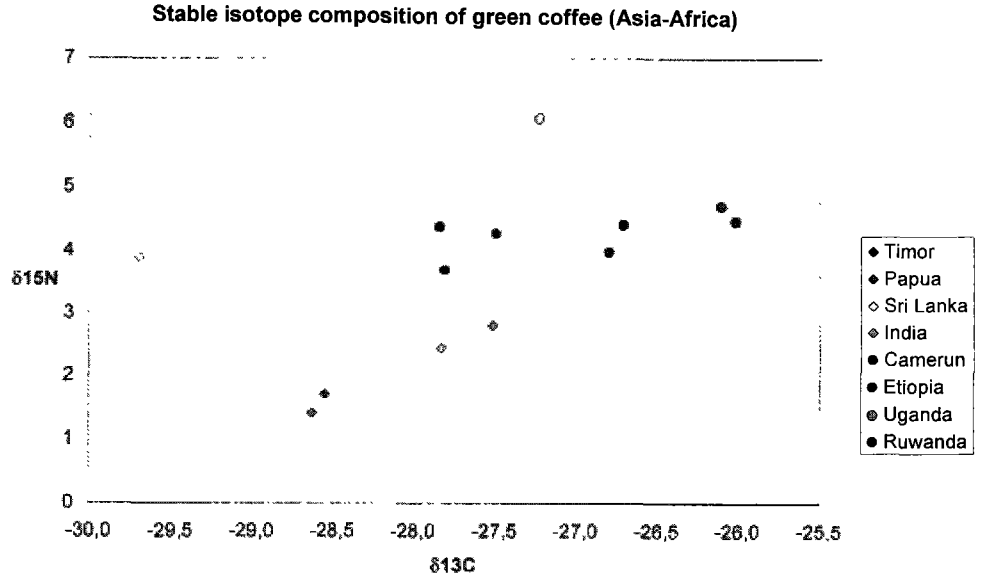
Then at a first glance, the conclusions could be that, there is something that induce variations in the photosynthetic and the nitrogen absorption activities, but these pushing factors cannot be used as markers for the geographic origin of the coffee.

But if we try to separate the samples according to nationality, instead of continental origin we can see that, at least for countries with numerous samples, an order can be found. Off course, countries'borders mean nothing in terms environmental and climatic factors, but still it is possible



to trace areas in which only one nationality can be found. Perhaps it is not possible to infer such an indication from a single result (i.e., nitrogen, or carbon) but the association of the two seems to be peculiar and quite defined.

These considerations are even more true if we consider the comparison between Asian and African samples, each of them relegated into their corner of the graph and without any overlapping.



Despite these results are not definitive and do not face or try to answer to the most fundamental problems of coffee physiology, they are quite encouraging for the continuation of the research. We are confident that analysis of the remaining isotopes (oxygen and sulphur) will offer a better prospective on the reliability of these techniques to coffee science and that the inter-correlation of more parameters will be able to define "the" marker for geographical origin of the product and possibly discriminate among varieties.

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Nonvolatile Compounds in Coffee

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The desirable color, aroma and taste of brewed coffee are formed by the roasting process that is applied to the green coffee beans. This report is focused on the macromolecules formed in coffee beans by roasting. The major reactions that occur during roasting are the Maillard reaction and the oxidative polymerization or degradation of phenolics.

Macromolecules

Extensive work has been done on the characterization of colored polymers in brewed coffee. Evidence of the Maillard reaction during the roasting of coffee beans has been reviewed in the passed ASICs by Dart,S.K. (1985), Ho,C.-T. (1993),and Reineccius, G.A. (1995). The formation of furans, pyrazines and aldehydes as aroma constituents supports the degradation of sugars by the Maillard reaction. Nevertheless, such phenolics as chlorogenic acids are degraded by roasting green coffee beans. A significant quantity of the chlorogenic acid lost during roasting remains in an uncharacterized form.

Leloup,V. *et al.* have monitored the fate of chlorogenic acids during medium-slow roasting of Colombia Arabica coffee at 240°C by a kinetic analysis. The chlorogenic acids rapidly disappeared during the roasting process, although not all chlorogenic acid isomers were similarly sensitive to the thermal treatment. Caffeoylquinic acid and dicaffeoylquinic acid decreased the most rapidly.
With a short roasting time, dicaffeoylquinic acid (diCQA) is partly hydrolysed into caffeoylquinic acid and the caffeic moiety. The caffeoylquinic acid isomers then rapidly undergo esterification with carbohydrate and protein, producing bound chlorogenic acids. With a longer roasting time, the phenolic moieties are rapidly degraded to generate such diverse phenolic components as vinyl catechol and catechol from the phenolic moiety. The quinic moiety degrades more slowly to generate hydroquinone, catechol, phenol and pyrogallol .

Heinrich,L. and Baltes,M. (1987) have prepared seven fractions of melanoidins from roasted robusta coffee beans by using a polyamide column. These melanoidins were degraded by Curie point pyrolysis, which was monitored by high-resolution GC/MS, to about 100 products, 33 phenols being found among them. The products were compared with the 37 products formed *via* model pyrolysis experiments on chlorogenic acid. Twelve compounds from coffee melanoidin were also found in the products from chlorogenic acid, suggesting that the chlorogenic moiety was involved in the coffee melanoidin.

A hot-water extract of roasted coffee has been analyzed by gel filtration column chromatography on Sephadex G 25 that was developed with water, four fractions being separated in order of molecular mass (Steinhart, H., 1993). These fractions were further separated into 3 or 4 bands by TLC on Sephadex, developing with a mixture of 25% ammonia and 1-propanol. Each band was hydrolyzed and analyzed for its sugar composition which differed according to the band. Mannose, arabinose, galactose and glucose were the major sugars, and rhamnose the minor.

The results of these works have not always shown whether the chromophore of brewed coffee melanoidin was of the Maillard or phenolic type. Since dialyzed brewed coffee and the further separated polymeric fraction showed the general absorption characteristics of model melanoidin in the visible range and a similar UV spectrum to that of chlorogenic and caffeic acids, brewed coffee is considered to contain both the sugar and phenolic types of melanoidin.

Microbiological characterization of melanoidin

The microbiological decolorization of brown pigments in foods has been tested to categorize the type of chemical structure of these brown pigments. The fungus, Paecilomyces canadensis NC 1, that can decolorize an instant coffee solution has been isolated from a glass bottle containing instant coffee of the freeze-dried type (Table 1). This glass bottle was left open for 2 weeks.

The fungus decolorized the instant coffee solution by 79% under the optimal conditions. The decolorized coffee solution was analyzed by gel permeation chromatography, and the absorbance was detected at 500 nm. Fig.1 shows the two chromatograms for the control solution and the decolorized one, indicating that the high-molecular-weight fractions had been decolorized (Terasawa, N., 1994). This same strain also decolorized black tea by >60%, and cocoa by 82%. The microorganism decolorized the model brown pigments formed from catechin by >90%, from chlorogenic acid by >30%, and from chlorogenic acid and sucrose by >40%. These samples cultured with two other microorganisms turned darker brown than the controls

Streptomyces werraensis TT14 (Murata, M., 1992A) and Coriolus versicolor IFO 30340 (Aoshima, I., 1985) have also been screened from soil by the decolorization rate of model melanoidin prepared from glucose and glycine. Three microorganisms, S. werraensis TT14, C. versicolor IFO 30340, and P. canadensis NC 1, have been cultured to compare the decolorization rate of model brown pigments and brown colored foods (Terasawa, N., 1996).

The resulting decolorization rates are summarized in Fig.2. This fungus decolored phenol-type brown pigments and is unique in comparison with the other two microorganisms. It follows that the major brown pigment in brewed coffee can be considered to be of the phenol type.

Table I. Decolorization Rate of Brown Pigments by *Streptomyces werraensis* TT 14, *Coriolus versicolor* IFO 30340, and *Paecilomyces canadensis* NC-1

Brown pigment	Decolorization rate (%) by		
	S. werraensis	*C. versicolor*	*P. canadensis*
Glc-Gly model melanoidin	38.9	68.1	38.0
Xyl-Gly model melanoidin	70.4	63.0	58.3
Instant coffee	−100[a]	−9.7[a]	60.8
Black tea	−68.9[a]	−109[a]	56.7

[a] Indicates colorization by the microbial treatment compared to the control.

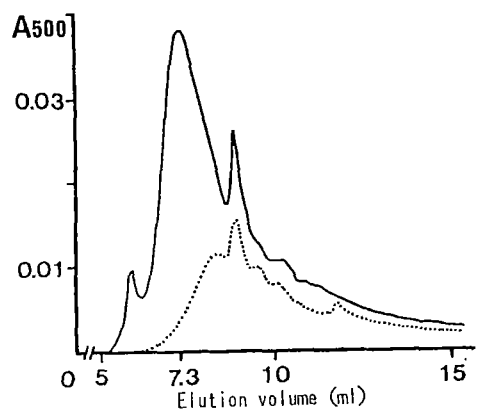


Fig. 1. Decolorization of Coffee by *Paecilomyces canadensis* NC-1 Analyzed by Gel Permeation Chromatography.

——, control; -----, decolorized coffee.

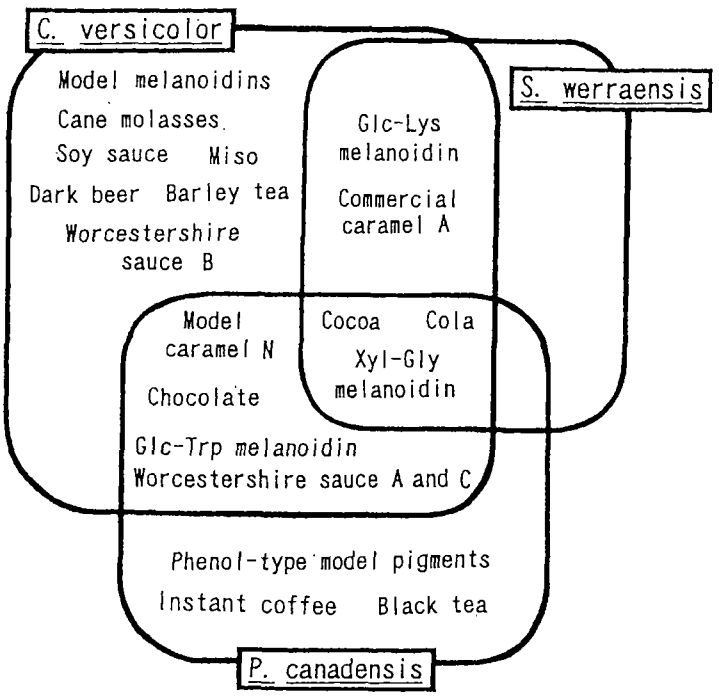
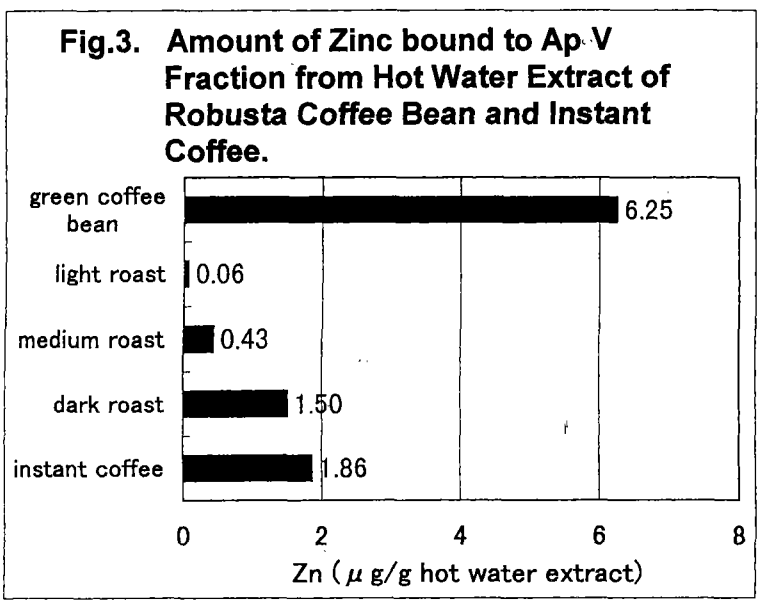


Fig. 2—Categorization of synthetic brown pigments and browned foods by statistical significance of microbial decolorization.



S.Homma et al., Unpublished data

Effect of coffee on the biological availability of minerals

The effect of brewed coffee on mineral nutrition has recently been noticed. A study on non-heme iron absorption by humans (Morck, T.A., 1983) has been reported to result in the reduction of non-heme iron availability. Brewed coffee has metal-chelating activity which results in a trace element deficiency in those geographical areas of malnutrition where coffee is consumed in a large quantity (Munoz, M.N., 1988). However, little effect of instant coffee on manganese absorption (91.7%) has been reported in the animal study by Fraile (1992), using suckling rats fed on $MnSO_4$ and polyphenol containing beverages such as tea, coffee, and red and white wines.

Separation of the zinc-chelating compounds from instant coffee

There have been a few studies on separating these metal- chelating compounds in roasted coffee. Homma *et al.* have reported a dissociation constant value of 3.4×10^{-5} [M] for the zinc-chelating activity of the instant coffee component having a molecular size of 3000 Da. Asakura *et al.* (1990) have shown that the ligands present in instant coffee that bind zinc(II) were acidic in nature and had a molecular size of less than 5000 Da by paper electrophoresis.

The zinc(II)-chelating compounds have been separated from instant coffee by coagulating with $ZnCl_2$, purifying by dissolving in 1% ammonia, and chromatographing in ion-exchange (Amberlite IRA 410 and IR 120) and cellulose columns, with development by a mixture of 1% ammonia and *n*-propanol. The yield from instant coffee of the active compound, Ap V, that was finally separated in the cellulose column was 0.3-0.4%.

This active compound was dissociated into its subunits by treating with EDTA. Ap V was polymerized by further exposure to Zn(II), and migrated to the Ap VI fraction, which is larger in molecular size than Ap V, by cellulose column chromatography (Homma, S., 1995).

Effect of roasting degree on the formation of metal chelating compounds (Homma, S., unpublished data)

The formation of metal-chelating compounds during the roasting process was monitored, and the chelating activity increased with increasing degree of roasting of the green beans. The chelating activity of brewed coffee was found to be greater with regular coffee than with instant coffee. It was also suggested that the green coffee bean has different active compound(s) from those formed in the roasted coffee bean (Fig.3).

General properties of the zinc-chelating compound (Homma, S., 1997)

Ap V was a brown amorphous powder and was soluble in water. Its molecular weight was estimated to be about 48,000 by HPLC, using proteins as standard markers. The apparent dissociation constants for Zn(II) measured from a Scatchard plot were 1.82×10^{-9} and 1.13×10^{-7} [M], and the numbers of binding sites were 1.05 and 1.98, respectively. This active compound also showed chelating activity for Cu(II) with apparent dissociation constants of 3.33×10^{-9} and 2.67×10^{-7} [M], and numbers of binding sites of 1.6 and 4.0, respectively (Homma, S., 1995).

The content of polyphenol was 30.4% by the Folin Denis method, and the contents of sugar and amino acid were about 3% and 4%, respectively. The chemical formula was experimentally found to be $C_{16}H_{21}O_9N_3$. The nitrogen content of more than 10% is indicative of the involvement of the Maillard reaction in the formation of the zinc(II)-chelating polymer. If phytate is involved in the formation of the active compounds while roasting green coffee beans, a strong contribution to chelation from the phosphorous group would be expected. However, phosphorous was hardly detectable by the modified Bartlett method.

Chemical structure and property of the zinc chelating compound (Homma, S., 1997)

The chemical structure of active compound Ap V has been investigated by characterizing the products formed through such degradative reactions on the Ap V sample as alkaline fusion (350°C) and alkaline decomposition (250°C) in glycerol.

Alkaline fusion of active compound Ap V gave about 11% of ether-soluble compounds, the major ones being low-molecular- weight polyphenols such as pyrogallol, protocatechuic acid, catechol, and *p*-hydroxybenzoic acid.

The acidic fraction shows the presence of polyphenolics, benzoic acid and its derivatives, and carboxilic acids with 4-5 carbon chains. The presence of amides in the basic fraction is indicative of the involvement of sugar and protein in the formation of Ap V.

Alkaline degradation in glycerol gave similar chromatographic patterns by LC-MS to those for alkaline fusion. Some peaks by LC-MS from the acidic fraction showed the connection of two adjacent benzene rings.
The oxidative degradation of Ap V with $KMnO_4$ and $NaIO_4$ gave different HPLC patterns after a prior treatment by methylation. This shows that Ap V contained a hydroxy group and a carboxy group which could be methylated. Degradation with $NaClO_2$ gave pyrogallol and caffeic acid, which is indicative of the presence of benzene rings connected with an ether bond.

Degradative reactions other than alkaline fusion gave fewer ether-soluble compounds than that by alkaline fusion, and similar phenolics were determined in the degradation products. This shows that the benzene rings involved in Ap V were connected with strong bonds which alkaline fusion could release to the greatest degree to produce phenolics.

Zn-chelating compound Ap V was found to be antioxidative toward linoleic acid by measuring the peroxide formed with ammonium thiocyanate and $FeCl_2$. Ap V was also found to show catalase and superoxide dismutase activities, although the specific activities were much less than those of the respective enzymes.

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Summary

The major reactions involved with the taste, aroma and color of brewed coffee are considered to be caramelization, the Maillard reaction and the oxidative polymerization of phenolics. These major reactions were reviewed.

Microorganisms were screened by their decolorizing effect on such brown-colored foods and model compounds as caramel, melanoidin and polymerized phenolics. It is possible to categorize or distinguish the brown pigments formed in foods according to the decoloration rate by these microorganisms. The brown color in instant coffee could be categorized as pigments rich in phenolics, being selectively decolorized by *Paecilomyces verraensis*.

For the interest in trace element nutrition Zn(II)-chelating compounds were separated from instant coffee by coagulating with $ZnCl_2$ and purified by chromatography in ion-exchange and cellulose columns, and the active compound was found to be a brown-colored polymer (m.w. 48000) of -log Kd = 8 for Zn(II) with a yield of 0.3-0.4% from instant coffee. The chemical composition of the Zn(II)-chelating compound was found to be 30.4% phenolics, 3% sugar, and 4% amino acid. Alkali fusion gave 10% of such ether-soluble compounds as pyrogallol, protocatechuic acid, catechol, and *p*-hydroxybenzoic acid as the major phenolics. The formation of the active compound was monitored during the roasting process, and the chelating activity increased with increasing degree of roasting of the green beans. The chelating activity of brewed coffee was found to be greater in regular coffee than in instant coffee.

Structural Aspects of Polysaccharides from Arabica Coffee

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Introduction

Polysaccharides constitute almost half of the dry weight of the coffee bean but there is still much to be discovered about their structural features and their biosynthesis.

Work by the groups of Thaler and Wolfrom (Table 1) has revealed the presence of mannans and arabinogalactans in green and roasted coffee beans (1) but it was only with Bradbury and Halliday's study in the 80's that structural data on these two polysaccharides was presented (2, 3).

The mannan purified by Bradbury *et al.* is composed of a 1,4-linked mannan chains with 1,6-linked single galactose residues every 100-130 residues (Table 1).

The arabinogalactan isolated by Bradbury *et al.* has an Arabinose/Galactose ratio of 0.4/1 and a main chain of 1,3-linked galactose with 3,6-branched galactose residues. The side-chains contain arabinose and galactose residues, these linkages are characteristic of type-II arabinogalactans (Table 2).

Table 1. Distribution of constituent monosaccharides in the "pure polysaccharide" of green Arabica coffee. Based on data summarised by Trugo (1).

Monosaccharide	**%w of total polysaccharide**
Mannose	53
Galactose	24
Glucose	18
Arabinose	5
Total polysaccharide	**38.7%**

Table 2. Glycosyl linkages of polysaccharides isolated from green Robusta coffee by Bradbury et al. (2,3).

Linkage	Mole %
Mannan	
t-man	1
4-man	12.3
4,6-man	0.1
t-gal	0.1
Arabinogalactan	
t-ara*f*	15.4
5-ara*f*	9.8
2-ara*f*	2.1
t-gal	5.0
3-gal	1.9
3,6-gal	21.5

Materials and Methods

Arabica Caturra from Ecuador was used for this investigation.

The cell wall material (CWM) was prepared and fractionated using methods adapted from existing methods (4).

For investigation of polysaccharide changes during development, cell wall materials were prepared from coffee grain endosperm at 3 stages of development: 12, 17 and 29 weeks after flowering.

All fractions were analysed for monosaccharide composition and glycosyl linkages.

Results

Mannose is the most abundant monosaccharide in the CWM followed by galactose, glucose and arabinose. Xylose is a minor component (Table 3).

The cell wall material contains a mixture of polysaccharides so a sequential extraction procedure was applied to try and separate them.

Table 3. Yield of CWM, monosaccharide composition and total carbohydrate content of the CWM from green Arabica coffee

Yield of CWM (%w)*	Monosaccharide (%w of CWM)					Total (%w of CWM)
	Ara	Xyl	Man	Gal	Glc	
78.6	5.8	0.5	33.6	17.5	11.9	69.3

*: % dry weight of green bean

Only about 20% of the CWM was solubilised by the sequential extraction procedure, a reflection on the intractability of the coffee bean. This procedure enabled the isolation of a soluble arabinogalactan and galactomannan.

The arabinogalactan was shown to have an Arabinose/Galactose ratio close to unity and a degree of branching of 2.3. The glycosyl residues identified are similar to those reported by Bradbury et al. (Table 4).
The galactomannan had a Mannose/Galactose ratio of 25:1 and a degree of branching close to 30 which is more highly branched than the coffee mannan reported earlier (Table 4).

Table 4. Glycosyl linkages of the soluble arabinogalactan and the galactomannan

Linkage	Mole %
Arabinogalactan	
t-rha	4.7
t-araf	28.6
5-araf	17.2
t-gal	3.9
3-gal	24.5
3,6-gal	21.1
Galactomannan	
t-gal	3.8
t-man	5.4
4-man	87.8
4,6-man	3.1

While it was difficult to derive structural information about the polysaccharides remaining in the residue, successive methylations revealed the presence of an arabinogalactan and a galactomannan with structural features similar to those of the compounds which were solubilised. This shows that the architecture of the cell wall rather than the primary structure of individual polysaccharides is responsible for the insolubility of the coffee bean polysaccharides.
The total polysaccharide content of the green arabica coffee bean was shown to be 56% of which over 50% was mannan or galactomannan, 22% was arabinogalactan and 20% was cellulose.

A key to better understanding coffee bean cell wall architecture may be obtained by determining the temporal sequence of polymer synthesis during growth.
The endosperm was isolated from Arabica coffee at 3 stages of development and the cell wall material purified.

Table 5. Main monosaccharides in polysaccharides of CWM of Arabica coffee at different stages of maturation

Stage	Monosaccharide (mole %)			
	Ara	Man	Gal	Glc
12 Weeks after flowering	17.3	2.9	15.4	51.7
17 Weeks after flowering	12.7	15.1	27.1	43.2
29 Weeks after flowering	8.9	48.7	24.2	17.3

There was a very low yield of CWM from the fresh tissue in the early stages and a 10-fold increase in yield of CWM between the 2 last stages reflecting a rapid dehydration of the endosperm coupled with increased biosynthesis (data not shown).
Cellulose and arabinogalactan predominated in the early stages of growth while mannan was deposited later during bean development.

Summary
The galactomannans and arabinogalactans which were isolated in this study in coffee were a heterogeneous mixture with varying degrees of branching. They generally exhibited higher degrees of branching than what was reported earlier.
Early in endosperm development cellulose and arabinogalactan synthesis predominated and mannan synthesis increased rapidly late in development
The difficulty in extracting the component polysaccharides demonstrated that their insolubility is more related to the overall architecture of the cell wall than to the primary structure of the polysaccharides.

Résumé
Les galactomannanes et arabinogalactanes purifiées au cours de cette étude constituent un mélange hétérogène possédant des degrés de substitution variables. Les degrés de substitution obtenus sont généralement supérieurs à ce qui a été publié précédemment.
La cellulose et les arabinogalactanes sont synthétisées tôt au cours du développement de l'endosperme alors que la synthèse des mannanes prédomine vers la fin du développement.
Le fait qu'il soit si difficile d'extraire les polysaccharides pariétaux démontre que leur insolubilité soit déterminée par l'architecture de la paroi cellulaire plutôt que par leur structure intrinsèque.

Acknowledgment

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Isolation and Characterization of a Foaming Fraction from hot Water Extracts of Roasted Coffee

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1. INTRODUCTION

There is little doubt that the main visual attribute of the espresso coffee brew is the foamy layer on top of the beverage [1], well known by the Italian term "*crema*". By definition [2], espresso is "a polyphasic beverage, prepared from roast and ground coffee and water alone, constituted by a foam layer of small bubbles with a particular tiger-tail pattern, on top of an emulsion of microscopic oil droplets in an aqueous solution of sugars, acids, protein-like material and caffeine, with dispersed gas bubbles and solids".

Taste-wise, espresso foam is of little sensory interest, but the colour, the texture and the persistence of the *crema* are important in recognising quality of the espresso coffee brew. Any error in grinding or in percolating, on temperature or extraction level, is immediately denounced by the characteristics of the foam. Another useful property of espresso foam is its action as an aroma-sealing lid, helping to keep in the cup the volatile compounds responsible for the odour of espresso brew.

More generally speaking, foam formation is a process that usually imparts desirable features to a wide range of food products.

In structural terms, foams are coarse dispersions of gas bubbles in a liquid continuos phase, usually water. They can be qualitatively classified as bubbly foams (e.g. ice-cream), in which the amount of gas incorporated is low enough for bubbles to retain their roughly spherical shape, or as polyhedral foams (e.g. beer froth), in which the gas-to-liquid ratio is so large that bubbles are pressed against one another in a honeycomb-type structure. In kinetic terms, one can notice a continuous range of stabilities: from unstable transient foams (e.g. champagne bubbles), whose lifetime is measured in seconds, to meta-stable permanent foams (e.g. *mousse au chocolat*), whose lifetime is to be measured in days [3].

To describe the foaming behaviour of different systems, it is convenient to make a distinction between foamability (i.e. the ease and extent of foam formation), on the one hand, and foam stability (i.e. the rate of loss of foam structure once formed), on the other. The presence of a surfactant - namely a chemical that adsorbs onto the air-water interface, thereby lowering the surface tension - is essential for making and stabilising a foam. However, this lowering is the prerequisite for foam formation, but not the cause of enhanced foam stability.

The vast majority of food foams are formed and stabilised by an adsorbed layer of macromolecules at the air-water interface [4].

Of the two main types of food macromolecules, polysaccharides and proteins, it is the proteins that dominate in most food foams. The dominance of proteins no doubt reflects their greater hydrophobicity as compared with polysaccharides, and in turn this reflects the wide spectrum in the chemical nature of the side-chains of the constituent aminoacids [5]. Moreover, any chemical or enzymatic modification that determines an increased adsorption of the protein at the air-water interface results in an increased foaming behaviour. The modifications include thermal denaturation, glycosylation, covalently linked substituents, etc. [6].

Due to their predominantly hydrophilic character, most polysaccharides have low surface activity at air-water interface, and are therefore not expected to form adsorbed layers in food colloids also containing proteins and low molecular-weight surfactants. Nevertheless, polysaccharides generally confer colloid stability through their thickening behaviour, and/or through interaction with adsorbed proteins to form protein-polysaccharide complexes [7].

The strongest type of protein-polysaccharide interaction is where there is a covalent linkage of protein and carbohydrate moieties to form a single hybrid amphiphilic macromolecule. *In situ* bond formation in food may occur, for instance, by Maillard reaction between ε-amino groups and reducing groups on polysaccharides. Most dairy foams (e.g. whipped cream) are also emulsions, and their structures are stabilised to a large extent by a matrix of partially aggregated fat globules at the air-serum interface [8].

However, it is common knowledge that the presence of fatty particles, from which surface-active material may spread at the air-water interface, has a destabilising effect on aqueous foams. One typical example of this effect is the poor foaming behaviour of whole milk with respect to skim milk [9].

Beer froth is no doubt one of the most investigated food liquid foams. The presence of a stable head of foam is a major factor in assessing beer quality. Beer foams are due to, and stabilised by, the presence of proteinaceous materials; these are derived from proteins that have undergone extensive proteolysis and heat denaturation during the brewing process [10]. It has been suggested that many high molecular weight proteins isolated from beer were surface active, and that these proteins were also glycosylated [11, 12]. Foam-active protein-polysaccharides complexes have been isolated from beer and characterised [13, 14]. Also evidence has been presented that a beer protein was glycosylated by the Maillard reaction [15]. A high molecular weight surface-active fraction with the characteristics of a melanoidin, was isolated from beer [16]. Foam stability was demonstrated to be due to molecular interactions between at least 12 polypeptides (and possibly other components such as carbohydrates) [17]. Hydrophobic proteins [18] and hydrophobic polypeptides [19] have been also recognised as key contributors to beer foam stability. In the latter study, the several fractions isolated by Hydrophobic Interaction Chromatography contained melanoidin-type molecules.

In spite of the important role played by foam in the espresso coffee brew, the chemical nature of surface-active agents has not so far been clarified. Little is known about the presence in roasted coffee of natural surfactants allowing its formation and stabilisation.

In comparison with the surface tension value of water-air interfaces (73 mN/m at 20°C), the much lower values found in espresso coffee (46 mN/m at 20°C for a pure Arabica blend espresso with 52.5 g/l total solids) are deemed to be necessarily due to the presence of some surface-active agents. It has been suggested that several classes of complex molecules, like glycolipids or glycoproteins, might be involved in the formation and/or stabilisation of the foam [20]. These surfactants may contribute to promote and/or to stabilise both foam and emulsion in the espresso coffee.

The proteins originally present in green coffee beans are strongly degraded, denaturated and chemically modified on roasting by pyrolitic reactions. Moreover, almost half of the green coffee bean (on a dry-weight basis) is made of polysaccharides, and the roasting process strongly alters and degrades the carbohydrate polymers inducing depolymerization, structural modifications and formation of condensation complexes with proteins, protein fragments and other breakdown products, along with the browning reactions giving melanoidins.

In a recent work, foamability and foam stability of espresso coffee have been determined as a function of the degree of roast, along with other dependent variables like total solids, pH, fat, protein and carbohydrate contents [21]. The principal component analysis indicated a correlation between foamability and protein content, and between foam stability and the fractions containing high molecular weight polysaccharides (or, better, complexes between polysaccharide, protein and phenolic compounds caused by the roasting process, probably products of Maillard reactions) [22].

Although no surface-active fraction has been isolated from espresso coffee, those results strongly suggest a possible similarity between beer and espresso coffee foams, as far as the chemical nature of the surface-active compounds is concerned.

According to the working hypothesis of similarities between beer and espresso coffee foams, we based our original method to isolate possible foaming fractions from hot water extracts of defatted ground dark roasted *Coffea arabica* blend on the procedures normally used to isolate foam-active high molecular weight material in beer.

After reviewing several techniques of isolation (gel permeation, ion exchange and affinity chromatography, isoelectric focusing), the classical precipitation was found to be most useful for a rapid isolation of possible foaming fractions. By following this approach high molecular weight material was precipitated from hot water extracts by saturation with ammonium sulfate.

The present study was largely stimulated by the isolation of a heavily foaming fraction, which has been characterised, and further sub-fractionated by means of isopropanol precipitation.

One sub-fraction, elsewhere described by our group [23], was found to be composed of about 80% (on molar basis) of mannan, containing small amounts of galactose and arabinose, and of about 20% of arabinogalactan. It is not yet clear if the two polysaccharides are the individual component of a physical mixture, or if they are associated to form a complex assembly. Foaming behaviour and surface activity of this sub-fraction have not been previously reported, and will be described herein after.

The other sub-fraction is way more effective in foam formation. Its chemical nature has been elucidated by several experiments and could be ascribed to the melanoidin-type compounds group.

In this article we present the characterisation of fractions and sub-fractions of hot water extracts prepared from defatted dark roasted coffee powder of a commercial blend, along with data deriving from the same treatment applied to coffees of different species and geographical origins.

2. RESULTS AND DISCUSSION

Isolation and characterisation of foaming fractions (**FFractions**)

The addition of ammonium sulfate to saturation of the hot water extract of defatted roasted coffee powder led to the formation of precipitate. The freeze-dried product, from now on referred to as ***FFraction Illy***, obtained after exhaustive dialysis of the precipitate was analysed as follows:

- elemental analysis C: 46.0%; H: 5.6%; N: 2.3%
- yield of defatted roasted coffee 5.2% w/w
- total carbohydrate 86.5%
- proteinaceous material 14.4%
- weight-average molecular weight <Mw> 26500

The FTIR spectrum is characterised by the following bands:

3400 cm^{-1}	(OH and NH stretching)
2900 cm^{-1}	(CH stretching)
1655 cm^{-1}	(Amide I band)
1524 cm^{-1}	(Amide II band)
1000-1150 cm^{-1}	(C-O-C stretching)
894 cm^{-1} and 876 cm^{-1}	(carbohydrate anomeric bands).

Moreover, ***FFraction Illy*** is characterised by certain proton signals in the ^{1}H-NMR spectrum, namely: carbohydrate proton signals (3-5 ppm), aliphatic proton signals (0.5 - 2.5 ppm), aromatic proton signals (around 7.0 ppm).

This material redissolved in water heavily foams on shaking: a foam volume of 110% has been measured. The surface tension of a 0.4% w/vol ***FFraction Illy*** aqueous solution was 52.1 mN/m.

Extraction and isolation of a foaming fraction has been carried out also on two different lots of unblended commercial Santos coffee (Brazilian *Coffea arabica*) obtaining ***FFractions SF*** and ***SV***, and on two unblended commercial *Coffea canephora var. robusta* of different origins (Uganda and Giava) obtaining ***FFractions UF*** and ***GV***.

Quite homogeneous results have been obtained on these fractions: in particular yield, elemental analysis, <Mw>, foam volume and surface tension of a 0.4% w/vol aqueous solution, reported in Table 1, suggest no evident correlation with coffee origin.

Fraction	yield [%w]	C [%]	H [%]	N [%]	<Mw>	foam volume [%v]	surface tension [mN/m]
SF	5.0	47.6	5.9	2.9	17930	116	52.1
SV	4.3	47.4	5.9	3.4	19420	122	50.7
UF	4.8	49.9	5.8	3.9	14420	116	52.9
GV	3.9	48.6	6.0	3.5	17040	110	51.5

Table 1. Chemical and chemico-physical data of unblended coffee fractions: Arabica (SF and SV: Brazil) and Robusta (UF:Uganda and GV: Giava)

Also FT-IR and ^{1}H-NMR spectra of the four fractions are almost identical to those of ***FFraction Illy***. A couple of FT-IR spectra (an Arabica and a Robusta) are shown in the following Fig.1:

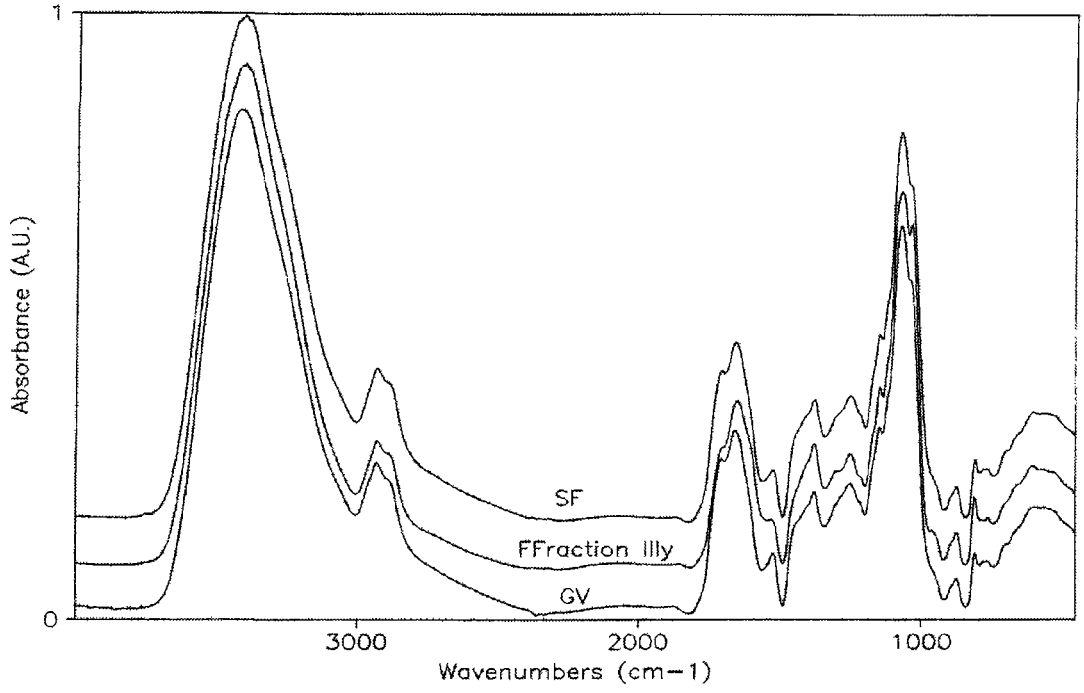

Fig.1

FFraction Illy has been further sub-fractionated by means of isopropanol precipitation, with 61.1% w/w of the total recovered products represented by ***FFraction A*** and the complement by ***FFraction B***.

Sub-fractionation has been carried out also on ***FFractions SF*** and ***SV***, leading to ***FFractions SFA*** (58.9% w/w of the total recovered products) and ***SVA*** (59.2% w/w), as well as to the complementary ***FFractions SFB*** and ***SVB***.

Polysaccharidic fractions (**FFraction A, SFA** and **SVA**)

FFraction A (elemental analysis C: 41.5%; H: 5.4%; N: 1.1%) is the high molecular weight component (<Mw> 34000) of the isolated foaming ***FFraction Illy***.

FFraction A has been the subject of our earlier structural investigation [23], where it was found to be polysaccharide material composed of about 80% mol of mannan (containing small amounts of galactose and arabinose) and of about 20% of arabinogalactan. ***FFraction A*** aqueous solutions do foam, on shaking, remarkably less than the starting material: a foam volume of 50% has been measured. The surface tension of a 0.4% w/vol ***FFraction A*** aqueous solution was 60.0 mN/m.

FFractions SFA and ***SVA*** are characterised by <Mw> values of 43390 and 46760, respectively. The ^{13}C-NMR spectra of the two fractions show an almost identical polysaccharide pattern, both in excellent agreement with that of ***FFraction A***. The chemical similarity of the three fractions may also be evinced by comparison of their FT-IR spectra, which are reported in the following Fig.2:

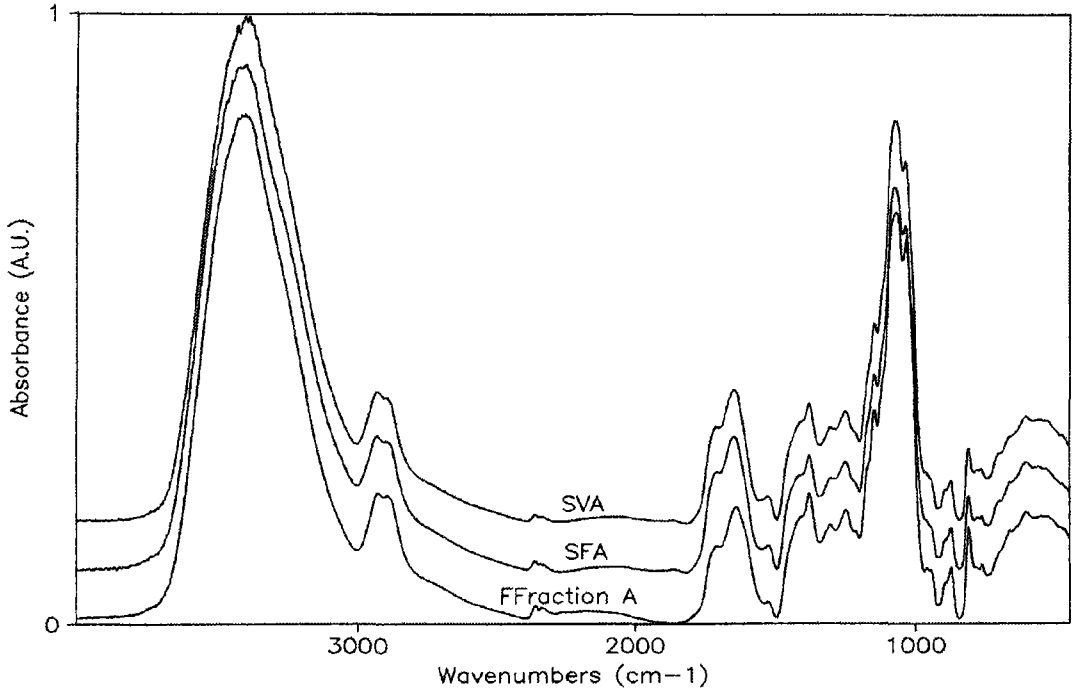

Fig.2

Foaming properties and surface activity of ***FFractions SFA*** and ***SVA*** are close to that of ***FFraction A***, being their foam volumes about 50%, and the surface tension of 0.4% w/vol of their aqueous solutions equal to 60.4 and 58.0 mN/m, respectively. The moderately low surface activity of these fractions is expected, on the basis of their polysaccharide chemical nature.

Proteinaceous fractions (**FFraction B, SFB** and **SVB**)

FFraction B (elemental analysis C: 46.7%; H: 5.3%; N: 4.3%) is the low molecular weight foam-positive component of the isolated foaming ***FFraction Illy*** (<Mw> 17000). Total carbohydrate content of 36.5% and proteinaceous material content (calculated as N * 6.25) of 26.9% were found.

When a more precise determination of the protein content was attempted, remarkable differences were observed in dependence of the method used for assaying protein. In particular the BCA method gave 3-4 times higher content than Bradford method, which gave 11.9% w/w content. The Bradford method involves measuring the extent to which the dye Coomassie Blue binds to a protein: the greater the degree of binding, the more protein is present. The BCA method, based on the measurement of reducing power, will register not only protein but also other types of molecule, such as melanoidins [19].

FFraction B contains 2,4-dinitrophenylhydrazine-positive substances as evidenced by abundant precipitate formation, denoting carbonyl groups.

UV absorption of ***FFraction B*** has a maximum at 278 nm with a shoulder centered around 330 nm. Tailing of this shoulder into the visible wavelenghts is responsible for the brown color of the material. Fluorescence spectra are characterised by excitation and emission maxima at 361 nm and 440 nm respectively. The UV-visible and fluorescent spectra of this fraction were similar to the spectroscopic properties of melanoidins and non-enzymatically glycosilated proteins [16, 24, 25].

The FTIR spectrum of ***FFraction B*** is characterised by the presence of bands (Amide I and II) which can be attributed to proteinaceous material, moreover some bands due to carbohydrates are also present.

The ^{1}H-NMR spectrum confirms the presence of carbohydrate moieties (proton signals in the range 3-5 ppm) and shows that the aliphatic proton signals (0.5 - 2.5 ppm) and the aromatic proton signals (around 7.0 ppm) are remarkably more intense than those in the starting ***FFraction Illy***. This feature indicates a more hydrophobic character for this fraction in comparison with that of the starting material. As far as the carbohydrate component is concerned, it was found to be constituted by arabinose, galactose and mannose in a molar ratio 1.9:3.3:1, respectively.

The whole set of experimental data strongly suggests that the chemical nature of ***FFraction B*** is of melanoidin type. This conclusion is based on indirect spectroscopic evidences. Direct chemical evidence of the composition or structure of any melanoidin is difficult to obtain [16]. What's more, melanoidins are usually a heterogeneous class of polymers, and preliminary findings indicate that this is the case for ***FFraction B.***

The foaming properties of ***FFraction B*** are close to those of ***FFraction Illy,*** but the surface tension is lower. Foam volume of 100% and surface tension of a 0.4% w/vol aqueous solution as low as 46.5 mN/m have been measured.

FFractions SFB and ***SVB*** are characterised by <Mw> values of 10140 and 8340, respectively. The FT-IR spectra of the two fractions reported in the following Fig.3 are almost identical and both similar to those of ***FFraction B***. The same may be said for the relevant ^{1}H-NMR spectra.

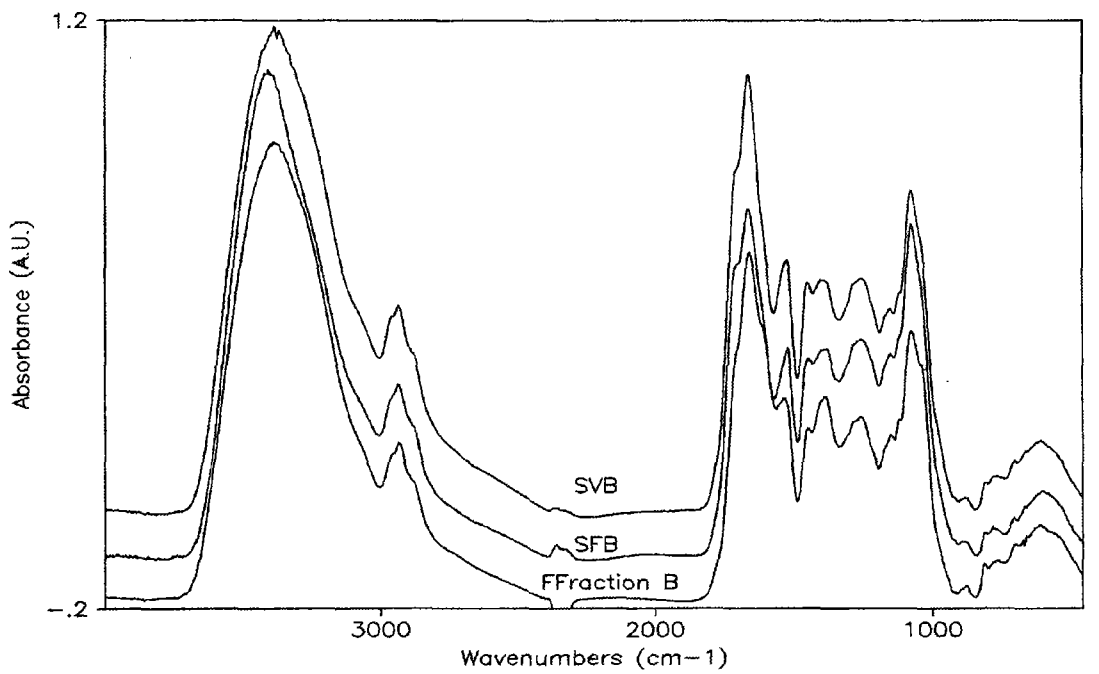

Fig.3

Foam volumes close to that of ***FFraction B*** have been measured. The surface tension of 0.4% w/vol aqueous solutions was found to be 45.4 and 47.3 mN/m for ***FFractions SFB*** and ***SVB***, respectively.

3. EXPERIMENTAL

MATERIALS

Extraction and isolation of a foaming fraction

Ground dark roasted *Coffea arabica* blend for espresso brewing technique, ICS grade produced by illycaffè company was used. 25 g of coffee powder were heated under reflux with n-hexane for 16 h within a Soxhlet apparatus. The defatted dried powder was extracted twice for 1 h with 200 ml Milli-Q water at 90°C. The solid residue was filtered off, ammonium sulfate was added to give a saturated solution and the mixture was maintained at 4°C overnight. The resulting precipitate was collected by centrifugation, dissolved in water and extensively dialyzed against (in this order): Milli-Q water, aqueous NaCl 1M, and Milli-Q water again. The non-dialyzable solution, after filtration, was freeze-dried to give a fluffy dark brown material (1.085 g recovered): ***FFraction Illy.*** This material heavily foams on shaking in aqueous solution.

Sub-fractionation

1 g of ***FFraction Illy*** was redissolved in 50 ml Milli-Q water, the solution was added to 2.5 volumes of isopropanol and the mixture was maintained at 4°C overnight. The resulting precipitate was filtered, dissolved in Milli-Q water and the solution dried under reduced pressure in a Rotavapor. The solid material was redissolved in 200 ml water and freeze-dried to give a brown fluffy material (560 mg recovered): ***FFraction A.*** The supernatant was dried under reduced pressure in a Rotavapor, dissolved in Milli-Q water and freeze-dried to give 356 mg of brown fluffy material: ***FFraction B.*** The latter in aqueous solution gives abundant foam on shaking.

Extraction and isolation of a foaming fraction has been carried out also on two different lots of unblended commercial Santos coffee (Brazilian *Coffea arabica*) obtaining ***FFractions SF*** and ***SV,*** and on two unblended commercial *Coffea canephora var. robusta* of different origins (Uganda and Giava) obtaining ***FFractions UF*** and ***GV.*** Subfractionation has been carried out on ***FFractions SF*** and ***SV,*** leading to ***FFractions SFA, SFB, SVA*** and ***SVB.***

METHODS

Protein content was estimated on the basis of elemental analysis (%N x 6.25, non corrected for caffeine) and was determined by the bicinchoninic acid (BCA) method as well as by the Bradford method. Bovine serum albumin was used as a standard protein in both cases.

Total carbohydrate content was determined by phenol-sulfuric acid colorimetric method [26]. Sugar composition was seen by gas chromatography of the alditol acetate derivatives of the hydrolysed samples (TFA 2 N, 100°C, 16 h). A Hewlett-Packard model 5890A equipped with a flame ionisation detector was used. The column was a 30 m x 0.25 mm SP-2330 Fused Silica (Supelco) with a temperature scan rate of 4°C/min in the range 150 to 250°C.

UV-vis absorption spectra were recorded by using a Varian Cary 3E UV-vis spectrophotometer. Fluorescence spectra were taken by using a Perkin-Elmer LS 30 luminescence spectrometer. FTIR spectra were recorded using a Perkin-Elmer 1750 spectrometer interfaced with a Perkin-Elmer 7300 data processor (128 scans at 2 cm^{-1} resolution). Pellets were prepared using 200 mg of IR spectroscopic grade KBr and 2 mg of freeze dried samples previously maintained for 24 h in a dessiccator under low pressure.

NMR spectra were recorded at 4.70 Tesla (^1H, 200.13 MHz and ^{13}C, 50.32 MHz) with a Bruker AC 200 instrument equipped with a multinuclear 5 mm probe. 10 to 15 mg of any sample were dissolved with 0.5 ml of D_2O directly inside the NMR tube and the analysis were performed at 60°C.

The weight-average molecular weight (<Mw>) was determined by high performance size exclusion chromatography (HP-SEC) using G6000, G5000 and G3000 TSK PWx1 columns (TosoHaas) in series, with a differential refractometer (Waters 410) in line. The samples were eluted with 0.15 M NaCl (0.80 ml/min). Calibration was performed by using pullulan standards (Polymer Lab.) in the <Mw> range 5.800-853.000.

Surface tension measurements were obtained using a De Nouy tensiometer (Kruss) thermostated at 25°C (Milli-Q water = 71.3 mN/m).

As far as the foaming properties are concerned, of the three general method of foam formation (bubbling, whipping or vibrating, and shaking), the one of shaking samples in a plugged graduated glass cylinder was found to be most useful for a rapid, quantitative method requiring only small volumes. The foam was characterised in terms of the volume of foam formed, expressed as a percentage of the volume of the liquid. These shaking experiments were performed using 20 ml of 0.4% w/vol fraction aqueous solutions, introduced in a plugged 50 ml glass cylinder. The cylinder was wrist shaken for 10 s and after 5 min at rest both total and liquid volumes were recorded.

4. CONCLUSIONS

Extraction and isolation of a high molecular weight foaming fraction from hot water extract of defatted dark roasted coffee powder has been described. Sub-fractionation gave two materials with different foaming properties and surface activity. One sub-fraction (about 60%) is constituted by polysaccharides already structurally characterised [23] and is poorly surface active, as expected. The other one (about 40%) is characterised by a lower molecular weight, is of melanoidin-type and is more surface active.

It is interesting to point out that the foam volume of the polysaccharide sub-fraction shows a very small decrease after 24 h from shaking, whereas that of the melanoidin-type one decreases about 50%. This observation suggests, in substantial agreement with the correlations found by Nunes et al. [21], that coffee foamability is mainly influenced by the melanoidin-type sub-fraction whereas foam stability by the other one.

No remarkable relationship has been noticed so far between chemical characteristics of the foaming fractions and coffee species or origin.

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5. SUMMARIES

A foaming fraction was isolated from hot water (90°C) extracts of defatted ground dark roasted coffee samples of different origin by means of ammonium sulfate precipitation. The freeze-dried product obtained after exhaustive dialysis against water was found to be a brown nitrogen- and carbohydrate-containing polymeric material able to give stable and abundant foam on shaking once redissolved in water, and to decrease significantly the surface activity of water.

Further fractionation by isopropanol precipitation afforded two different sub-fractions. The one obtained in higher yield (about 60%) is a polymeric material that foams very little on shaking once redissolved in water: this sub-fraction was found to be composed of about 80% of mannan (containing small amounts of galactose and arabinose) and of about 20% of arabinogalactan, perhaps associated to form a complex assembly. The other sub-fraction is characterised by lower molecular weight, and is much more active in foaming and in surface activity. The experimental data suggest that its chemical nature is of melanoidin-type.

The two sub-fractions seem to play a different role in the foaming properties. In particular foamability seems to be related to the melanoidin-type sub-fraction, whereas foam stability relates to the polysaccharide one.

A partir des extraits à l'eau chaude (90°C) de cafés d'origines variées, fortement torréfiés, moulus et déshuilés, on a isolé à l'aide d'une précipitation par sulfate d'ammonium une fraction moussante. Ce produit, obtenu après dialyse poussée avec eau et lyophilisation, a été caractérisé comme du matériel polymère contenant de la matière azotée et des carbohydrates. Ce matériel forme une mousse abondante et stable quand dissous en eau et agité, et il diminue significativement la tension superficielle de la solution.

En le fractionnant ultérieurement par précipitation a l'isopropanol, on obtient deux fractions bien différentes: la plus abondante (rendement 60%) c'est un matériel polymère qui forme a peine de la mousse quand agité. Il est composé surtout de mannane (80% environ, avec des faibles teneurs en galactose et arabinose) et du arabinogalactane, peut-être associés à former un ensemble complexe. L'autre fraction exhibe un poids moléculaire plus faible, et une activité tensioactive et moussante beaucoup plus élevée: on pourrait l'attribuer au groupe des melanoïdines.

Ces deux fractions semblent jouer des rôles distincts en ce qui concerne la mousse du café: en particulier l'aptitude à former de la mousse parait dériver de la fraction melanoïdinique, alors que la fraction polysaccharidique facilite sa stabilité.

Effects of Saliva and Milk Additives on the Coffee Flavour Release in the Oral Cavity

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Introduction

The analysis of the aroma substances contributing to coffee flavour has shown that about 30 volatile compounds were substantially responsible for its flavour (1, 2, 3). Some changes might occur due to the addition of milk and/or sugar to the coffee beverage. They are added in order to develop a desirable colour change, to impart a body to the coffee beverage, to reduce bitter and sour tastes and to reduce the astringency of the coffee. Some ingredients of these additives such as lipids, proteins and carbohydrates interact with flavour impact volatiles (4). Consequently, the interactions affect the retention of these volatiles, and thereby the quality and quantity of coffee aroma in the headspace. Furthermore, flavour release is influenced by human saliva, the composition of which is individual to a human being.

Direct injection of a headspace sample onto a GC capillary column gives the most accurate composition of the volatile compounds present. However, working with static headspace (gastight syringe) was not adequate here, because only very low amounts of aroma compounds could be collected. Therefore, a new device (external dynamic sampling device) was developed and combined with GC-FID / olfactometry, and GC-MS / olfactometry. Oral vapour gas chromatography (OVGC) was considered to be a valuable method to analyse the release of volatile compounds from the beverages by human volunteers.

The major objective of this work was to investigate the influence of different milk additives, one coffee whitener and saliva on the release of flavour impact compounds from coffee beverages.

Experimental

As previosly described (5) the two economically important coffee species were used: one Arabica coffee and one Robusta coffee. Five products, UHT-milk (3.5% fat), Condensed milk (10% fat), Coffee creamer (10% fat), Whipping cream (30% fat) and Coffee whitener (vegetable, 34% fat), were selected as typical coffee additives (5).

Sample Preparations

The beans were stored at -17°C and ground directly prior to use in a coffee grinder. The brew was prepared in a coffeemaker with 12g coffee powder and 225g tap water.

Coffee brew (50g) was placed in the external dynamic sampling device (Fig. 1), with a headspace volume of 275mL, and 10g artificial saliva (water, inorganic salts, mucin, α-amylase) or human saliva were added. The temperature of the water bath was 40°C. With a flow of 40mL/min for 30 minutes, nitrogen was flushed above the coffee surface, and the volatiles were collected on Tenax TA tubes. Coefficients of variance of 8 GC-MS replications ranged between 2 and 20% for most of the peak areas of volatile compounds.

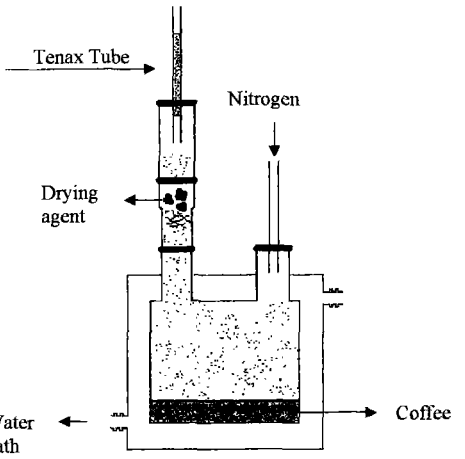

Figure 1. External Dynamic Headspace device

Oral Breath Sampler

Five assessors took 18mL of the freshly brewed beverage into their mouths, and kept it there during OVGC sampling. Figure 2 shows the Oral Breath Sampler (6).

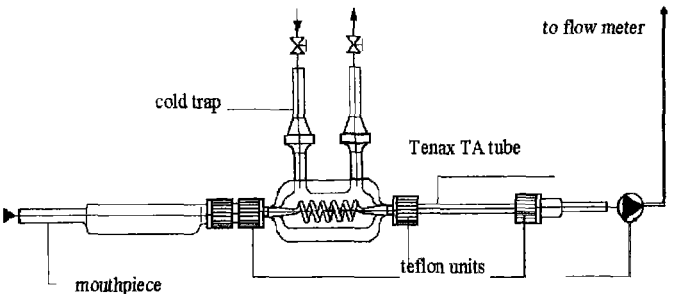

Figure 2. Oral Breath Sampler of ROOZEN and LEGGER-HUYSMAN (6)

The assessors had to place a mouthpiece between their lips in such a way that released volatile compounds were directed onto a Tenax TA tube by a vacuum pump with a constant flow (142mL/min) for 6 minutes. A cold trap was used for freezing out water vapour. Reproducibility of the method was checked by analysing the same kind of beverage in five replicates with subsequent comparison of the chromatograms. The volatile compounds were analysed on a Carlo Erba MEGA 5300 GC equipped with a FI-Detector. The volatiles were desorbed by a thermal desorption device and injected onto a Supelcowax 10 fused silica capillary column.

GC-FID, GC-MS, GC-Olfactometry and GC/O-analysis

The same instrumental equipment and the same kind of GC/O-analysis was used as described previously (5).

Results and Discussion

Oral Breath Sampler

Four characteristic impact compounds of the coffee beverage, 2-methylbutanal (2-MB), 3-methylbutanal (3-MB), 2,3-butanedione (2,3-B) and 2,3-pentanedione (2,3-P), were selected for data analysis (Figure 4).

These highly volatile compounds dominate the first aroma impression of coffee brews. Furthermore their concentration in the oral cavity enables detection and data evaluation. Control investigations with additives only revealed that except for 2,3-butanedione (whipping cream, coffee whitener), none of the additives had any relevant concentration of volatiles which occurred in the black coffee beverage. The FID chromatograms of the coffee beverages with additives showed a decrease of the four compounds, caused by the ingredients of these additives.

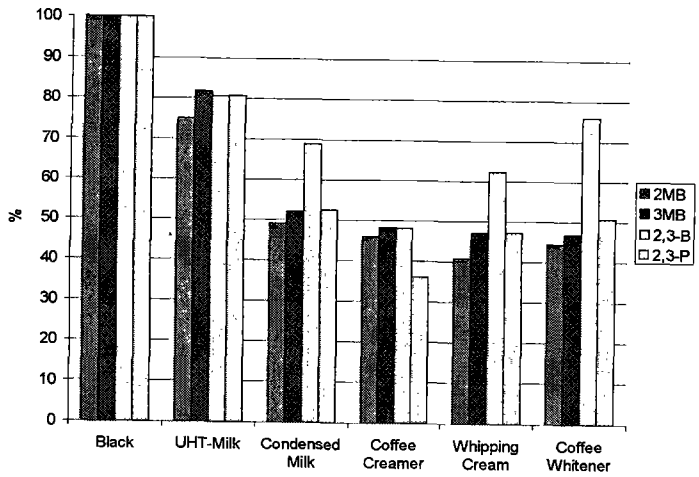

Figure 3. *Effect of additives on aroma retention in Robusta coffee beverage: combined peak areas of five panelists for five additives shown as percentage related to black coffee*

GC-FID, GC-MS and GC-Olfactometry

More than 50 potent odorants were recognized at the sniffing port. The identification of these volatiles verified mostly the contributors to the coffee aroma described in literature (1, 2, 3). The amount of volatiles in the headspace was influenced by the artificial saliva. Most of the descriptors showed a signicant decrease or increase (Figure 5).

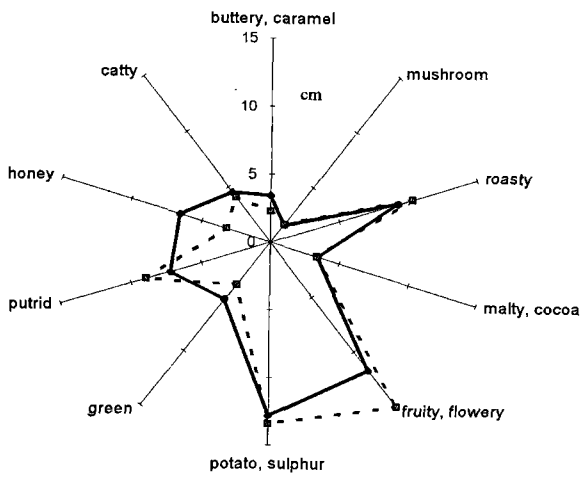

Figure 4. *Influence of artificial saliva (·····) on the intensity of GC-O descriptors used for coffee volatils (Robusta coffee ——)*

These results obtained were confirmed by GC/MS-analysis of the beverages. Figure 6 illustrates the effect of human saliva on the release of furfurylthiol, a potent odorant with a roasty aroma quality. With the saliva of the assessors the concentration of furfurylthiol in the headspace increased. This increase was typical for each panelist and was not dependent on the sex of the panelists.

The changes in the concentration of coffee volatiles with saliva could be caused by several effects. The inorganic salts of saliva could have a salting-out effect and increase the amounts of volatiles in the headspace / oral cavity. In contrast mucin, a glycoprotein, could be involved in the retention of coffee volatiles. Each human saliva is composed of different high amounts of these components. Furthermore the flow rate of saliva differs from person to person. Therefore the perception of aroma substances may be unique for each consumer.

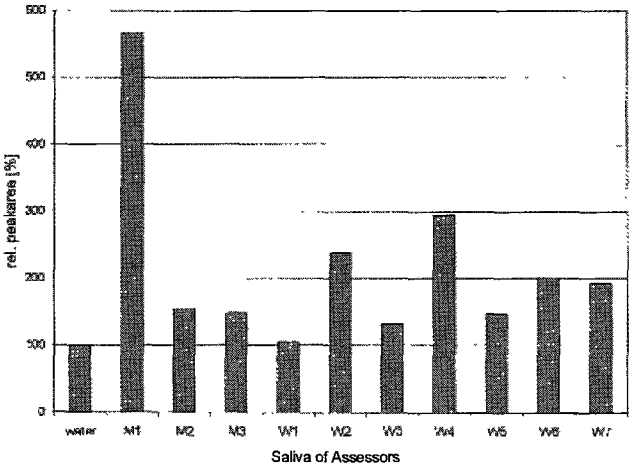

Figure 5. *Influence of human saliva (m: man / w: woman) on the release of furfurylthiol. Results are expressed relative to the peak area of the targetion (GC/MS) of black Robusta coffee*

Acknowledgements.
The technical assistance of A. Legger, J. L. Cozijnsen and K. Meier is gratefully acknowledged.
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Summary

Milk and vegetable products as an additive for coffee beverages have an effect on the release of aroma substances in the brew through their lipid, protein and carbohydrate components. Furthermore the flavour release is influenced by human saliva. For the investigation of these effects an external dynamic headspace sampling technique was developed. With this technique the most potent odorants of the coffee beverage were determined. Analyses were performed by gas chromatography/olfactometry, FI- and MS-detection. To characterize the odour profiles of the different beverages GC/O analysis was used. Artificial or human saliva changed the odour profile of the coffee beverage.
The release of volatile compounds of the beverage in the oral cavity of human volunteers was measured by oral vapour gas chromatography. These investigations showed that all beverages with a milk or vegetable additive had a reduced, but typical odour profile.

Zusammenfassung

Milchprodukte bzw. Kaffee-Weißer als Zusatz für Kaffee haben einen Einfluß auf die Freisetzung der flüchtigen Aromastoffe des Kaffeegetränkes. Dies ist auf die Inhaltsstoffe der Zusätze, wie Lipide, Kohlenhydrate und Proteine, zurückzuführen. Weiterhin wird die Freisetzung dieser Aromastoffe in der Mundhöhle durch den Speichel beeinflußt. Für die Untersuchung dieser Effekte wurde die Methode der externen dynamischen Headspace entwickelt. Mit dieser Technik konnten die wichtigsten Aromastoffe bestimmt werden. Die Detektion bei diesen gaschromatographischen Untersuchungen erfolgte mittels FI-Detektor, MS-Detektor und Sniffing-Port (GC/Olfaktometrie). Mittels der olfaktometrischen Untersuchungen konnte gezeigt werden, daß sich das Aromaprofil nach Zusatz von künstlichem oder menschlichem Speichel veränderte.
Messungen über die Freisetzung der Aromastoffe in der Mundhöhle des Menschen wurden mittels der Oral Vapour Gaschromatographie durchgeführt. Durch den Zusatz von Milchprodukten bzw. Kaffee-Weißer wurde die Freisetzung von Aromastoffen des Kaffees im Mundraum der Probanden reduziert.

Evaluation of Acidity and Bitterness of Coffee Brew

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INTRODUCTION

The perceived acidity and bitterness of coffee brew have been recognized as important attributes of coffee quality. The intensity of acidity and bitterness of coffee brew have been assessed largely by sensory tests involving a rating method or a scoring method. However, evaluated values obtained from these kinds of sensory tests are regarded as results from a nominal or an ordinal scale, and such quantitative results are sometimes considered not enough. Therefore, a higher quantitative scale is expected for use on sensory test of coffee brew.

For the purpose of improving the quantitative nature of sensory testing, gust scale (a ratio scale of taste) was used to evaluate the intensity of acidity and bitterness of coffee brew. The relationship between evaluated gust values and physico-chemical properties was also investigated.

In this paper, an evaluation method to estimate gust values for perceived acidity and bitterness of coffee brew from titratable acidity and luminosity will be described.

MATERIALS AND METHODS

Materials

For the acidity experiment, light roasted coffees of 6 trade varieties (Brazil No.2, Colombia Supremo, Java WIB-1, Tanzania AA, Toraja TLA, and Zimbabwe AA+) and 8 different roast degrees of Brazil No.2 and 5 different roast degrees of Java WIB-1 were used.

For the bitterness experiment, 5 different roast degrees of arabica (Brazil No.2) and robusta coffee (Java WIB-1) were used.

Roast and ground coffee was extracted with ion-exchanged water using a coffee brewer. The brew was cooled in an ice-water bath to room temperature. The concentration of coffee brew was adjusted to brix 1.50%.

Sensory test

The sensory test was conducted with a panel of 10 trained testers. The gust scale was used to

evaluate the intensity of coffee brew acidity and bitterness, i.e., the coffee brew was placed in front of 9 standard solutions arranged in intensity from left (low) to right (high). The tester choose the solution best matching the coffee brew for intensity of acidity or bitterness. Concentrations of standard solutions are listed in Table 1.

Table 1 Concentrations of standard solutions for gust scale

Acidity		Bitterness	
Gust value	Tartaric acid conc. (w/v%)	Gust value	Quinine sulfate conc. (w/v%)
1.0	0.00848	2.0	0.00051
1.5	0.01215	4.0	0.00137
2.0	0.01569	6.0	0.00182
2.5	0.01913	8.0	0.00254
3.0	0.02249	10.	0.00329
3.5	0.02579	14.	0.00486
4.0	0.02903	18.	0.00650
4.5	0.03223	22.	0.00820
5.0	0.03539	26.	0.00995

Measurements of physico-chemical properties

Brix was determined using a digital refractometer. Titratable acidity was measured by neutralizing 100 ml of coffee brew with N/10-NaOH. Luminosity (L-value in Lab chromaticity diagram) of coffee brew was measured using a color and color difference meter.

RESULTS AND DISCUSSION

Gust values for perceived acidity and titratable acidity

The relationship between titratable acidity and the intensity of acidity is shown in Fig. 1. Titratable acidity was positively and significantly related to the logarithm of gust value for perceived acidity (log Ga). A linear relation was observed for a correlation coefficient about 0.90. Since the acidity of dark roasted Brazil No.2 coffees and medium to dark roasted Java WIB-1 coffees could not be perceived, these data are omitted from Fig. 1.

Gust values for perceived bitterness and luminosity

As shown in Fig. 2, luminosity related well to the logarithm of gust value for perceived bitterness (log Gb) linearly. The correlation coefficient was close to 0.97.

Estimation of gust values

Gust values were estimated using the following equations.

$$Ga = 10^{0.103T-0.380}, \qquad Gb = 10^{-0.032L+2.142}$$

Ga: gust value for acidity — Gb: gust value for bitterness

T: titratable acidity (ml) — L: luminosity (L-value)

The gust scale is based on ratio judgements, so the equality of ratio is guaranteed. Therefore, the impression of ratio for the intensity of taste between 2 coffee brews α and β is given by $G\alpha/G\beta$.($G\alpha$:gust value for coffee brew α; $G\beta$:gust value for coffee brew β)

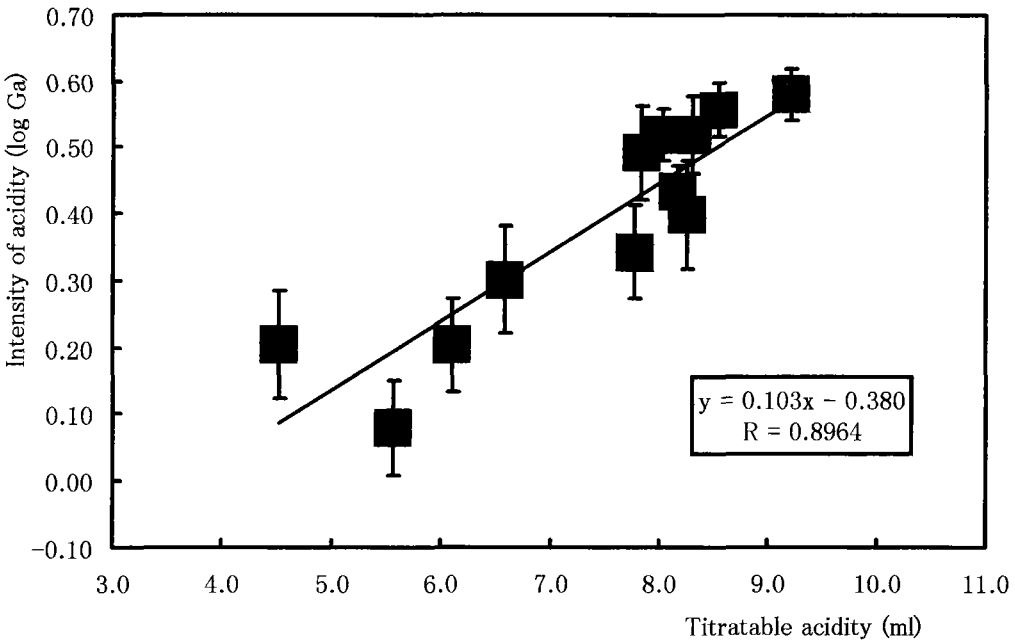

Fig.1 Relationship between titratable acidity and intensity of acidity

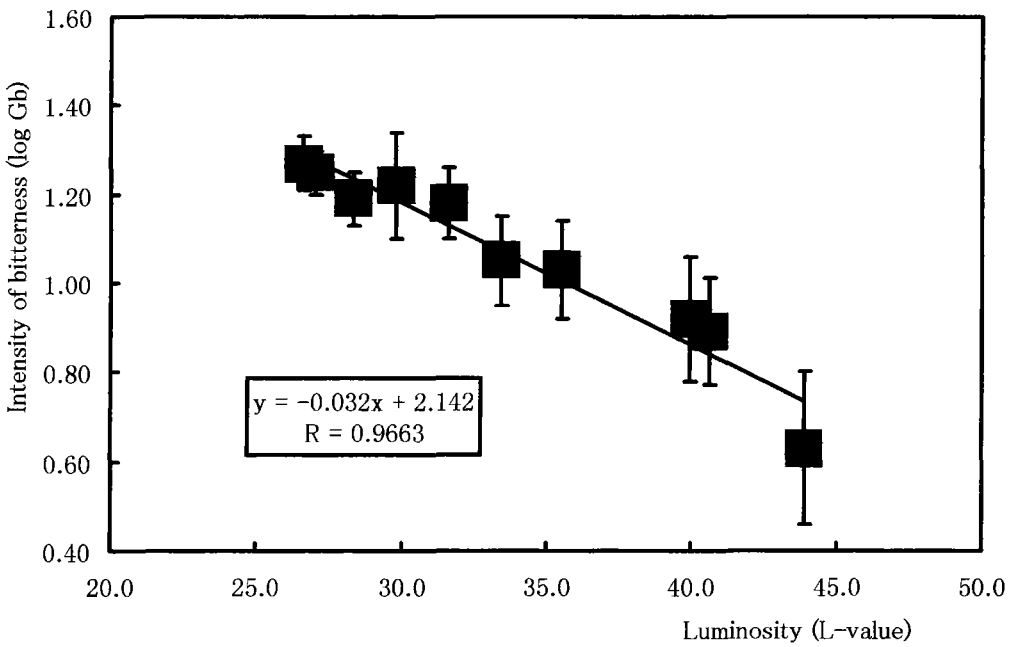

Fig.2 Relationship between luminosity and intensity of bitterness

Application

Estimating gust values from physico-chemical properties is applied to the quality control and the development of coffee products, a study of market trends, and so on. As an example, the intensity of taste of 24 different roast coffees, 5 domestic and 19 foreign, is compared in Fig. 3.

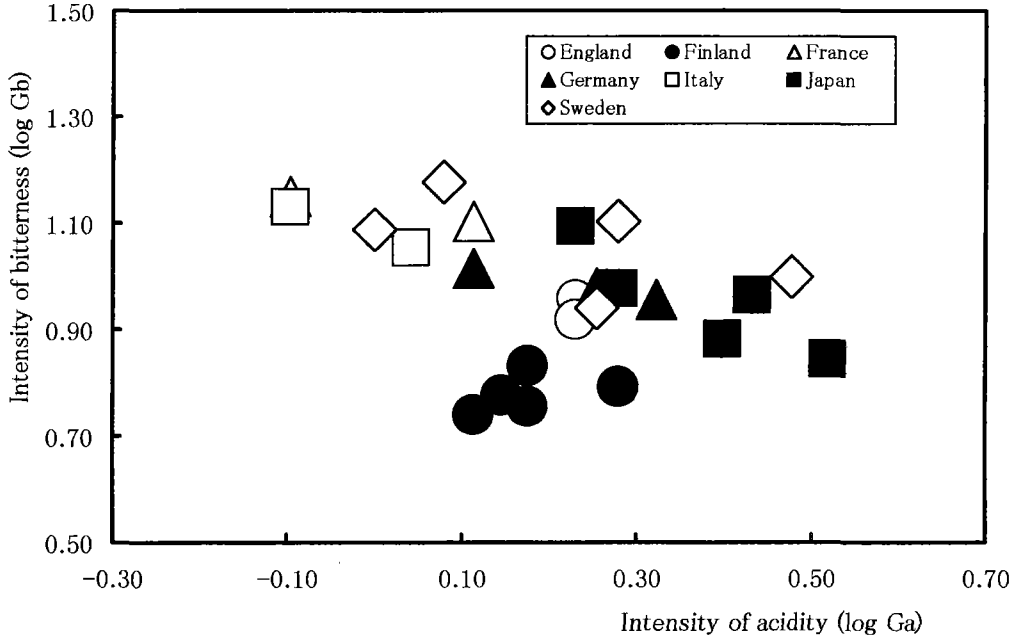

Fig.3 Comparison of intensity of taste of coffee products

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SUMMARY

Using the gust scale on sensory tests, we evaluated the intensity of acidity and bitterness of coffee brew given quantitative values with an equi-ratio property. The relationship between titratable acidity and the logarithm of gust value for acidity, luminosity and the logarithm of gust value for bitterness were linear with high correlation. According to experimental equations, gust values were estimated from physico-chemical properties.

RÉSUMÉ

Nous avons effectué des essais sensoriels pour évaluer le degré d'acidité et d'amertume du café préparé. Pour cela, nous avons préparé une échelle de goût avec des valeurs quantitatives basées sur des coefficients égaux. Nous avons pu constater qu'il existait un rapport linéaire avec forte corrélation entre l'acidité titrable et le logarithme des valeurs gustatives de l'acidité et de la luminosité, et le logarithme des valeurs gustatives de l'amertume. Les équations qui ressortent des essais montrent qu'il est possible de donner des valeurs gustatives à l'acidité et à l'amertume du café à partir des propriétés physico-chimiques.

A new Method for the Determination of 16-o-Methylcafestol in Roasted Coffee

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Because of its stability during the roasting process, the diterpene 16-O-methylcafestol (16-OMC) is the ideal indicator for safely detecting the addition of Robusta to Arabica coffee [1,2].
Its determination is carried out using the DIN method No. 10779, which includes several analysis steps (see Figure 1, left side) [3].
Although reliable, the method is very time-consuming especially the extraction in a separatory funnel with multiple extraction steps, standing overnight for phase separation due to persistent emulsions, followed by washing, and drying. Therefore, some efforts have been made in order to simplify the method and minimize analysis time [4].

In our research team, an extraction column filled with Extrelut® from Merck has successfully been applied for clean-up to determine pesticides in blood [5]. The principle is very easy: The aequous layer and all watersoluble components are retained within the material, whereas lipophilic substances can be eluated with organic solvents.
Therefore, it seemed to be obvious to replace the time-consuming liquid/liquid extraction step by using this short extraction column (see Figure 1, right side).
Having changed the organic eluent it was now possible to analyse 16-OMC in roasted coffees in half of the time (Figure 2).

In order to compare the new method with the DIN method with respect to correctness and reproducibility, we analysed several commercial coffee samples and mixtures of an Arabica coffee with various Robusta proportions using both methods, and found similar contents. For example the reproducibility for an Arabica coffee with a 10 % Robusta addition is shown in Table 1.
In comparison to the DIN method, the detection limit for the new method is about two times higher under the chosen conditions (25 mg/kg coffee) and in addition, there are sometimes problems with a non-uniform chromatographic baseline. On the other side the advantages are time savings, solvent savings, and a proper performance due to a total lack of emulsions.

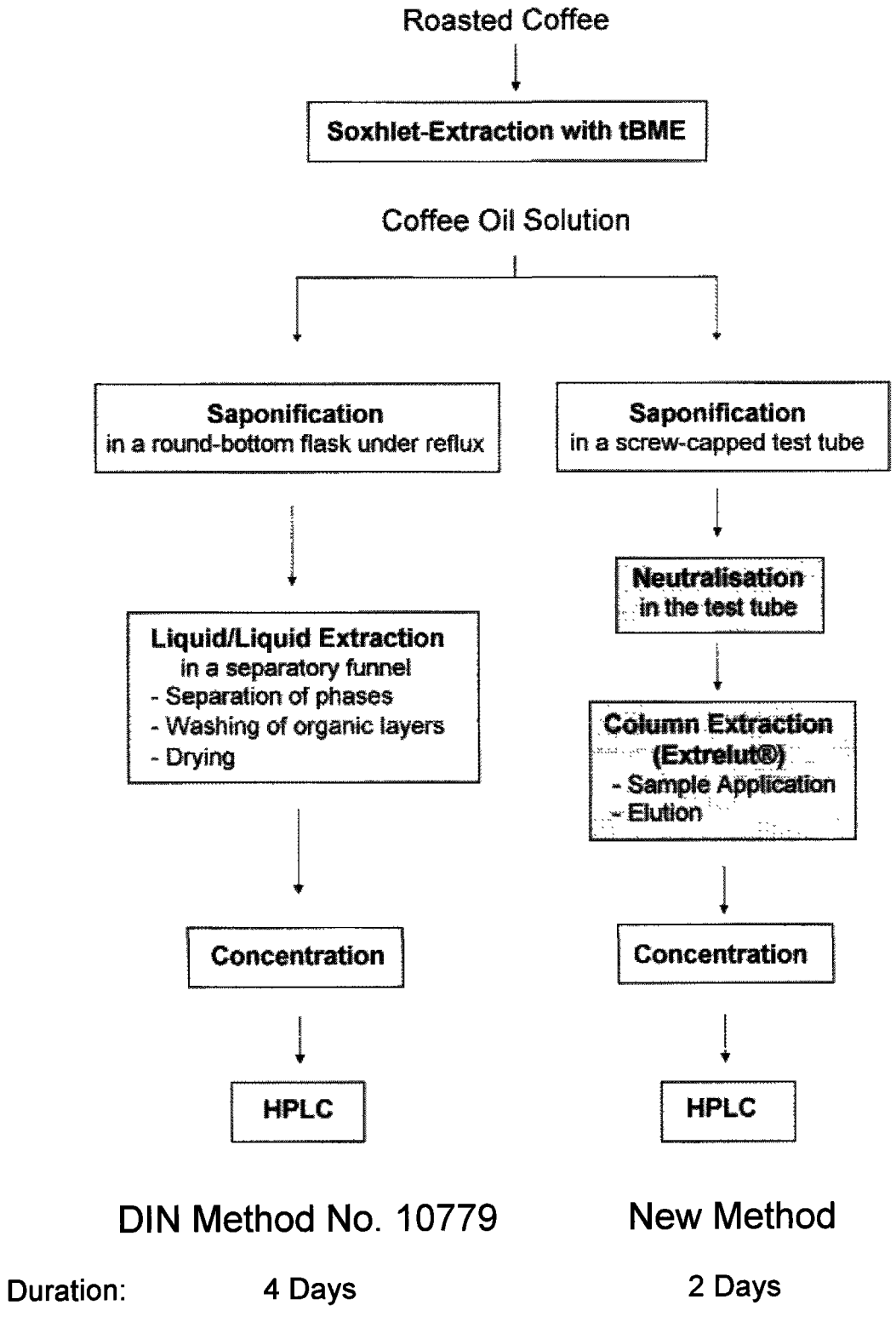

Figure 1. Analysis scheme for the determination of 16-OMC

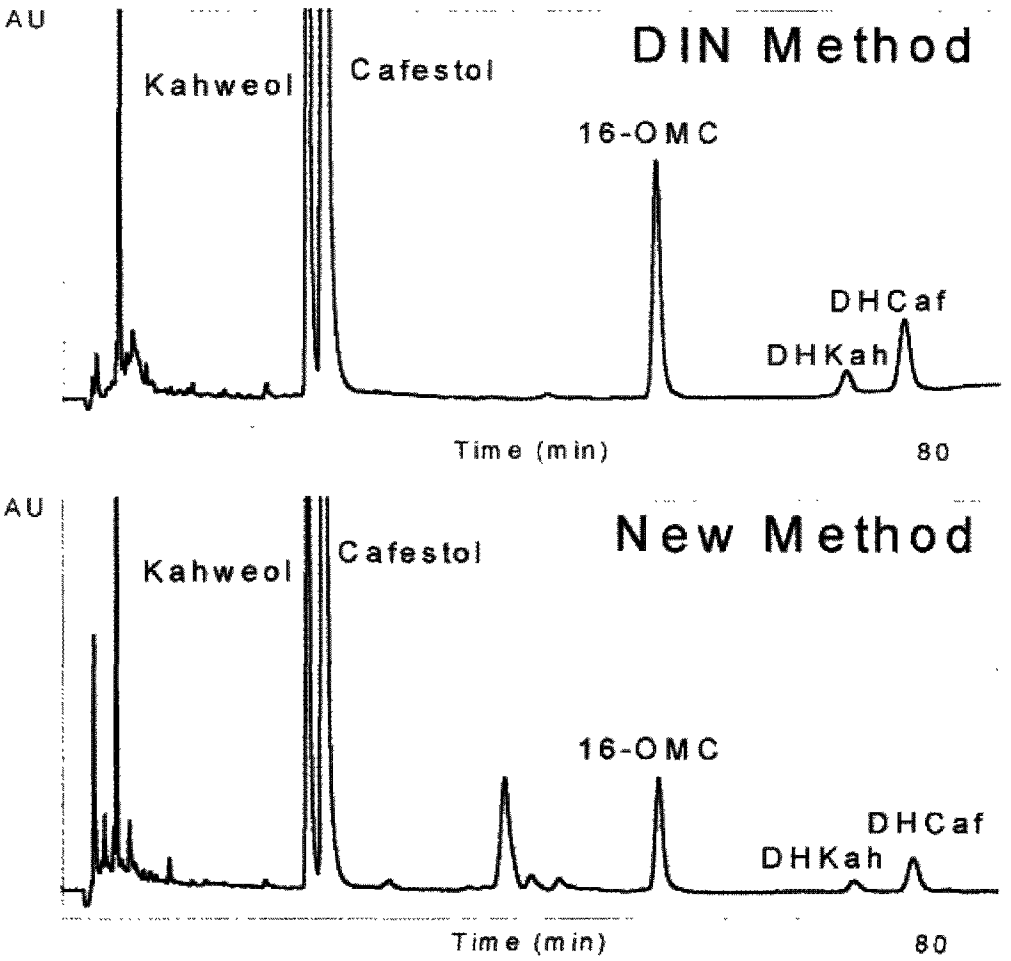

Figure 2. HPLC chromatograms of a commercial coffee sample with 640 mg 16-OMC per kg; DHCaf: Dehydrocafestol, DHKah: Dehydrokahweol

Table 1. Results of a five-fold determination of an Arabica coffee with 10% Robusta (16-OMC content obtained with DIN method: 147 mg/kg)
cv: coefficient of variation

Sample	16-OMC [mg/kg]	Reproducibility [%]
1	149	101.3
2	151	102.7
3	147	100.0
4	148	100.7
5	150	102.0
mean value	149	-
cv [%]	1.06	-

Summary
A new method is presented which permits the determination of 16-O-methylcafestol in roasted coffee in half of the time required for the DIN method No. 10779. This was achieved by replacing the time-consuming liquid/liquid extraction step of the DIN method with a clean-up using an Extrelut® column. Comparing the new method with the DIN through the analysis of several coffee samples, similar data with high reproducibility were obtained.

Zusammenfassung
Es wird eine Methode vorgestellt, die die Bestimmung von 16-O-Methylcafestol in Röstkaffee in der Hälfte der Zeit im Vergleich zur bisherigen DIN Methode Nr. 10779 gestattet. Dies wurde erreicht durch Austausch des zeitaufwendigen Analysenschrittes der Flüssig-Flüssig-Extraktion im Scheidetrichter durch Clean-up an einer Extrelut®säule. Die Analyse verschiedener Kaffeeproben nach der DIN-Methode und nach der neuen Methode führte zu gleichen Gehalten mit hoher Reproduzierbarkeit.

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Determination of the Relationship between Phosphate Concentration and perceived Acidity in Coffee

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INTRODUCTION

In 1997 Kenya Kagumo coffee was purchased in Nairobi at a record high price due to its pronounced acidity.[1] After initial analysis by Michelson Laboratories using ion exclusion chromatography, it was concluded that the phosphoric acid concentration was significantly higher in the Kenya Kagumo sample than in other Kenyan coffees.[2] It was hypothesized that the increase in hydrogen ion concentration from the dissociation of phosphoric acid governs the perceived acidity of coffee.[3]

Currently, two positions are held on the role of phosphoric acid in coffee. One explanation is that phosphoric acid is present in coffee, but is neutralized by excess potassium in solution and therefore makes no direct contribution to the perceived beverage acidity.[4] Another explanation is that phosphoric acid is present in coffee and strongly influences flavor as a result of the low pK_a of phosphoric acid.[3,5] No studies have reported a precise comparison of the phosphate content of coffees of different origins.

Given the uncertainty regarding phosphate levels in coffee and the influence of phosphate on the taste of coffee, we have measured phosphate concentrations in Kenyan Kagumo (1997), Kenyan Mweiga (1998), Aged Java Old Brown (1996/1997), Sumatran Mandheling Golden Pwani (1998/1999), Costa Rican Tarrazu Papagayo (1998/1999), and Indian Cherry Robusta (1998/1999) coffees to determine if there is a correlation between perceived acidity and phosphate concentration. The perceived acidity is also strongly affected by the degree of roasting of the coffee. The possible connection between roasting, acidity, and phosphate concentration was studied by determining the phosphate concentration in Colombian La Vareda (1999) coffee of different roast degrees.

Upon extraction of a coffee, acid-base reactions involving phosphate and numerous other species occur, ultimately leading to a coffee solution of approximately pH 5. Phosphoric acid has dissociation constants[6] of 7.1×10^{-3}, 6.3×10^{-8}, and 4.2×10^{-13}; therefore, in a coffee solution one will find almost exclusively the

dihydrogen phosphate ion, irrespective of whether the phosphate originally existed as phosphoric acid or as a phosphate salt. For this reason, we have focused our investigation on the total concentration of phosphate in coffee and have not attempted to identify the original form in which the phosphate existed.

EXPERIMENTAL METHODS

Materials. ACS reagent grade standards of acetic acid, citric acid monohydrate, formic acid, lactic acid, malic acid, oxalic acid, potassium nitrate, pyruvic acid, quinic acid, sodium dihydrogen phosphate monohydrate, sodium chloride, sodium sulfate, sodium sulfite, and succinic acid were used as received from the supplier. Acid-base buffers for pH calibrations were obtained from Fisher.

Coffee Samples. Coffee samples were roasted by the Specialty Coffee Institute in a STA Impianti (Bologna, Italy) laboratory roaster and used within 36 hours of roasting. Table 1 shows the Agtron number, roasting time, the initial and final temperatures, and percent weight loss for the Colombian La Vareda coffee. All other coffees were roasted to a medium-brown roast (Agtron # 55 ± 2). Coffee samples were ground on a Ditting grinder setting 8.

Table 1. Roasting specifications for the Colombian La Vareda coffee.

Agtron Number	Roast Time (min)	Initial Temperature (°C)	Final Temperature (°C)	Percent Weight Loss
74.0	9.3	176.6	179.4	8.77
65.1	10.1	176.6	185.0	11.13
55.0	10.8	176.6	196.1	13.71
43.7	11.5	176.6	198.3	15.90
36.7	12.2	176.6	205.0	18.09
26.5	13.0	176.6	208.9	23.27

Coffee Extraction. Ground coffee (5.500 g) was submitted to extraction by pouring 100.0 mL of boiling de-ionized water over the ground coffee in an insulated beaker. The coffee was extracted for 4.5 minutes with constant stirring and isolated by suction filtration. The coffee sample was cooled to 25°C, and the pH was determined using an Accumet AR10 pH meter equipped with a standard single-junction glass-Ag/AgCl combination electrode, which was calibrated at pH 4 (0.5 M potassium hydrogen phthalate buffer) and pH 7 (0.5 M potassium dihydrogen phosphate/sodium hydroxide buffer). The percent extracted solids was determined by drying 20.0 mL of coffee extract in a pre-weighed beaker to a constant weight. Each coffee was brewed twice, and two trials were performed with each extract. Coffee extraction was performed five minutes prior to analysis.

Qualitative Analysis of Phosphate. Activated carbon (5.0 g) was rinsed (5x) with boiling de-ionized water to remove residual phosphate. The coffee was de-colorized by mixing 10 mL of coffee extract with 2.5 g of the phosphate-free activated carbon (2x) for 5 minutes. Nitric acid (3 mL, 6 M) and ammonium molybdate (3 mL, 2 M) reagents were added to the decolorized filtered coffee extract.

Anion Exchange Chromatography. Anion analysis was performed using a Dionex DX-100 Chromatograph equipped with a conductivity detector. A Dionex IonPac AS14 4-mm (10-32) anion exchange column, a Dionex AG14 4-mm (10-32) guard column and a Dionex ASRS-I 4-mm self-regenerating suppression system were used. All samples were filtered through a 0.2 μm Nylon membrane filter (Millipore) and 25.0 μL of the each sample was injected onto the column. The aromatic anions present in coffee did not elute under normal chromatographic conditions. In order to maintain good column performance, the columns were cleaned every 25 trials with de-ionized water for 15 minutes, a solution of

5% acetonitrile and 20% sodium chloride (1 M, adjusted to pH 2 with HCl) for 10 min, a solution of 80% acetonitrile and 20% sodium chloride (1 M, adjusted to pH 2 with HCl) for 60 minutes, and de-ionized water for 180 minutes. Calibration standards were re-run after each cleaning.

Anion standards were run individually by diluting 5 mL of coffee extract to 10 mL using 150 mg L^{-1} solutions of either acetic acid, citric acid, formic acid, lactic acid, malic acid, oxalic acid, potassium nitrate, pyruvic acid, sodium chloride, sodium dihydrogen phosphate, sodium sulfate, sodium sulfite, succinic acid, or quinic acid. Chromatographic analysis for standard additions of mono-anions was performed using a sodium hydroxide (1 mM) mobile phase, and analysis for standard additions of di-anions and tri-anions was performed using a mobile phase of sodium carbonate (1.75 mM) and sodium hydrogen carbonate (0.50 mM) at a flow rate of 2.0 mL min^{-1}.

Quantitative analysis of phosphate was performed using a 6.0 mM sodium hydrogen carbonate and 0.6 mM sodium carbonate mobile phase at a flow rate of 1.5 mL min^{-1}. Phosphate standards ranging from 40-130 mg L^{-1} phosphate in increments of 30 mg L^{-1} were prepared from successive dilutions of a standard 200 mg L^{-1} phosphate solution prepared from sodium dihydrogen phosphate monohydrate. Two runs were made with each phosphate standard.

RESULTS AND DISCUSSION

Qualitative Analysis of Phosphate. The presence of phosphate can be confirmed by treating a solution with nitric acid and ammonium molybdate. Upon addition of the ammonium molybdate, a bright yellow complex of triammonium dodecamolybdophosphate ($(NH_4)_3[PO_4 \cdot 12\ MoO_3] \cdot 12\ H_2O$) is formed if phosphate is present.[7]

$$PO_4^{3-} + 12\ H_2MoO_4 + 3\ NH_4^+ \rightarrow (NH_4)_3[PO_4 \cdot 12\ MoO_3] \cdot 12\ H_2O$$

Prior to subjecting a coffee solution to this test, it proved necessary to decolorize the coffee with activated carbon in order to permit careful observation of the yellow $(NH_4)_3[PO_4 \cdot 12\ MoO_3] \cdot 12\ H_2O$ solid. To ensure that the activated carbon did not contain phosphate, 5 mL of boiling water was added to 5.0 g of the rinsed activated carbon, and allowed to sit for 5 minutes while boiling. The extract was isolated by suction filtration and analyzed both by ion chromatography and by adding nitric acid and ammonium molybdate reagents. No peak corresponding to phosphate ion was observed in the ion chromatogram, and the qualitative test did

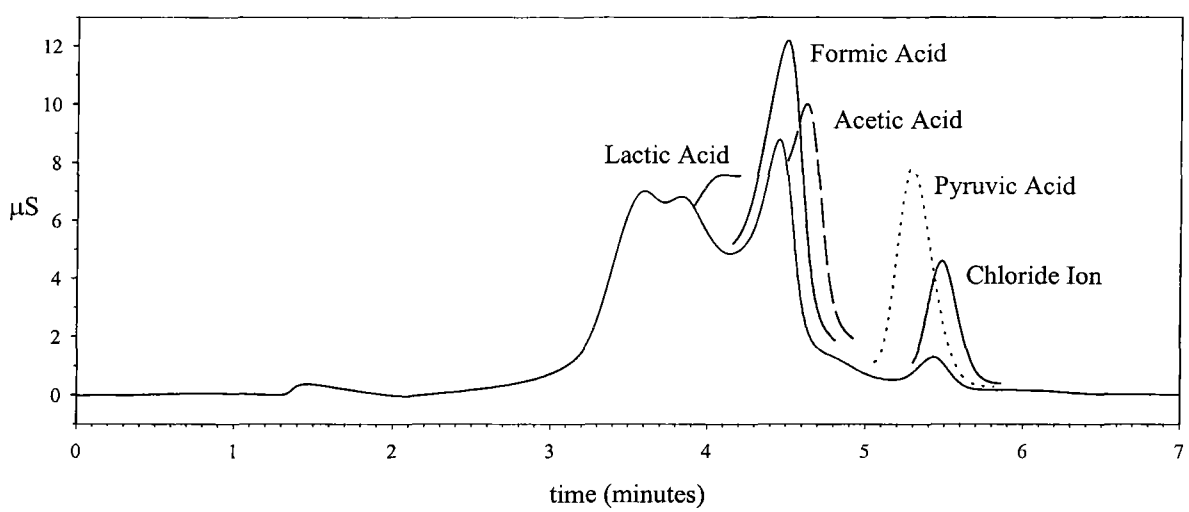

Figure 1. Chromatogram of Kenyan Kagumo coffee with standard additions of lactic acid, formic acid, acetic acid, pyruvic acid, and sodium chloride using a sodium hydroxide (1 mM) mobile phase at a flow rate of 2.0 mL min^{-1}.

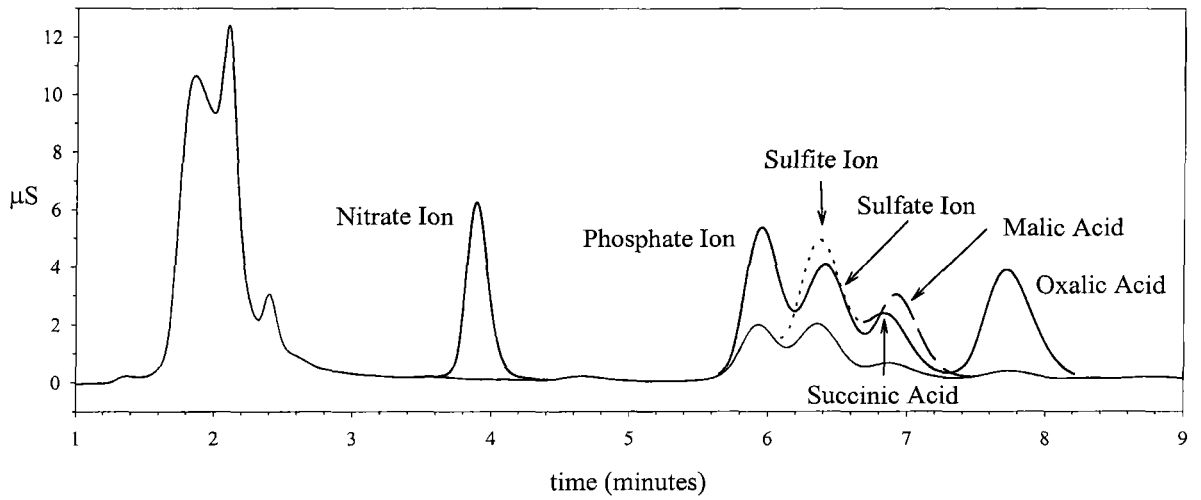

Figure 2. Chromatogram of Kenyan Kagumo coffee with standard additions of potassium nitrate, sodium dihydrogen phosphate, sodium sulfite, sodium sulfate, succinic acid, malic acid, and oxalic acid using a sodium carbonate (1.75 mM) and sodium hydrogen carbonate (0.50 mM) mobile phase at a flow rate of 2.0 mL min^{-1}.

not result in the formation of a yellow solid, verifying that no phosphate was released from the activated carbon. When nitric acid and ammonium molybdate were added to a decolorized solution of Kenya Kagumo coffee a bright yellow solid precipitated verifying the presence of phosphate in the coffee.

Ion Chromatography. Optimal chromatographic conditions were determined using Kenyan Kagumo coffee. Coffee contains a complex mixture of anions, and it proved impossible to clearly resolve all detectable components. Figures 1 and 2 show representative chromatograms of the mono-anion and di-anion regions, respectively. Tri-anions and aromatic anions did not elute under the chromatographic conditions that were employed. These analytes were removed by frequent washing of the columns as described in the Experimental section.

Attempts were made to determine whether any common anions co-eluted with phosphate ion by co-injecting known anions with the coffee sample. Figure 1 shows the chromatogram of the Kenyan Kagumo coffee with standard additions of lactic acid, formic acid, acetic acid, pyruvic acid, and sodium chloride overlaid on a chromatogram of pure coffee to facilitate comparison. Figure 2 shows the chromatogram of the Kagumo coffee with standard additions of potassium nitrate, sodium dihydrogen phosphate, sodium sulfite, sodium sulfate, succinic acid, malic acid, and oxalic acid. No standard co-eluted with the phosphate standard.

The composition of the mobile phase and the flow rate were varied to shift the phosphate peak relative to other di-anionic components in order to check for unidentified co-eluting components. The integration of the phosphate peak remained constant under all chromatographic conditions, indicating that no significant component co-eluted with phosphate and that phosphate concentrations could be accurately determined. No peaks were observed in the mono- or di-anion region when a blank sample of de-ionized water, treated using the same extraction procedure but without the addition of coffee grounds, was injected onto the column. Thus, no extraneous phosphate was introduced during the handling of the samples.

Quantitative analysis of phosphate concentrations were performed using 6.0 mM sodium hydrogen carbonate and 0.6 mM sodium carbonate mobile phase and a flow rate of 1.5 mL min^{-1}. A calibration curve was created from peak area and concentration data, and a second-order polynomial was fit to the data as shown in Figure

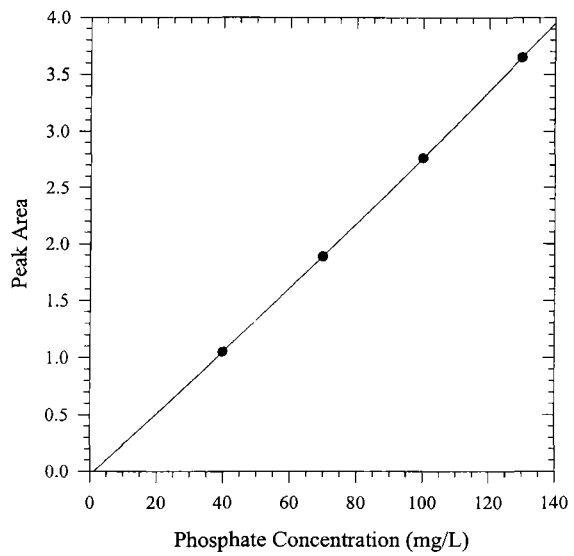

Figure 3. Calibration curve prepared from peak areas and concentration of phosphate standards. The solid line is a second-order polynomial fit to the experimental points. Two independent trials are plotted at each concentration.

3. A representative chromatogram of the di-anion region of coffee under these chromatographic conditions is shown in Figure 4.

Concentration of Phosphate in Coffees. The phosphate concentration of each coffee was determined to examine the relationship between phosphate concentration and perceived acidity. Phosphate concentrations were found to vary nearly linearly with the amount of extracted solids within the small range of concentrations employed in this study. In order to account for variations in phosphate concentration with the effectiveness of the extraction, the phosphate concentrations were normalized to 20 % extracted solids. The experimental results are given in Table 2. The average phosphate concentrations (at 20% extracted solids) were found to be: Kenya Kagumo 81.7 ± 2.7 mg L^{-1}, Costa Rica Tarrazu Papagayo 89.5 ± 0.8 mg L^{-1}, Kenya Mweiga 94.0 ± 1.2 mg L^{-1}, Sumatra Mandheling Golden Pwani 104.3 ± 2.3 mg L^{-1}, Aged Java Old Brown 128.0 ± 0.5 mg L^{-1}, and Indian Cherry Robusta 136.8 ± 0.8 mg L^{-1}.

The Kenya Kagumo sample, which was prized for its high acidity, had the lowest phosphate concentration. The Sumatra Golden Pwani, which was valued for its lack of acidity, had a relatively high phosphate concentration. Indeed, the coffee with the lowest perceived acidity, the Aged Java Old Brown, had the second largest phosphate concentration. In terms of the taste of the coffee, acidity was inversely correlated with the phosphate concentration. The coffees regarded as highly acidic had lower phosphate levels than those coffees known for being less acidic. On the other hand, the concentration of phosphate in the coffee

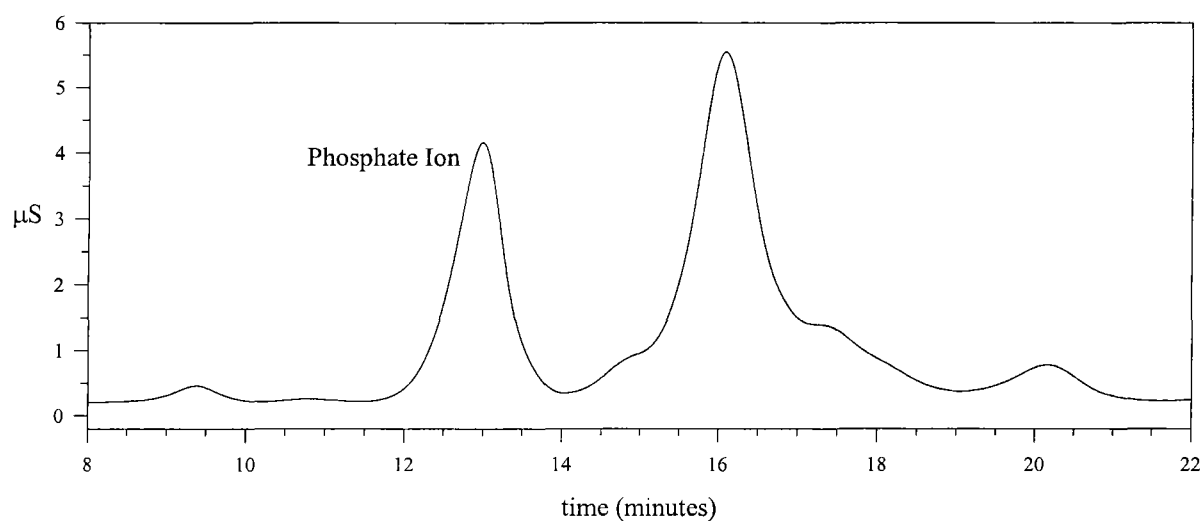

Figure 4. Di-anion region of Kenyan Kagumo coffee using 6.0 mM sodium hydrogen carbonate and 0.6 mM sodium carbonate mobile phase at a flow rate of 1.5 mL min^{-1}.

Table 2. Experimental phosphate concentration, pH, percent of solids extracted, and normalized phosphate concentration for coffees roasted to an Agtron value of 55 ± 2. Each coffee was extracted twice (indicated by the 1 or 2) and two trials (a or b) were performed with each extract.

Coffee	Extract (Trial)	Phosphate Concentration (mg L^{-1})	pH	Percent Solids Extracted	Normalized Phosphate Concentration (mg L^{-1})
Kenya Kagumo	1(a)	81.1	4.97	20.46	79.3
Kenya Kagumo	1(b)	81.1	4.97	20.46	79.3
Kenya Kagumo	2(a)	82.4	4.95	19.57	84.2
Kenya Kagumo	2(b)	82.1	4.95	19.57	83.8
Costa Rica	1(a)	78.6	5.13	17.65	89.0
Costa Rica	1(b)	78.7	5.13	17.65	89.1
Costa Rica	2(a)	78.7	5.13	17.62	89.3
Costa Rica	2(b)	79.9	5.13	17.62	90.7
Kenya Mweiga	1(a)	88.1	4.90	18.89	93.3
Kenya Mweiga	1(b)	87.7	4.90	18.89	92.8
Kenya Mweiga	2(a)	88.2	4.91	18.45	95.6
Kenya Mweiga	2(b)	87.2	4.91	18.45	94.5
Sumatra Golden	1(a)	88.1	5.07	17.10	103.1
Sumatra Golden	1(b)	86.9	5.07	17.10	101.7
Sumatra Golden	2(a)	91.4	5.08	17.20	106.3
Sumatra Golden	2(b)	91.4	5.08	17.20	106.2
Aged Java	1(a)	119.5	5.05	18.72	127.7
Aged Java	1(b)	120.2	5.05	18.72	128.4
Aged Java	2(a)	118.7	5.05	18.63	127.5
Aged Java	2(b)	119.6	5.05	18.63	128.4
Indian Robusta	1(a)	131.1	5.48	19.28	135.9
Indian Robusta	1(b)	132.9	5.48	19.28	137.8
Indian Robusta	2(a)	127.4	5.49	18.66	136.6
Indian Robusta	2(b)	127.6	5.49	18.66	136.8

was uncorrelated with the pH of the coffee, suggesting that phosphate species play a relatively minor role in establishing the pH of the coffee. The observed pH of coffee (4.9-5.5) lies outside the effective buffering regions of phosphate species (1.1-3.1 for $H_3PO_4/H_2PO_4^-$, 6.2-8.2 for $H_2PO_4^-/HPO_4^{2-}$, and 11.4-13.4 for HPO_4^{2-}/PO_4^{3-}), indicating that phosphate species do not provide any significant buffering action in coffee.

Effect of Roast Degree on Phosphate Concentration. A study of the variation in extractable phosphate concentration with roast degree was performed using Colombian La Vareda coffee. During the course of our study, it was observed that larger volumes of coffee grounds were brewed at the darker roasts, because each coffee was weighed out to 5.500 g, and the density of the grounds decreased with darker roasts. In order to account for this effect, phosphate concentrations were normalized to a constant mass of coffee prior to roasting. Each extraction was performed using 5.500 g of roasted coffee. If w represents the fraction of weight lost during roasting, then the original mass of the 5.500 g roasted sample prior to roasting would have been 5.500 g/(1-w). Over the relatively narrow range of conditions employed in this study, the phosphate concentration was found to vary approximately linearly with the mass of coffee brewed. The phosphate concentration was therefore multiplied by (1-w) in order to normalize each concentration to the value expected had 5.500 g of unroasted coffee been roasted and then extracted. The variations of phosphate concentration and pH with roast degree for the Colombian La Vareda coffee are given in Table 3.

The extractable phosphate concentration increased significantly early in the roast when other chemical changes such as fat hydrolysis, oxidation of acids, thermal degradation of esters and autoxidation of

Table 3. Agtron number, percent weight loss during roasting, percent solids extracted in each brew, phosphate concentration, pH, and normalized phosphate concentration for Colombian La Vareda coffee at various roast degrees. Each coffee was extracted twice (indicated by the 1 or 2) and two trials (a or b) were performed with each extract.

Agtron Number	Extract (Trial)	Percent Weight Loss	Percent Extracted Solids	Phosphate Concentration (mg L^{-1})	pH	Normalized Phosphate Concentration (mg L^{-1})
74.0	1(a)	8.77	18.30	64.1	4.90	58.5
74.0	1(b)	8.77	18.30	64.9	4.90	59.2
74.0	2(a)	8.77	19.47	65.0	4.85	59.3
74.0	2(b)	8.77	19.47	64.8	4.85	59.1
65.1	1(a)	11.13	19.01	77.5	4.89	68.9
65.1	1(b)	11.13	19.01	77.9	4.89	69.2
65.1	2(a)	11.13	18.43	77.7	4.89	69.1
65.1	2(b)	11.13	18.43	77.7	4.89	69.1
55.0	1(a)	13.71	20.55	89.3	5.08	77.1
55.0	1(b)	13.71	20.55	89.5	5.08	77.2
55.0	2(a)	13.71	20.57	89.8	5.06	77.5
55.0	2(b)	13.71	20.57	89.3	5.06	77.1
43.7	1(a)	15.90	20.05	92.1	5.37	77.5
43.7	1(b)	15.90	20.05	92.4	5.37	77.7
43.7	2(a)	15.90	20.29	92.8	5.36	78.0
43.7	2(b)	15.90	20.29	92.9	5.36	78.1
36.7	1(a)	18.09	20.81	93.8	5.71	76.9
36.7	1(b)	18.09	20.81	93.3	5.71	76.4
36.7	2(a)	18.09	20.39	95.6	5.72	78.3
36.7	2(b)	18.09	20.39	95.4	5.72	78.1
26.5	1(a)	23.27	21.25	99.2	6.13	76.1
26.5	1(b)	23.27	21.25	99.2	6.13	76.1
26.5	2(a)	23.27	21.28	99.9	6.13	76.6
26.5	2(b)	23.27	21.28	100.7	6.13	77.3

aldehydes and ketones are known to occur.[8] The normalized phosphate concentration at the lightest roast (Agtron # 74.0) was determined to be 59.0 ± 0.4 mg L^{-1}. At the next degree of roast analyzed (Agtron # 65.1), the extractable phosphate concentration increased to 69.1 ± 0.1 mg L^{-1}. At roasts darker than Agtron number 55.0, the concentration of extractable phosphate stabilized to approximately 77 mg L^{-1}.

It is probable that the increase in phosphate concentration is the result of the decomposition of inositol-hexaphosphoric acid or other phosphate containing organic compounds.[9,10,11] A recent study[10] reported that the concentration of phosphoric acid concentration increased as the concentration of inositol-hexaphosphoric acid decreased during extended storage of brewed coffee at 60 °C, while another study[9] indicated that inositol-hexaphosphoric acid is decomposed at darker roasts. In both studies, higher temperatures resulted in increased phosphate concentrations in coffee.

This trend in phosphate concentrations, however, runs opposite to the observed decrease in perceived acidity at darker roasts. The relatively acidic lightly roasted coffees displayed the lowest phosphate levels, while the less acidic darker roasted coffees were found to possess the highest phosphate concentrations, consistent with the trend observed in the preceding section. Interestingly, the light roast coffees (with the highest perceived acidities) also displayed the lowest pH (highest chemical acidity), and the chemical acidity was observed to decrease (as evidenced by an increase in pH) as the roast degree increased. In this case, the pH

trend correlates well with the trend in perceived acidity. Phosphate concentrations, however, were observed to increase as the coffee pH increased.

CONCLUSIONS

The experimental data indicates that phosphate concentrations are relatively low in coffees of high perceived acidity, and phosphate concentration increases with an increase in pH as detailed in the roast study. While our data clearly indicates an inverse correlation between perceived acidity and phosphate concentration, this evidence is not sufficient to conclude that phosphate directly lowers the perceived acidity. When examining several different coffees of the same roast, no correlation between coffee pH and phosphate concentration was observed, despite relatively large variations in phosphate concentration. Conversely, the series of measurements made on Colombian La Vareda coffee at different roast degrees showed a strong inverse relationship between pH and phosphate concentration, with the phosphate concentration increasing and the chemical acidity of the coffee decreasing as the roast degree increased. This behavior is likely attributable to thermal degradation of organophosphorus compounds. If this degradation releases PO_4^{3-}, the basic PO_4^{3-} would quickly become protonated to form $H_2PO_4^-$, thereby increasing the pH of the coffee. It is also possible that the pyrolysis of the coffee releases other bases, which might also contribute to the change in pH.

Although we did not undertake a detailed study of the other anions, the chromatographic data clearly shows coffee to contain significant concentrations of numerous carboxylic acids. It should be noted that conductivity detection under the conditions employed in this study displays a higher sensitivity for the phosphate ion than for carboxylic acids. One can conclude, consistent with other findings[4], that carboxylic acids such as citric acid, malic acid, and the chlorogenic acids are much more important sources of hydrogen ions in coffee than is the phosphate ion. Since phosphoric acid is only a minor source of hydrogen ions it is reasonable to expect that it will not govern the acidity of coffee. The measured pH of coffee is very close to the pK_a values of carboxylic acids, indicating that these carboxylic acids are only partially deprotonated in coffee solution. Carboxylic acids undoubtedly play the dominant role in establishing the pH of coffee and in buffering the coffee.

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SUMMARY

The phosphate concentration of Kenyan Kagumo, Kenyan Mweiga, Aged Java Old Brown, Sumatran Mandheling Golden Pwani, Costa Rican Tarrazu Papagayo, and Indian Cherry Robusta coffee was determined using anion exchange chromatography. The change in extractable phosphate with roast degree was determined using Colombian La Vareda coffee at roasts ranging from very light to very dark (Agtron # 75-25). Using a medium-brown roast (Agtron # 55 ± 2), the average phosphate concentrations (normalized to 20 % extractable solids) were: Kenyan Kagumo 81.7 ± 2.7 mg L^{-1}, the Costa Rican Tarrazu Papagayo 89.5 ± 0.8 mg L^{-1}, the Kenyan Mweiga 94.0 ± 1.2 mg L^{-1}, the Sumatran Mandheling Golden Pwani 104.3 ± 2.3 mg L^{-1}, the Aged Java Old Brown 128.0 ± 0.5 mg L^{-1}, and the Indian Cherry Robusta 136.8 ± 0.8 mg L^{-1}. It was observed that the concentration of extractable phosphate increased significantly during the beginning of the roasting process. The normalized phosphate concentration at the lightest roast (Agtron # 74.0) was determined to be 59.0 ± 0.4 mg L^{-1}, while the concentration at the darkest roast (Agtron # 26.5) was determined to be 76.5 ± 0.6 mg L^{-1}.

RÉSUMÉ

La concentration en phosphate dans les cafés "Kenyan Kagumo", "Kenyan Mweiga", "Aged Java Old Brown", "Sumatran Mandheling Golden Pwani", "Costa Rican Tarrazu Papagayo", et "Indian Cherry Robusta" a été déterminée par chromotographie d'échanges d'ions. La variation en phosphates extractibles avec le degré de torréfaction a été obtenue en utilisant le café "Colombian La Vareda" à des niveaux de torréfaction allant de clair à très foncé (Agtron #75-25). En utilisant un degré de torréfaction moyen (Agtron #55 ± 2), les concentrations moyennes de phosphate mesurées (normalisées à 20% des solides extractibles) étaient: "Kenyan Kagumo" 81,7 ± 2,7 mg L^{-1}, "Costa Rican Tarrazu Papagayo" 89,5 ± 0,8 mg L^{-1}, "Kenyan Mweiga" 94,0 ± 1,2 mg L^{-1}, "Sumatran Mandheling Golden Pwani" 104,3 ± 2,3 mg L^{-1}, "Aged Java Old Brown" 128,0 ± 0,5 mg L^{-1}, et "Indian Cherry Robusta" 136,8 ± 0,8 mg L^{-1}. Nous avons observé que la concentration en phosphate extractible croit de manière significative au début du processus de torréfaction. Pour le degré le plus faible de torréfaction (Agtron #74,0), la concentration normalisée en phosphate a été trouvée égale à 59,0 ± 0,4 mg L^{-1}, tandis que pour le degré de torréfaction le plus élevé (Agtron #26,5) elle était de 76,5 ± 0,6 mg L^{-1}.

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Structural Properties of Coffee Beans as Influenced by Roasting Conditions

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Introduction

Roasting of coffee beans not only forms color and flavor compounds, but also leads to a complete alteration of the bean microstructure. The pore structure presents a crucial factor of final product quality in roasted beans and is important as well in case of further processing (extraction). It controls mass transfer phenomena during roasting and storage such as oil migration and gas desorption. The loss of flavor compounds and the change in flavor profile during storage (staling) are probably related to the extent of exposure of inner surface and to oxygen accessibility. Therefore, the selection of appropriate roasting conditions to optimize microstructure presents a major goal in roasting technology.

The large volume increase of coffee beans during roasting is accompanied by the formation of excavated cells. This macropore system of roasted beans has been described extensively using light microscopy, scanning electron microscopy (SEM) and image analysis procedures (Bürgin, 1969; Dentan, 1977; Dentan and Illy, 1985; Gutiérrez et al., 1993; Illy and Viani, 1995; Massini et al., 1990; Puhlmann et al., 1986; Wilson, 1997). Radtke (1975) reported bean porosity values ranging from 0.38 to 0.49 depending on the origin and pretreatment of coffee. Kazi and Clifford (1985) found different average cell sizes for „high yield" (34 ... 40 µm) and „regular" coffees (21 ... 23 µm), respectively.

So far, very little is known on the influence of process conditions on the development of the micropore system within the cell walls. According to Dentan (1977) cell walls of green beans are crossed by numerous plasmodesmata. Saleeb (1975) concluded from gas adsorption measurements that the macropores of roasted beans are accessible through very narrow micropores of molecular magnitude (2.8 nm) which form a so-called ink-bottle structure. In contrast, Wilson (1997) found no evidence of pre-existing channels within the cell walls of green beans, but two different types of micropores of an average diameter of 100 nm and 10 nm, respectively, in roasted beans.

A direct microscopic visualization of micropores in the cell wall is difficult. On the other hand, mercury porosimetry allows the determination of pore volume and pore size distribution (Adamson, 1990; Perren and Escher, 1997). In the application of mercury porosimetry to coffee beans this method has already proved to be a valid and very useful tool for investigation of pore structure (Schenker, 1998). Our objective was to investigate the influence of different roasting conditions on the development of the micropore system of coffee beans during roasting.

Experimental

Two wet-processed *Coffea arabica* Linn. varieties from Colombia and Costa Rica were obtained from a Swiss import company. Roasting experiments were carried out with a fluidized-bed hot air laboratory roaster in batches of 100 g green beans. For the present experiments samples were roasted in a high-temperature short-time roasting process (HTST) and a low-temperature long-time process (LTLT) according to the process characteristics as given in Table 1.

Roasting was monitored and controlled by online temperature measurements. To describe roasting dynamics, coffee samples were removed at regular intervalls during roasting and analysed. In order to be able to compare the two processes roasting was targeted to the same degree of roast,

Tab. 1: *Roasting parameters for HTST and LTLT process and typical properties of roasted products*

	HTST roasting	LTLT roasting
Process parameters:		
Hot air flux (m^3s^{-1})	0.01885	
Hot air temperature (°C)	260 ±0.5	220 ±0.5
Roasting time (s)	155 ... 180	540 ... 720
Cooling	cold air, no water, bean temperature < 40 °C reached within 60 s	
Product properties (typical values):		
Color (L*/a*/b*)	24.06 / 9.26 / 11.33	24.02 / 9.27 /11.17
Roast loss	15.33	15.81
Water content (g H_2O/100g coffee)	2.68	2.15

based on overall weight loss and final product color. All samples of final products for porosimetric analysis had a roast loss of 15% which in practice corresponds to a „medium degree of roast". Color was measured with a colorimeter Chroma Meter CR-310 (Minolta, Japan) with a measured area of 19.6 cm^2. Ground coffee samples were placed in a petri dish and gently pressed with standardized force to form an even surface. Results were depicted in the CIE $L^*a^*b^*$ color space.

A displacement method based on a system described by Mohsenin (1986) was used to determine bean volume and bean density. A small container was filled with peanut oil and placed on a balance. 30 g roasted beans were weighed into a wire basket which was suspended on a support beside the balance. The basket was immerged into the oil and moved up and down for 15 s in order to release air bubbles trapped between the beans. Immersion was carried out likewise with the empty basket. From the weight difference of the immersed basket with and without coffee beans and using oil density the bean volume was calculated. Relative bean volume at progressing roasting was based on the volume of green beans taking the weight loss during roasting into account.

Porosimetry was carried out using a mercury-porosimeter Carlo Erba 2030 (Carlo Erba Strumentazione, I-Rodano). About 0.4 g roast coffee was placed in a dilatometer and evacuated for some 15 min. The dilatometer was carefully filled with mercury. The pressure in the micropore unit was gradually increased to 400 MPa (4000 bar) during 45 min and the volume of intruded mercury recorded. Pressure values were converted into values of „equivalent pore radius" r, based on the Washburn equation (Adamson, 1990) which describes a linear relationship between the size of an intrudable circular pore and the appplied mercury pressure. The pore radius corresponding to the maximum in the distribution function is defined r_{main} = maximum dV/d log (r/r_0), whereby V = cumulated pore volume and r_0 = 1 m (normalization, dimensionless exponent). Porosity ε was defined as the ratio of absolute volume per g bean and absolute volume of intruded mercury per g bean.

The bean pore structure was investigated by cryo-scanning electron microscopy (cryo-SEM), with a Philips 515 microscope (Philips, The Netherlands) equipped with a SEM cryo unit (SCU 020, Bal-Tec, Balzers, Principality of Liechtenstein). Pieces of beans were frozen in liquid nitrogen, fractured by a scalpel and transfered to the cold stage of the preparation chamber. The samples were exposed to -80 °C for 10 min under $p<2 \cdot 10^{-4}$ Pa and cryo-sputtercoated with 15 nm platinum. The specimen were examined at a temperature below -130 °C at an accelerating voltage of 12 kV.

Sample preparation for transmission electron microscopy (TEM) was carried out with a modified procedure according to Angermüller and Fahimi (1982). Ultrathin sections of small and fixed bean pieces were stained and examined in a Hitachi H-600 transmission electron microscope at 100 kV. Images were digitally recorded using a Gatan slow-scan CCD camera.

Results & Discussion

The temperature curves for the HTST and the LTLT roasting process are given in Figure 1. As the air-to-bean-ratio in the laboratory roaster is high the two processes are characterised by rapid heat transfer as compared to industrial processes. However, during the LTLT process the actual bean temperature did not exceed 210 °C.

The development of bean volume during roasting is shown in Figure 2. Volume increase presents a steady change in both processes as no instantaneous expansion is observed which would lead to a discontinuity in the curve. High temperature conditions resulted in much higher expansion rates as compared to low temperature conditions. The development of volume increase is affected by a driving force and, opposed to it, by structure resistance.

The profound alteration in the bean tissue microstructure during roasting is documented for the LTLT process in Figure 3. The dense tissue structure of the green bean developes to a porous structure built by the cell wall framework and the remainings of cytoplasm forming a thin layer adjacent to it. As transport of coffee oil and gases across this structure takes place during storage the existance of a cell wall micropore network allowing for mass transfer must be assumed.

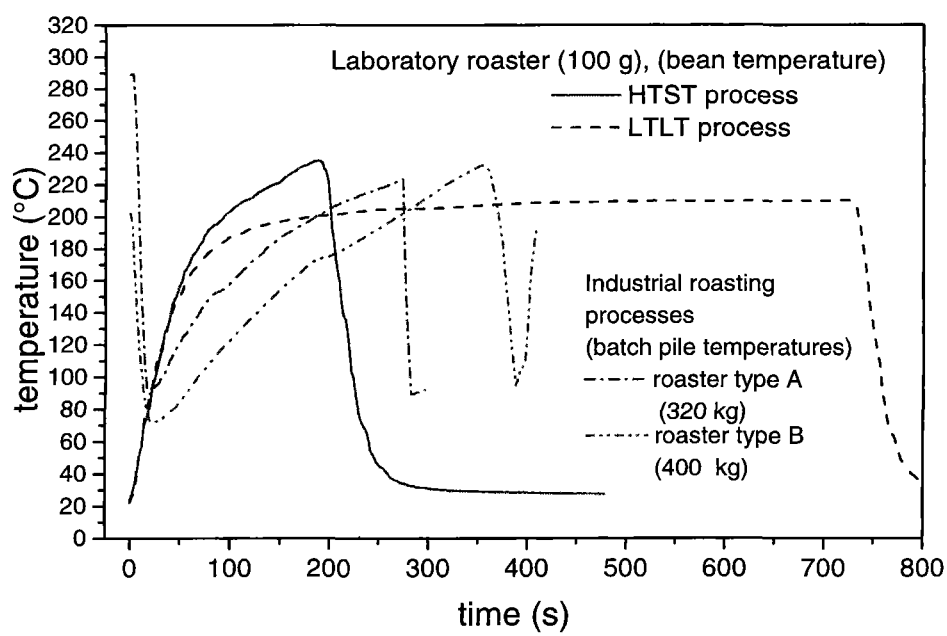

Fig. 1: *Temperature curves measured inside the bean in batches of 100 g green coffee during the High-temperature short-time roasting process (HTST) and the Low-temperature long-time (LTLT) process. Batch pile temperatures during industrial roasting in two different roaster types are given for comparison.*

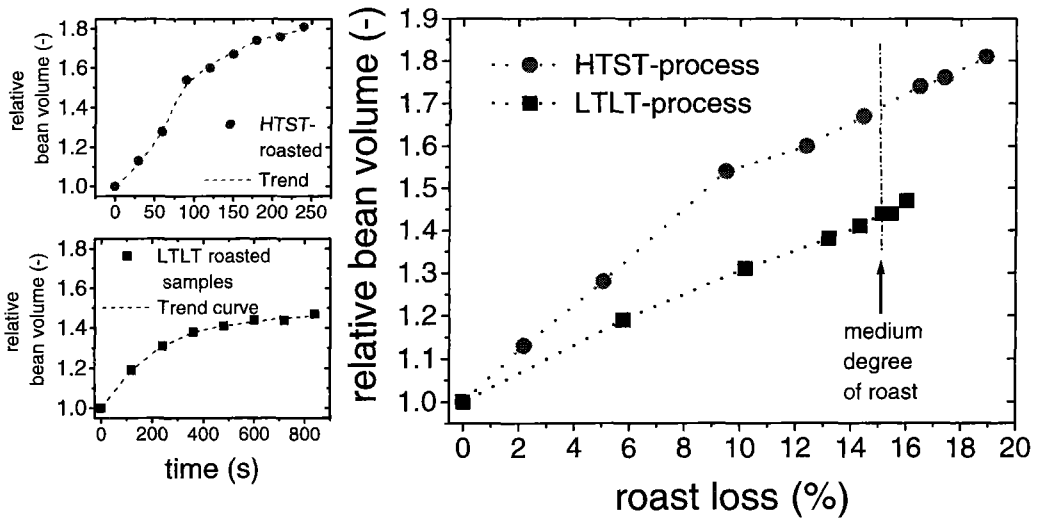

Fig. 2: *Development of bean volume during high and low temperature roasting. Left: Development of bean volume as a function of time. Right: Identical data as a function of roast loss.*

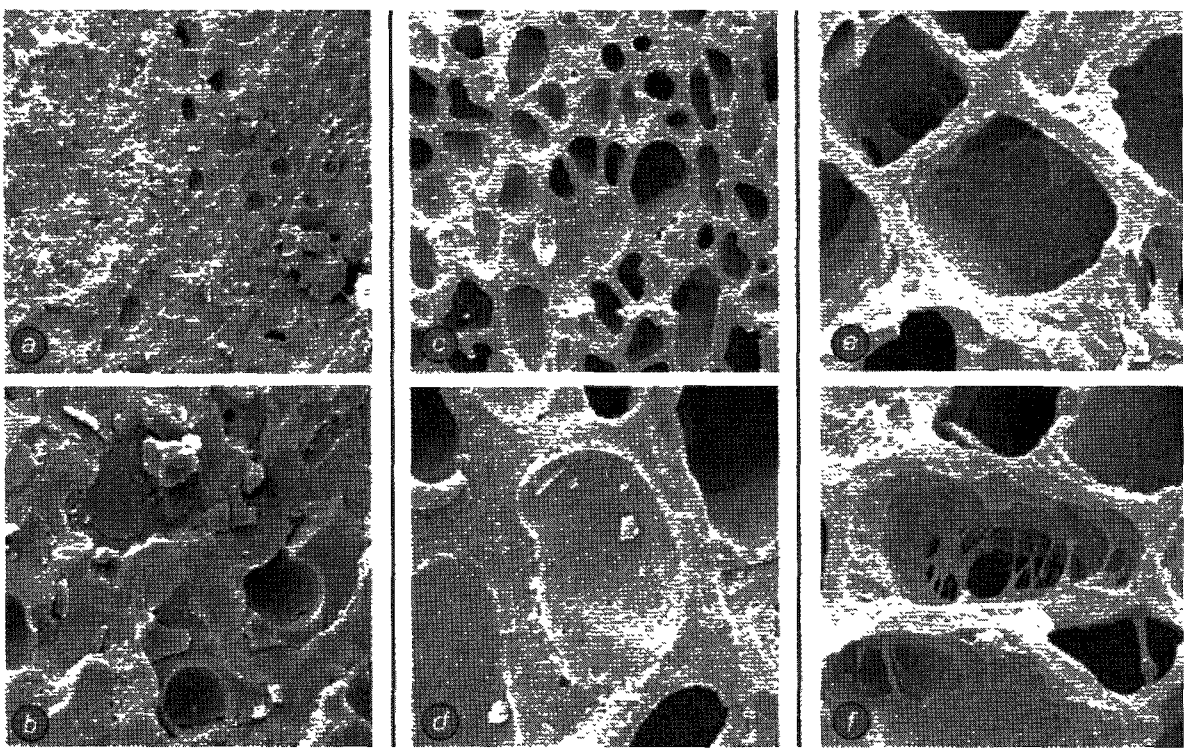

Fig. 3: *Cryo-SEM micrographs of the microstructure in green coffee beans (a and b) and in LTLT roasted beans in an early stage of roasting after 60 s (c and d) and in the fully roasted bean after 600 s (e and f) with a medium degree of roast. 3a: Dense green bean tissue structure (image width [i.w.] 282 µm). 3b: Some single cells (i.w. 85 µm). 3c: tissue structure (i.w. 282 µm). 3d: single cell (i.w. 70 µm). 3e: single cell (i.w. 71 µm). 3f: single cell (i.w. 71 µm).*

Plasmodesmata channels were found in the green as well as in the roasted state in some spots of the cell wall (Figure 4). However, these channels are cell to cell connections and do not provide access to the bean surface. Moreover it is not clear, wether these channels are free for mass transfer or congested by denatured proteins. Thus they do not seem to play a key role in mass transfer.

In the initial stage of the oil migration process little oil droplets emerge everywhere on the cell surface (Figure 5). These findings support the hypothesis of an overall permeable three-dimensional microfibril network of polysaccharides in the cell wall of roasted beans.

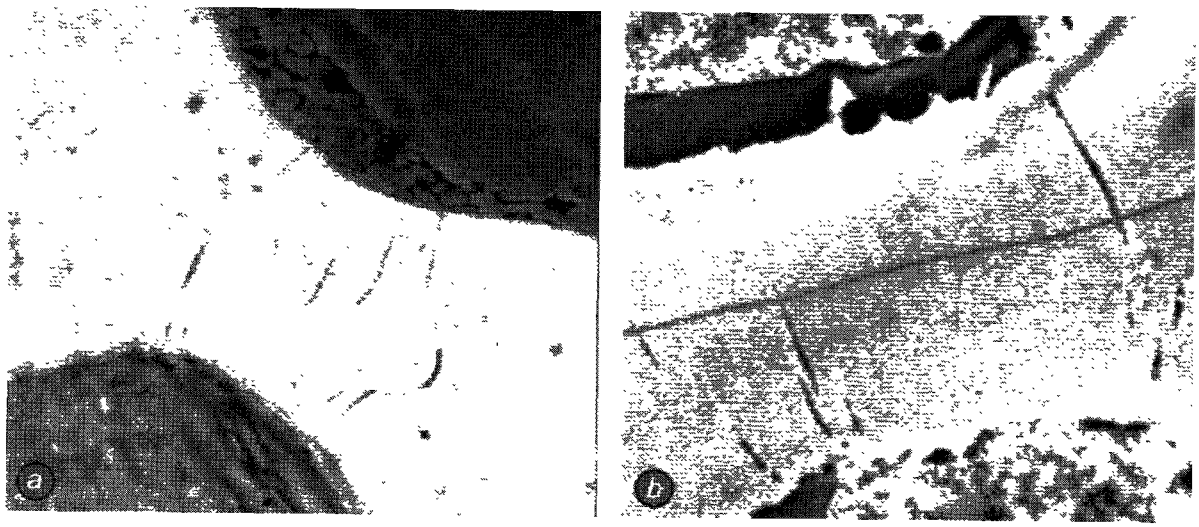

Fig. 4: *TEM micrographs of the coffee bean cell wall. 4a: Green bean (image width 12.1 µm). Thin, continuous black line: Middlelamella. Adjacent to it on each side: Cell wall (bright) and cytoplasm (dark). Perpendicular to the middlelamella: Plasmodesmata channels. 4b: Partially roasted cell wall (image width 9.06 µm).*

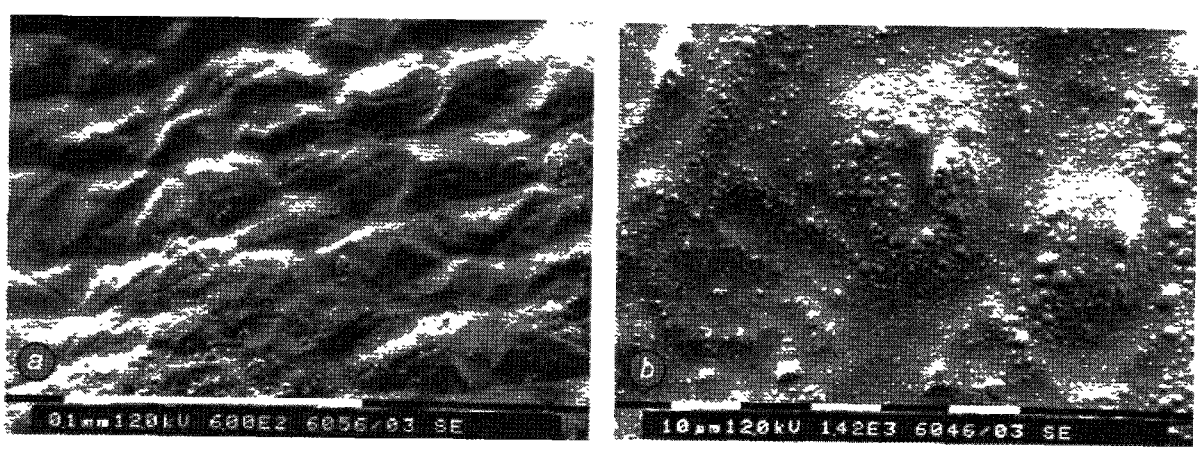

Fig. 5: *Surface structure of the HTST roasted coffee bean. 5a: Immediately after roasting (scale bar: 100 µm). Smooth and even surface. 5b: 24 hours after roasting (scale bar: 10 µm). Surface of a single cell showing the initial stage of oil migration: Hundreds of little oil droplets emerge on the surface.*

The pore size distribution in roast coffee was dominated by small micropores in a very narrow diameter range of 20 to 50 nm (Figure 6). Access for mercury to the cell lumina is provided by small micropores in the cell walls. Therefore, only a high pressure corresponding to the small size of the entrance pores allows for mercury-penetration of the cell lumina. Consequently, high values for apparent pore volume of micropores of the cell wall were obtained, while this corresponded to the filling of the cell lumina. Hence, the pore size at the maximum of the distribution function (r_{main}) represents the size of cell wall micropores. The overall cumulated pore volume is represented by the end point of a porosimetric curve.

As the bean volume increases during roasting greater values for cumulated pore volume were observed (Figure 6). Moreover, with higher degrees of roast greater values for r_{main} were found. Therefore, an enlargement of the cell wall micropores and a trend to a narrower size distribution during roasting can be stated.At equal degree of roast porosimetric curves were also influenced by the roasting conditions (Figure 7). High temperature roasted samples showed significantly greater values for r_{main} than low temperature roasted samples (13.45 nm versus 11.22 nm). A substantial difference between the two roasting processes was also found for the overall cumulated pore volume.

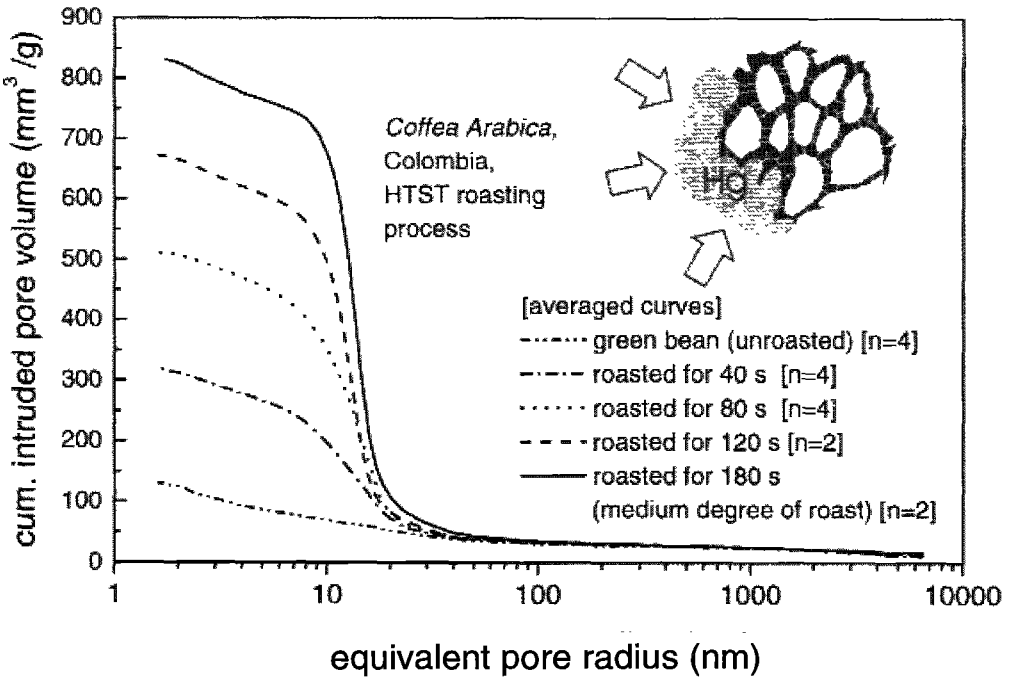

Fig. 6: *Development of bean microporosity during HTST roasting. Greater values of overall cumulated pore volume and greater r_{main} values (point of sharpest increase in porosimetric curves) are observed with higher degrees of roast. Access for mercury to the cell lumina is provided by small micropores in the cell walls (illustration on top right).*

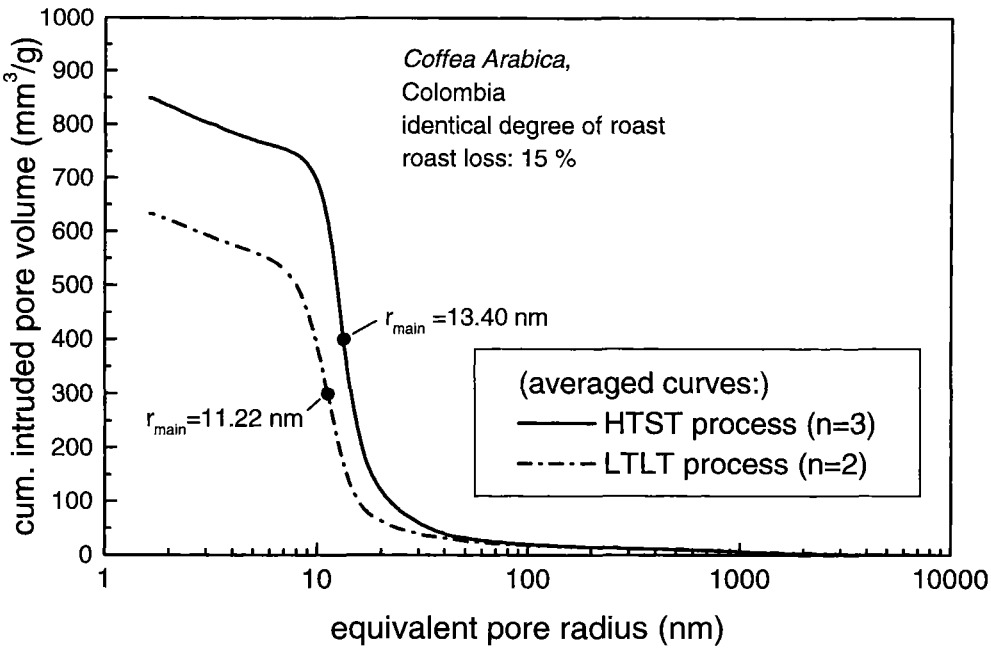

Fig. 7: *Dependancy of bean microporosity on the type of roasting process. High temperature roasted beans show greater overall cumulated pore volume and greater values for* r_{main}.

Conclusions

Mercury porosimetry is a valid and useful method for investigating pore structure of roast coffee. Cell wall micropores in the range of 20 to 50 nm diameter allow for mercury-penetration of the cell lumina. An enlargement of cell wall micropores takes place during roasting. At the same degree of roast, roasting conditions have a major impact on the structural product properties of coffee beans. High-temperature roasted beans exhibit greater bean volume, cumulative pore volume and larger micropores in the cell walls as compared to low-temperature roasted coffees. Larger micropores may promote faster gas desorption and oil migration as well as enhanced oxygen accessibility and accelerated loss of flavor compounds. The origin and structural organisation of the cell wall micropore network remains unclear.

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Summary

Hot air roasting of coffee beans not only forms color and flavor compounds, but also leads to a profound alteration of the bean microstructure. The resulting pore structure controls mass transfer phenomena during roasting and storage. The principal objective of the present project was to investigate the influence of different roasting conditions on volume increase and pore structure development. Coffee beans were roasted in two different, well-defined processes to equal degree of roast. Volumetry, mercury porosimetry and electron microscopy were employed to study structural product properties. The roasting conditions were found to have a major impact on microstructure. High-temperature roasted coffees had greater bean volume, pore volume and larger cell wall micropores as compared to low-temperature roasted beans.

On-Line Analysis of Food Processing Gases by Resonance Laser Mass Spectrometry (RREMPI-TOFMS): Coffee Roasting and Related Applications

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Introduction

The combination of laser induced resonance-enhanced multiphoton ionization (REMPI) and time-of-flight mass spectrometry (TOFMS) represents a highly selective as well as sensitive analytical technique, well suited for species selective real-time on-line monitoring of trace-products in complex gas mixtures as e.g. in combustion flue gases [1]. Here we discuss a few applications of a newly developed mobile REMPI-TOFMS device, for direct on-line monitoring of target volatile compounds, on the released of volatiles during food processing. The typical rich flavor of coffee is mainly formed by the mild pyrolysis of the green coffee beans during the coffee roasting process. One class of typical flavor compounds are the substituted phenols like guaiacol or 4-vinylguaiacol. These are thermal decomposition products of quinic acids, which are present at relative high levels in green coffee. The 4-vinylguaiacol molecule for example is the decarboxilation product of ferrulic acid. With increasing degree of roasting the chemical environment in the coffee beans changes and other chemical reactions occur. Therefore, the relative pattern of the phenolic compounds in the pyrolysis gas characteristically changes with the roasting time. REMPI-TOFMS allows a selective on-line detection of phenolic compounds and other flavor active substances (depending on the laser wavelength). From the pattern of the phenolic compounds the roast-degree can be determined. Thus REMPI-TOFMS can be readily applied for on-line process monitoring in food industry, for instance for quality control and feed-back steering of roasting, baking or fermentation processes.

Experimental

On-line measurements of trace compounds in complex exhaust gases require methods that combine selectivity with sensitivity. A soft ionization without fragmentation is decisive for successful on-line mass spectrometry. The combination of laser induced resonance-enhanced multiphoton ionization (REMPI) and time-of-flight mass spectrometry (TOFMS) represents a highly selective and sensitive two-dimensional analytical technique. The REMPI selectivity can be tuned from substance class selectivity to isomer selectivity by variation of the laser wavelength and the molecular beam source. The MPI-technique combines UV-spectroscopy and mass

spectrometry [2-5]. The intensity of e.g. a two-photon absorption is enhanced by several orders of magnitude if the wavelength of the laser is in resonance with the excitation energy of an UV-transition of the target molecule. The intermediate states of target molecules can be selectively excited by absorption of a laser photon (i.e. the wavelength of a tunable laser is set in resonance with the respective UV-transition). They are typical for each compound. The excited molecules subsequently are ionized by absorption of an additional laser photon if the energy of the absorbed two photons exceeds the molecular ionization potential. By the resonance condition, the laser UV-spectroscopy is directly involved into the ionization process. Thus the high selectivity of REMPI-TOFMS instruments allows even a direct, on-line monitoring of trace chemicals from real-world samples (e.g. on-line monitoring of traces of organic compounds in coffee roasting off-gases [2, 6] or waste incineration off-gases [7]).

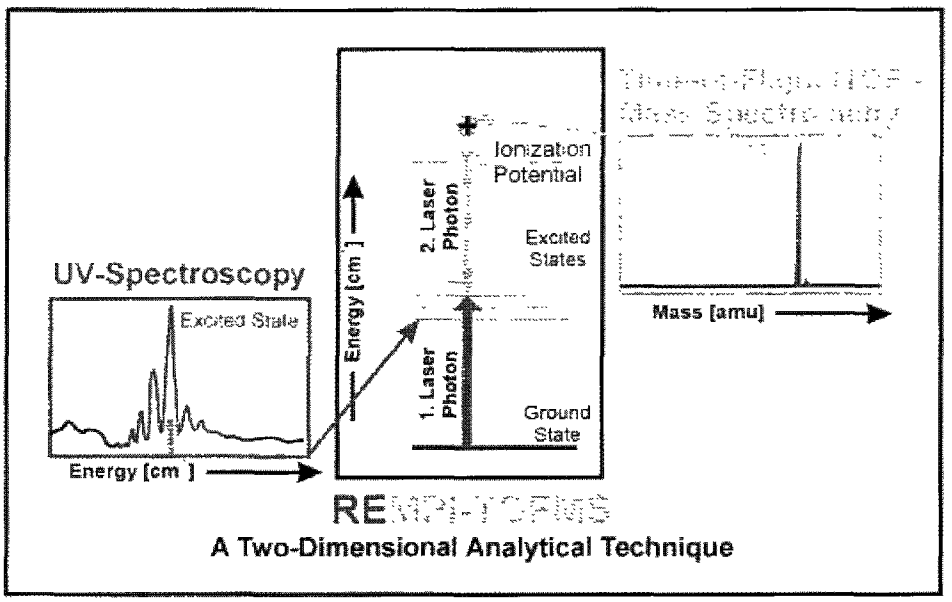

Figure 1: *Schematic representation (energy diagram) of the resonance-enhanced multiphoton ionization process (REMPI, middle). Two-photon REMPI ionization occurs when the laser photon energy (i.e. the wavelength) is tuned in resonance with an UV-spectroscopic transition of the molecule of interest and if the energy of two laser photons exceeds the ionization potential. Via the resonance condition the selectivity of laser UV-spectroscopy is involved into the ionization process (schematic UV spectrum, left). REMPI is a soft ionization technique. This means that most compounds are ionized without or only with marginal fragmentation. The formed ions subsequently are mass analyzed in a time-of-flight mass spectrometer (TOFMS). This adds the mass spectrometric selectivity (schematic mass spectrum, right).*

For the REMPI detection of aromatic compounds, usually a one photon resonant / two photon ionization process is used. For molecules the selectivity can be tuned by variation of the temperature of the molecules, which determines the number of populated rotational and vibrational states in the molecular ground state. The temperature is determined by the inlet technique used. Here, two different approaches are possible. If specific compounds or isomers are to be analyzed, the more sophisticated, supersonic molecular jet inlet technique may be applied. However, despite of isomer selectivity simultaneous ionization of other unwanted molecules cannot be suppressed in complex gas mixtures with thousands of compounds. For this reason the second mass spectrometric dimension is necessary, even if a supersonic beam inlet system is used. An other approach is to use effusive molecular beams (no cooling). In opposite to cold molecules, rotational and vibrational states are excited in hot molecules, resulting in broad absorption bands. For a given wavelength a whole substance class may have overlapping absorption bands and will be ionized effectively by REMPI.

Developing a mobile REMPI-TOFMS device for on-line measurements at industrial plants primarily requires the development of a very robust device for operation in a hot, dusty and vibrating environment. Thus using a small and reliable fixed frequency laser with simple and robust effusive molecular beam inlet, is most convenient. A very important part of the instrumental design is the setup of the probing and sample inlet system for real-time on-line acquisition of the complex sample. The gas consists of a variety of organic substances of different volatility

and may be loaded with dust particles. The requirements for direct probing of analyt gas from the roasting off-gas are i) avoiding of condensation of low volatile compounds, ii) minimization of memory effects for the semi-volatile compounds, iii) reduced catalytic activity of the surface and iv) a rugged and reliable design. The sample inlet system is built with quartz glass surface from the source right into the MS. It consists of a quartz glass tube, a quartz wool filter paper and a capillary. The whole inlet system is heatable up to 200°C to avoid memory effects. For target analysis of aromatic hydrocarbons and nitrogen or oxygen containing heterocyclic compounds there are two common commercially available fixed frequency lasers emitting in the suitable range: the KrF-excimer laser with emission at 248 nm and the Nd:YAG, using the forth harmonic at 266 nm. The individual ionization yield for aromatic molecules may be different. For instance toluene can be detected more effectively with the KrF laser while the sensitivity for benzene is higher with the 4th harmonic of the Nd:YAG. Thus many heterocyclic compounds can be ionized with the laser wavelength 248 nm or 266 nm, while the majority of the other compounds like aliphatic hydrocarbons, most carbonyl compounds, inorganic substances as well as many substituted aromatic compounds remain "invisible". The setup for the on-line monitoring or headspace measurements of coffee is schematically depicted in Figure 2. The lower part of the figure shows the laser mass spectrometer with data acquisition.

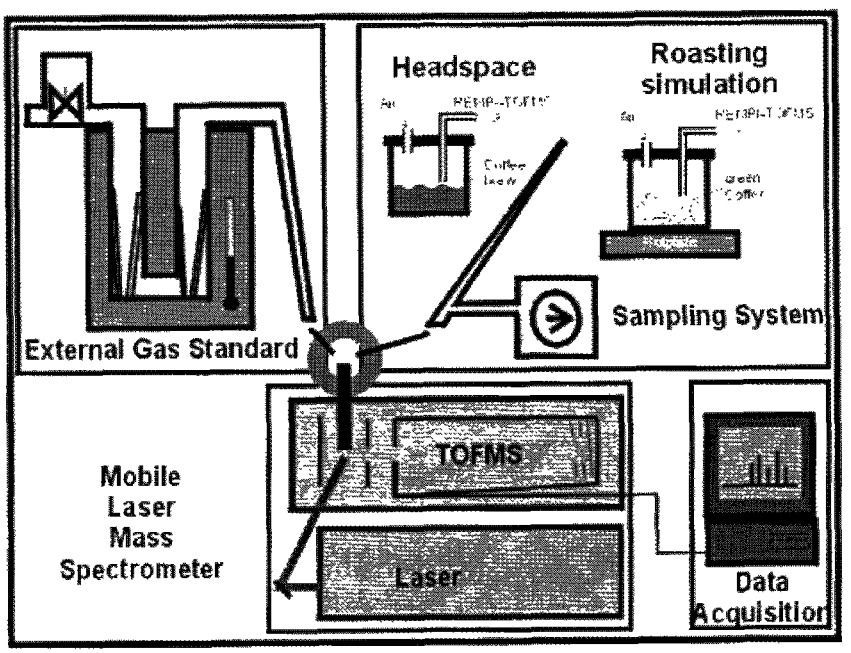

Figure 2: *Scheme of the laser mass spectrometer with data acquisition (lower part), the calibration gas standard (upper left) and the headspace / roasting simulation setup with sampling system (upper right). The heatable sample inlet of the laser mass spectrometer can be coupled to either the dynamic gas standard (diffusion / permeation tubes under constant gas flow in a water bath) or the off-gas sampling system (quartz glass tube and pump depicted) of the experimental setup.*

With the storage of the whole mass spectrometric information, there is no limitation in the subsequent data processing, allowing e.g. addition of n mass spectra for enhancement of the signal to noise (S/N) ratio with the square root of n, and preserving the possibility of finding unexpected peaks in the MS, which is impossible using selected ion monitoring with a limited number of integrators. In these experiments the mass spectra have been recorded with a repetition rate of 10 Hz, corresponding to a time resolution of 100 ms.
The Nd:YAG laser at 266 nm was used for ionization and most of the mass spectra are averaged over 50 laser shots with a laser repetition rate of 10 Hz. With the applied wavelength some aromatic and heterocyclic molecules are ionized. Aliphatic molecules without any chromophores exhibit a much larger energy gap between S1 and S0 (excitation step) and a higher ionization potential. Further on, chromophores have a distinct spectral region of absorption [8]. Therefore most organic molecules can not contribute to the REMPI mass spectrum at a given laser wavelength if they do not fulfill the resonance conditions. For example, at a given laser wavelength, over 95% of the volatiles present in coffee can easily be ruled out by the argument that adequate chromophores for REMPI

ionization are missing. Aromatic molecules in general are likely to be ionized with wavelengths of 248 nm or 266 nm, but many substituted aromatics can be excluded due to spectroscopic reasons. In the mass spectra small peaks due to the bulk constitutions of the sample gas (e.g. N_2 and CO, 28 amu) appear also (see Figure 3). These molecular ions are not formed by resonant multiphoton ionization but by laser induced electron ionization. Since the laser crosses the molecular beam right under the needle tip electrons are released by photons from the edge of the laser beam.

The whole instrument, including the laser, vacuum pumps and electronics is setup in a rack with 0.8 x 1.0 m length and 1.5 m height. The weight is about 150 kg and the box has wheels and hooks for crane lifting. Additionally a PC is required for data acquisition. The modular setup can be equipped with compact Excimer or Nd:YAG Lasers and an additional tunable dye laser.

Results and Discussion

A typical averaged REMPI @ 266 nm – TOFMS mass spectrum of arabica coffee roasting off-gas is shown in Figure 3. As mentioned above, the phenolic compounds are very prominent in the REMPI @ 266 nm mass spectrum. Several phenolic compounds as well as nitrogen or oxygen containing heterocyclic compounds are detectable (e.g. phenol, cresol, dihydroxybenzene, guaiacol, 4-vinylguaiacol, 4-ethylguaiacol, indole, methylindole, caffeine, phenylacetaldehyde, furfurol,...). Note that there are several hundered compounds present in the coffee roasting off-gas within the ppb to ppm concentration range [6]. A non selective ionization technique would generate congested mass spectra. The compounds are identified using the molecular mass and the REMPI wavelength as parameters. The coffee roasting simulation was performed in a heatable quartz glass tube. The roasting temperature in the tube was maintained at about 200 °C (Figure 3) or 400 °C (Figure 5). Four green arabica coffee beans were placed in the hot tube. A sample of the roasting off-gas was taken continuously at the top of the tube and immediately analyzed in the REMPI-TOFMS device.

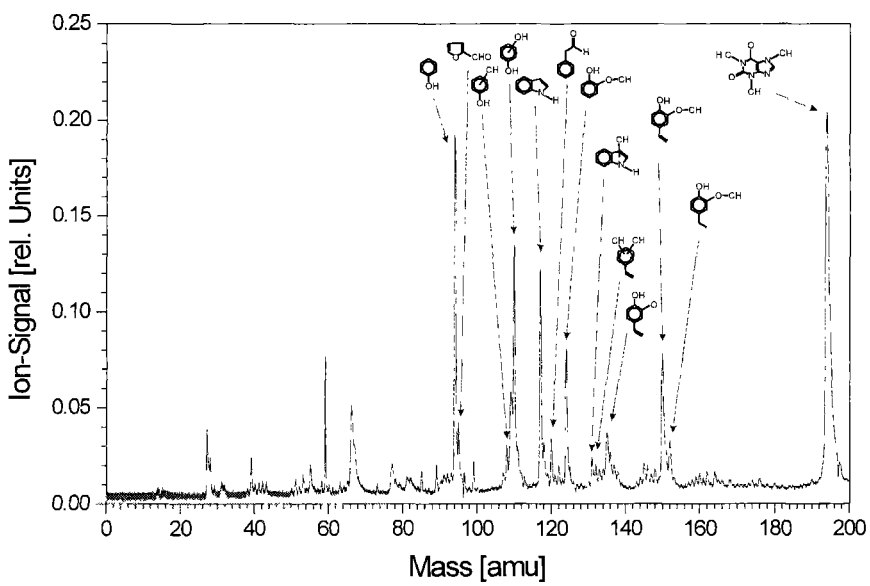

Figure 3: *Averaged mass spectrum of a coffee roasting simulation at 200 °C recorded with REMPI @ 266 nm. Several phenolic (like phenol, cresol, dihydroxybenzene, guaiacol, 4-vinylguaiacol, 4-ethylguaiacol,..), nitrogen (like indole, methylindole, or caffeine) and oxygen (phenylacetaldehyde, furfurol) containing heterocyclic compounds are identified.*

Some phenolic compounds which are partly flavor active exhibit a characteristic release dynamic during the roasting process. This effect can be used for an on-line determination of the roast degree. Figure 4 shows the time-intensity profiles of the phenol and the 4-vinylguaiacol signals during a coffee roasting simulation. The two compounds exhibit characteristic and significant differences in the time-intensity profile. After an initial latency

period, caused by the warming up time of the beans under water vaporization, the concentrations of the phenolics start to increase. Firstly, the 4-vinylguaiacol concentration rises, slightly delayed also the phenol. The considered phenolic components are generated from the decomposition of the polymeric lignocellulose [9] as well as, predominantly in the case of coffee roasting, from degeneration of chlorogenic-, caffeic-, ferrulic- and quinic acids [10, 11]. These organic acids are present in relativly high amounts in the green coffee beans and readily decarboxilate and further decompose under mild pyrolytic conditions [11]. Later, the 4-vinylguaiacol concentration drops again, probably caused by a decreasing formation rate due to precursor depletion and thermal decomposition. One likley decomposition pathway is the abstraction or reduction of the vinylic group, leading to guaiacol or ethyl guaiacol, respectively. The REMPI-signal of phenol remains high throughout the experiment time. The ratio between the signals of phenol and 4-vinylguaiacol represents an indicator parameter that reflects the actual degree of roasting. This is in coincidence with literature data [11]. This behavior suggests an application of the technique for a real-time on-line control of technical coffee roasting processes. In future a feedback steered process control involving REMPI-TOFMS data might be possible.

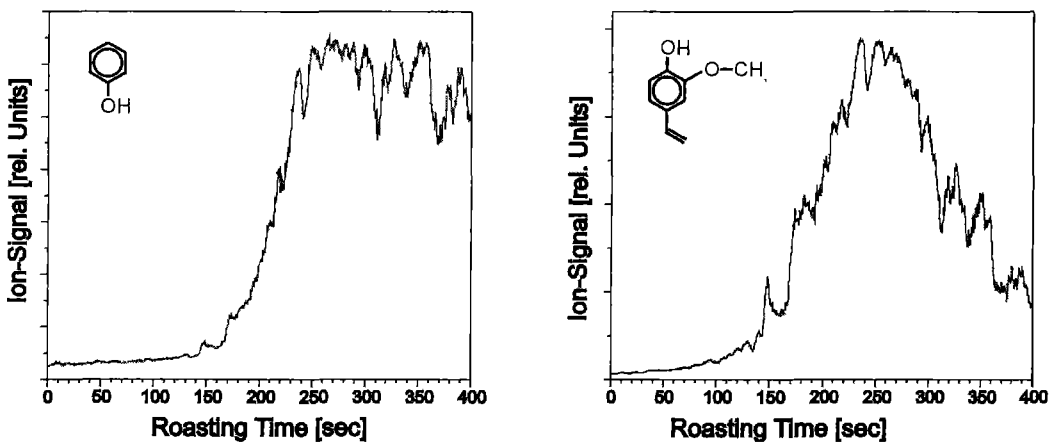

Figure 4: *REMPI @ 266 nm -TOFMS time-intensity profile of phenol and 4-vinylguaiacol measured during a coffee roasting simulation. The different time-intensity behavior of these compounds suggest an application of the REMPI-TOFMS technique for real time on-line control of technical coffee roasting. The relationship between phenol and 4-vinylguaiacol is already known as an indicator parameter for the roast degree.*

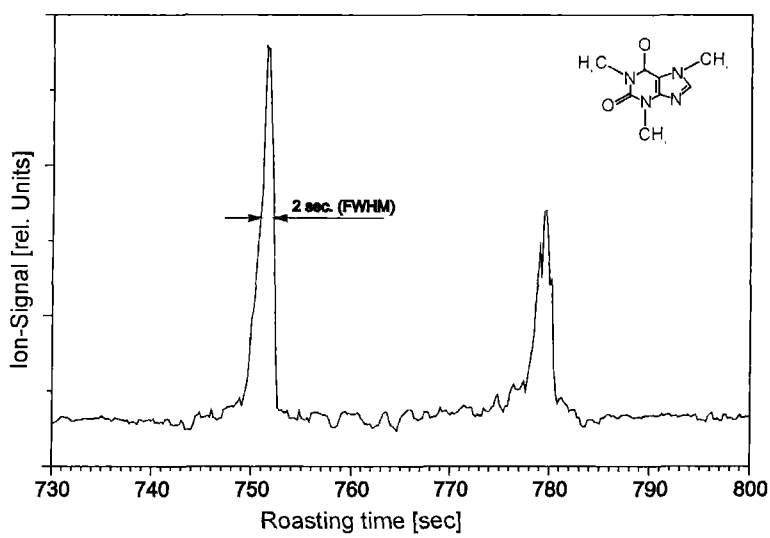

Figure 5: *On-line registered REMPI @ 248 nm – TOFMS time-concentration profile of coffeine measured during a coffee roasting simulation.*

The typical flavor active compounds from roast and ground coffee are not present in the green coffee beans. The multidude of the flavor active compounds first is formed during the pyrolysis of precursor compounds [10-13]. The involved volatilization and decomposition processes and subsequent chemical reactions are very complex (e.g. Maillard reaction or Strecker degradation [11, 12]). However, the involved processes are not stationary, as the temperature and the chemical composition in the green coffee beans changes continuously during the roasting process. Thus also the formation of the pyrolysis products during the coffee roasting process is of highly dynamic and transient nature. An example for the highly dynamic nature is the time-concentration profile of caffeine measured with REMPI @ 248 nm – TOFMS at about 400 °C shown in Figure 5. After a short latency period the beans start to emit cracking sounds, indicating "popping-effects" due to carbon dioxide, mostly formed according to the pyrolysis of organic acids. During each "popping" caffeine enriched carbon dioxide gas is emitted. These highly dynamic caffeine eruption are clearly visible in the time-intensity profile, demonstrating the high time resolution and the appropriate design of the heatable inlet system which avoids memory and catalytic effects in the probing system.

An other application of REMPI-TOFMS is the headspace measurement over brewed coffee. Figure 6 shows the time-concentration profile of 4-vinylguaiacol during brewing of roast and ground coffee. For this experiment 20 ml hot water at 90°C were added to 320 mg coffee powder (arabica coffee). The gas phase over the coffee beverage was continuously extracted, directly injected into the TOF mass spectrometer via a capillary restrictor and subsequently analyzed by REMPI-TOFMS. The first 15 sec of the profile represent the coffee powder signal without water. Then the coffee brewing process occurs. As shown in Figure 6 the first contact of the hot water with the powder causes an intense transient release of volatiles ("aroma flash"). The duration of this aroma flash is about 15 sec. After that time the signal decreases to the new equilibration level.

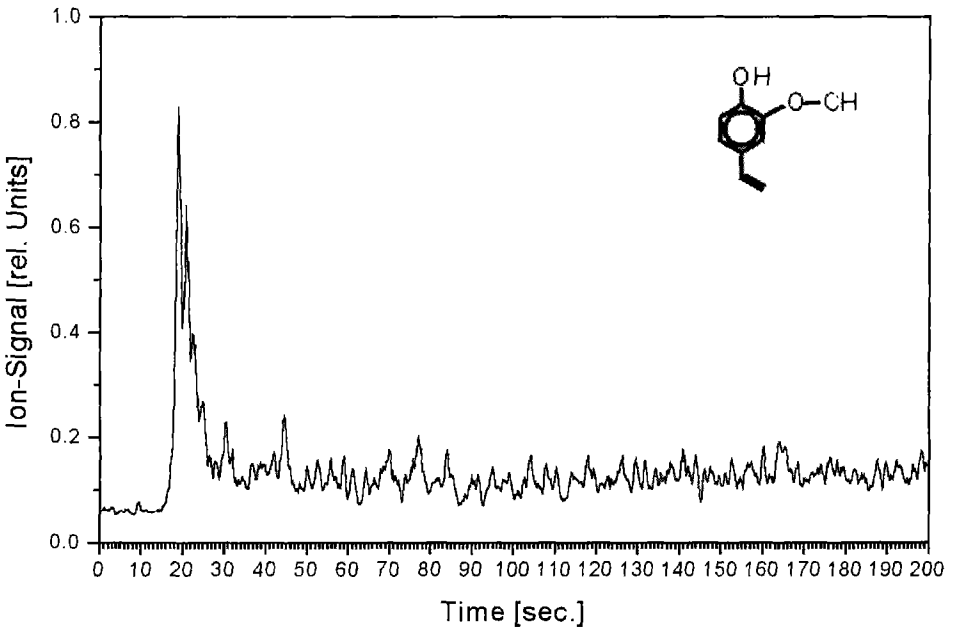

Figure 6: *On-line REMPI @ 266 nm time-concentration profile of 4-vinylguaiacol recorded during a brewing coffee simulation (headspace experiment). The first contact of the hot water with the coffee powder causes an intense transient release of volatiles („aroma flash").*

Conclusion and Outlook

As shown here, REMPI-TOFMS is well suited for on-line real-time measurements in food industrial processes. It is a very fast, sensitive and very selective (depending an the inlet technique) technique. First coffee roasting simulations and headspace measurements demonstrated that REMPI-TOFMS is able to monitor on-line and in real-time several important flavor active volatiles such as aromatics and oxygen or nitrogen containing heterocyclic hydrocarbons. Thus it would be possible to build up a feedback steered process control system with respect on flavor active hydrocarbons.
The technique allows the study of highly dynamic processes. Using the laser wavelength 266 nm for the REMPI-process several phenolic compounds can be detected. The ratio of phenol and 4-vinylguaiacol is identified as an indicator parameter for the actual roasting degree, in agreement with literature data. Further applications in process control, quality management and fundamental research are possible.

Acknowledgements

R. Dorfner thanks the Max-Buchner-Forschungsstiftung for the sholarship.

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Evaluation of a Microwave Sensor for Inline Measurement of Roasted and Green whole Coffee Bean Moisture

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INTRODUCTION

Reducing the standard deviation of a manufacturing process is a typical goal of food manufacturers because it can result in improved product quality and economics. To continuously adjust a process and successfully reduce the finished product analytical standard deviation, knowledge of the current, in-process, analytical properties is required [1]. This knowledge can be obtained through the use of in-line sensors. Success in reducing the standard deviation of food manufacturing processes through application of in-line sensors has been limited for several reasons including the high degree of variability of the agricultural materials used [2]. Within coffee manufacturing, an opportunity exists to reduce the standard deviation of roasted whole bean (RWB) moisture and subsequently ground coffee moisture. This could allow improved conformance to manufacturing specifications and reduced costs. Reduced costs could be achieved through decreasing the amount of product which is rejected for being out of specification and through reduced labor costs for laboratory moisture testing [3]. Better conformance to the moisture specification could also result in improved product flavor quality through increased storage stability [4, 5].

An in-line microwave moisture sensor was evaluated as a method to measure the moisture of green whole bean (GWB) and RWB coffee. A microwave sensor was selected for evaluation due to the reported advantages of the technology. These advantages include selectivity for moisture whether distributed throughout the product or on its surface. The technology is also reported to be independent from variations in product density, surface color, texture, emissivity, and composition [3, 6, 7]. The goal of this work was to determine if the sensor could be used to predict GWB moisture for purchasing or quality measures or to predict RWB moisture. RWB moisture knowledge could be used in a feedback loop to control the amount of quench water added to the RWB at the exit of a roaster. This paper summarizes the learnings and observations from the calibration of the in-line microwave moisture sensor for this application.

MATERIALS

Five lots of Arabica coffee beans were used. The five lots were made up of three lots of Colombian and two lots of Brazilian GWB. The Tews Elektronik MW2300 (Hamburg, Germany) microwave moisture measuring system was used to develop the calibration and validation curves in this work. Different batches of coffee beans were prepared for the development and validation of the GWB and RWB calibrations.

EXPERIMENTAL

GWB Drying

To develop the GWB calibration curve, the moisture content of the GWB was decreased from the initial moisture to the target moisture by slowly evaporating moisture from the beans in 1500 gram batches. The GWB were placed in a 12.75" long, 8.5" wide, 2" deep glass baking pan and spread to a uniform thickness of approximately 1.5" depth. The pans were then placed one at a time into a model 1630 VWR Scientific (Greenbelt, MD) atmospheric pressure oven, which was preheated to 70°C, until the required loss of moisture was achieved. The GWB were then cooled by convection at approximately 24°C. The GWB were then placed in a 2.5 gallon sealed glass jar which was left undisturbed at approximately 24°C for 72 hours to equilibrate.

GWB Moisturizing

Similarly, the moisture content of GWB was increased by adding the required amount of distilled water to 2000 gram batches of GWB and continuously mixing in a sealed 2.5 gallon glass jar on a model RM101 jar rotator (U.S. Best, New Albany, IN) for 30 minutes until the water was absorbed. The GWB were then left undisturbed at approximately 24°C for 72 hours in the sealed jar to equilibrate.

Roasting

GWB were roasted in a Neuhaus Neotec (Bremen, Germany) model RFB-6 pilot scale roaster which had been preheated to 285°C inlet air temperature. The roaster was charged with 2500 gram batches. Upon completion of roasting, the RWB were discharged to the cooling section and automatically quenched with non-distilled water. The application of quench water varied between 19 and 25 seconds to achieve the desired range of RWB moisture. The RWB were placed in a sealed 2.5 gallon glass jar and held until measured.

Calibration Development and Validation

After completing the moisture equilibration, the GWB were filled into the measurement cavity of the sensor and measured, as specified by the sensor manufacturer, to develop and validate the calibration. The value reported by the sensor for each calibration sample, a measure of microwave attenuation and phase shift, was plotted against the moisture of the sample as measured by the reference laboratory method. Sensor validation samples were measured in triplicate and averaged to minimize the standard deviation of the sensor.

At the completion of each roast batch, the RWB were filled into the measurement cavity of the sensor and measured, as specified by the sensor manufacturer, to develop and validate the calibration. The value reported by the sensor was plotted against the moisture of the sample as measured by the reference laboratory method. The RWB were measured for calibration development at the following time intervals: 1 minute, 1 hour, and 24 hours after roast. Validation samples were measured in triplicate and averaged to minimize the standard deviation of the sensor.

GWB and RWB Moisture Determination

The moisture content of the GWB and RWB was determined by atmospheric oven method. 10 grams of GWB or RWB were weighed into a 57mm Fisher Scientific (Pittsburgh, PA) aluminum weigh dish and placed into a model #1630 VWR Scientific oven which was preheated to 107±2°C. The samples were dried for 16±1 hours and then placed in a desiccator to cool for 15 minutes. The total weight loss on drying was assumed to be moisture. Samples were analyzed in triplicate and averaged to minimize the standard deviation of the method. The average oven method standard deviation for GWB and RWB was 0.087 and 0.093, respectively.

RESULTS

GWB Calibration

The sensor was successfully calibrated for Arabica GWB coffee. The calibration curve was approximately linear across the calibration rage of 6.5% to 14.5% moisture (Figure 1). The resulting moisture prediction curve was linear with a slope of approximately 1.0 and an intercept of approximately zero for a plot of predicted moisture vs. oven method actual moisture (Figure 2). Triplicate measurement of GWB with the sensor resulted in a standard deviation of 0.084 for the sensor. The standard error of prediction (SEP) was 0.126 and the root mean standard deviation (RMSD) was 0.138 compared to the reference oven method.

RWB Calibration

Calibration of the sensor for Arabica RWB was unsuccessful. The calibration curve for RWB measured 1 minute after roast (Figure 3) was concave in the region of less than 5% moisture and was relatively flat, with a slope approaching zero, in the region of primary interest, 5 to 7% moisture. The inadequate slope resulted in a large change in predicted moisture for a small change in microwave attenuation. This resulted in poor prediction of RWB moisture by the sensor (Figure 4). Triplicate measurement of RWB with the sensor resulted in a standard deviation of 0.125 for the sensor. The SEP was 0.321 and the RMSD was 0.514 compared to the reference oven method with a bias of approximately 0.3% over the range of 3.5 to 6.5% moisture.

The shape of the calibration curve, surprisingly, changed when the same RWB were measured 60 minutes and 24 hours after roast. The calibration curve was approximately linear for RWB measured 60 minutes after roast (Figure 5) and was convex for RWB measured 24 hours after roast (Figure 6). These findings concur with unpublished work performed at Kraft Jacobs Suchard on Arabica and Robusta RWB coffee [8].

Table 1: Calibration Statistics Summary and Moisture Prediction vs. Reference Oven Method

Calibration Statistics Summary	***Green Whole Bean***	***Roasted Whole Bean***
Reference Method Standard Deviation	0.087	0.093
Sensor Standard Deviation	0.084	0.125
Sensor SEP vs. Reference Method	0.126	0.321
Sensor RMSD vs. Reference Method	0.138	0.514

For a Desired Confidence Interval, the True Moisture is Within:	***Green Whole Bean***	***Roasted Whole Bean***
67% Confidence Interval (± 1 RMSD)	Predicted Moisture ± 0.14	Predicted Moisture ± 0.51
95% Confidence Interval (± 2 RMSD)	Predicted Moisture ± 0.28	Predicted Moisture ± 1.03
99% Confidence Interval (± 3 RMSD)	Predicted Moisture ± 0.41	Predicted Moisture ± 1.52

Figure 1: GWB Calibration

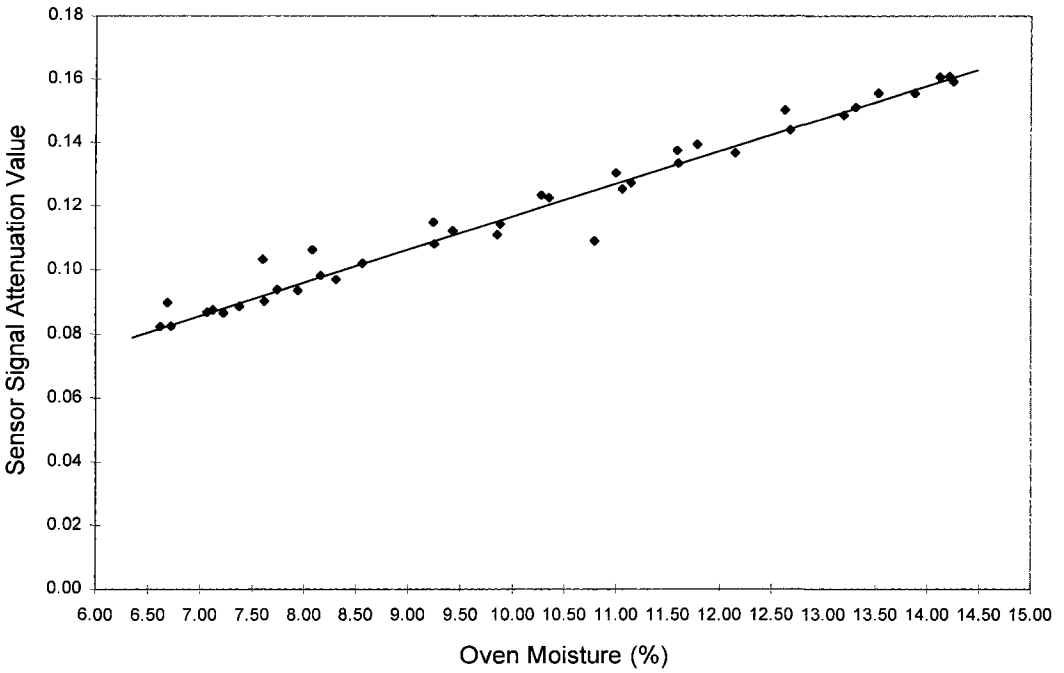


Figure 2: GWB Moisture Prediction

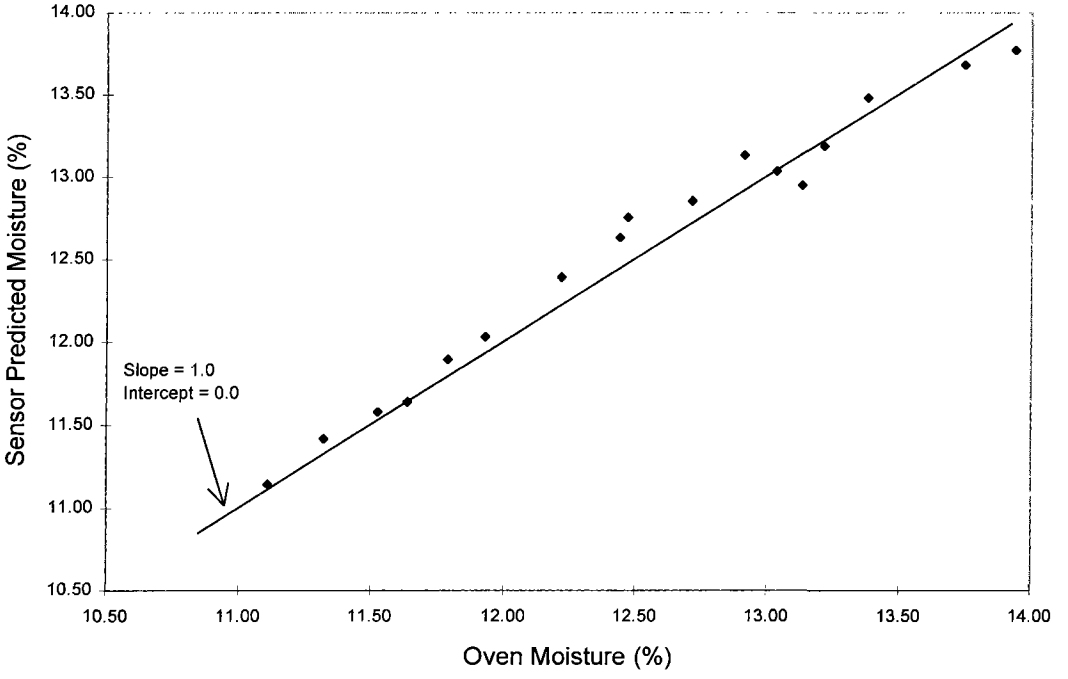


Figure 3: RWB Calibration, 1 Minute After Roast

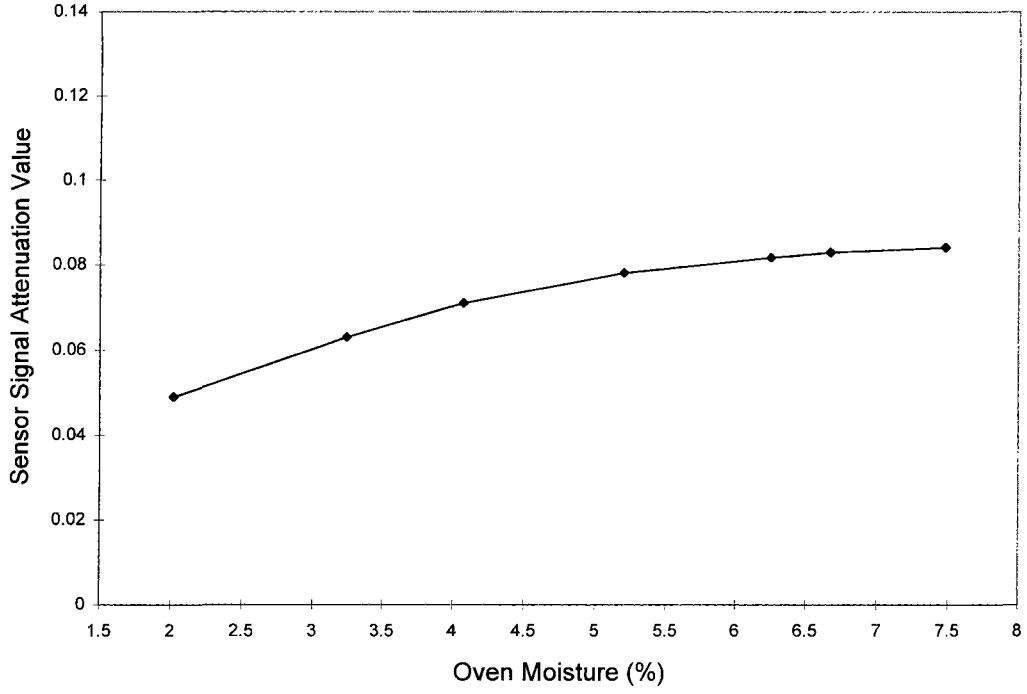


Figure 4: RWB Moisture Prediction

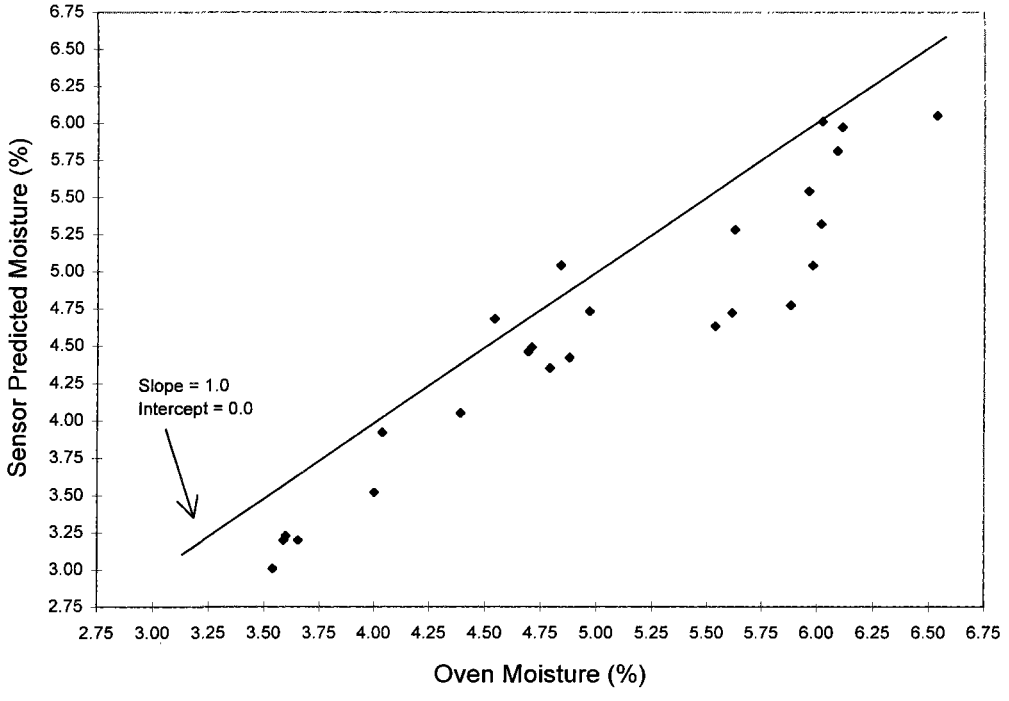


Figure 5: RWB Calibration, 60 Minutes After Roast

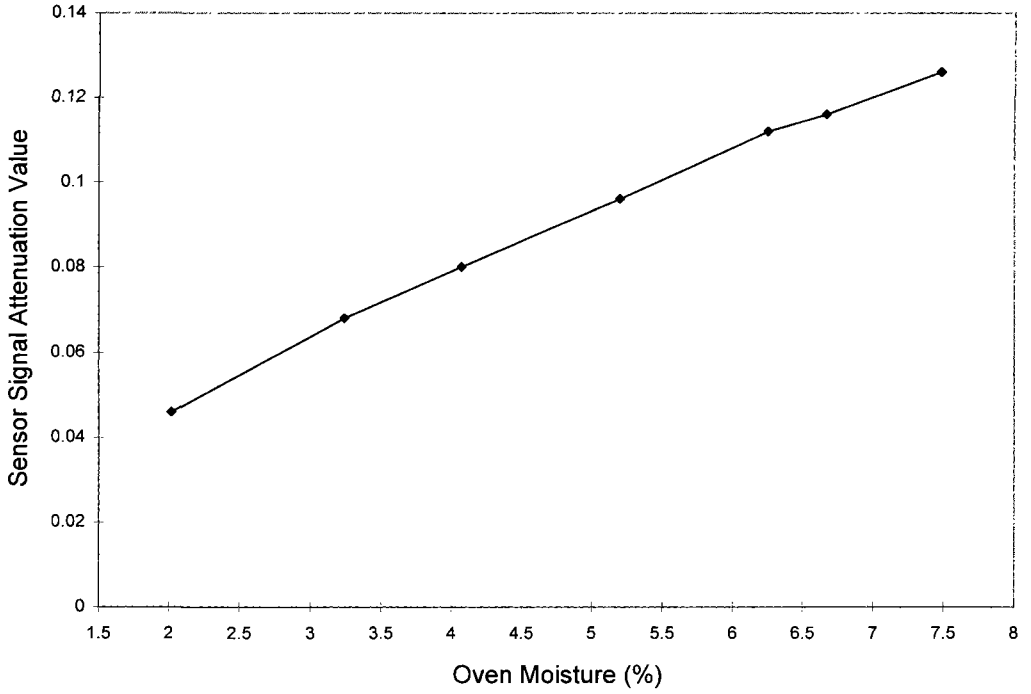
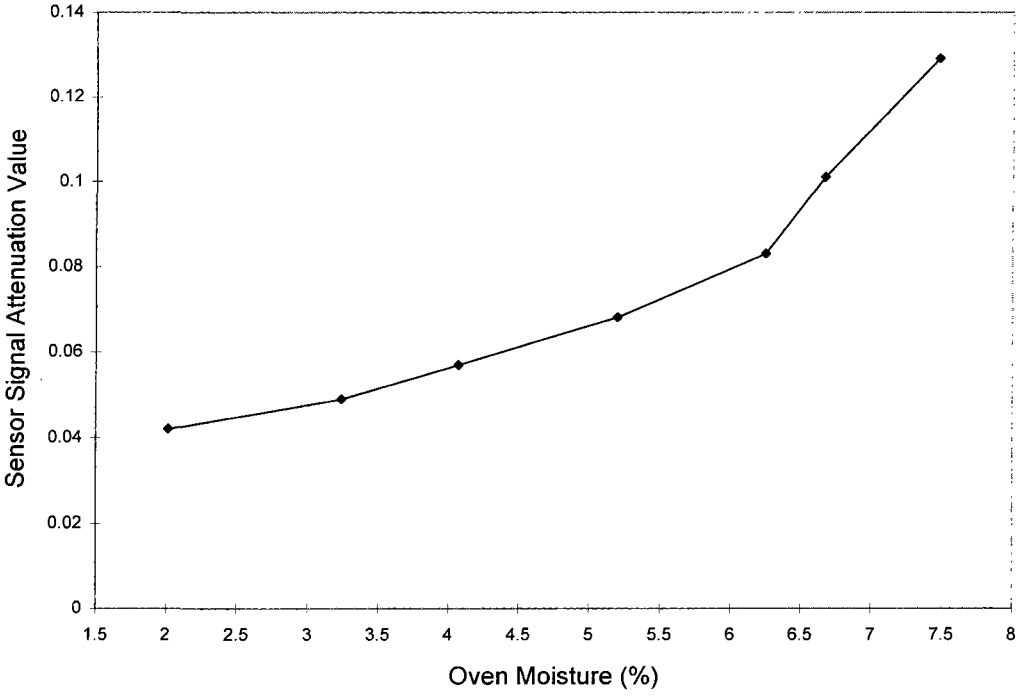


Figure 6: RWB Calibration, 24 Hours After Roast



CONCLUSIONS

The in-line microwave moisture sensor was successfully calibrated to measure the moisture of GWB Arabica coffee. The SEP was 0.126 and the RMSD was 0.138 compared to the reference oven method. Therefore, for a three sigma, or 99%, confidence interval, the sensor provided a predicted moisture that was within ±0.41 of the true moisture of the GWB sample. Therefore, the sensor could likely be successfully used to predict the moisture of GWB entering a plant or roaster.

Attempts to calibrate the sensor for RWB Arabica coffee were not successful. The calibration slope was approximately zero in the range of 5 to 7% moisture. The SEP was 0.321 and the RMSD was 0.514 compared to the reference oven method with a bias of approximately 0.3% over the range of 3.5 to 6.5% moisture. Therefore, for a three sigma, or 99%, confidence interval, the sensor provided a predicted moisture that was within ±1.52 of the true moisture of the sample. Therefore, the sensor would likely not be used successfully in a feedback control loop to control the amount of quench water added to the RWB at the exit of a roaster.

For unknown reasons, the shape of the RWB calibration curve changed when the same RWB were measured 60 minutes and 24 hours after roast. The calibration curve was approximately linear for RWB measured 60 minutes after roast and was convex for RWB measured 24 hours after roast. This change in the calibration curve may be due to the instrument measuring changes in moisture binding, moisture equilibration, evolution of CO_2, or other chemistry changes within the RWB. Further work is required to determine the cause of this effect and its potential applicability to coffee processing.

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SUMMARY

An opportunity exists to reduce the standard deviation of roast and ground coffee moisture by using in-line sensors to measure and control the amount of quench water added to roasted whole beans (RWB) at the exit of a roaster. An in-line microwave moisture sensor was evaluated as a method to predict the moisture of green whole bean (GWB) and RWB coffee. The sensor was successfully calibrated to predict the moisture of GWB Arabica coffee. Attempts to calibrate the sensor for RWB Arabica coffee were not successful. The calibration slope for RWB measured 1 minute after roast was approximately zero in the range of 5 to 7% moisture. For unknown reasons, the shape of the RWB calibration curve changed when the same RWB were measured 60 minutes and 24 hours after roast. The calibration curve was approximately linear for RWB measured 60 minutes after roast and convex for RWB measured 24 hours after roast. This change in the calibration curve may be due to the instrument measuring changes in moisture binding, moisture equilibration, evolution of CO_2, or other chemistry changes within the RWB. Further work is required to determine the cause of this effect and its potential applicability to coffee processing.

The Relation between Volatile Retention and Movement of Ice Front during Freeze Drying of Coffee

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Abstract

This paper relates mass transfer of water with the desorption of certain aroma compounds during the freeze drying of coffee. A computer-based model simulates the movement of the ice front during the sublimation stage of the freeze-drying. Results from an experimental study on the retention of some aroma compounds are presented. The data are analyzed on the basis of the existence of a relationship between the movement of the ice front and the amount of desorbed volatile compounds.

The experimental procedure involved removing a majority of the aroma compounds present in the coffee samples using vacuum distillation. Five selected aroma compounds were then added to the stripped coffee, four of which can be naturally found in roasted coffee and a fifth was used as a marker. Coffee solutions, having the same amount of added aroma per kilogram of solids, were freeze dried in trays under different processing conditions (pressure, heating temperature, freezing rate, initial solid contents, and thickness of the sample), using a pilot plant scale equipment. The retention of each volatile was quantified using GC-MS.

The results obtained are useful to observe the influence of the process parameters on the retention of each volatile compound. More information on the diffusion coefficients of each aroma compound in the coffee is needed in order to complete a mathematical model for volatile retention.

Introduction

Freeze drying process essentially involves consists a product and removing the ice by sublimation under reduced pressure and at low temperature. There are two different stages of the drying. In the primary stage, the ice sublimes leaving behind a porous layer. The secondary stage commences when all the ice disappears (Mellor 1979). In this latter stage, the water that did not freeze earlier desorbs through the pores of the material.

Soluble coffee is one of the most successful freeze-dried products available in he market. The retained aroma is the main reason for its success. Coffee extract has a large number of organic compounds, which gives it the characteristic flavor and odor. Recent studies on impact odourants of coffee listed twenty-two compounds as potent odourants (Semmerloch P and Grosch 1996). It is known that all the volatile compounds are not being perceived by human senses. It is therefore important to study the retention of volatiles individually, rather than lump their effects together.

Two basic mechanisms have been proposed to explain the retention of volatile compounds during the drying of liquid materials:

- Microregion entrapment (Flink and Karel 1975).
- Selective Diffusion (Thijssen and Rulkens,1969)

Both mechanisms give partial complementary explanations. Microregion entrapment takes a microscopic qualitative approach, while Selective Diffusion build on a mathematical and macroscopic approach. This latter theory can be directly applied to equipment design and process modelling, and this forms the basis of our project.

Selective diffusion theory is based on the differences between the diffusivity of water and volatile compounds in concentrated solutions. The diffusivity of water and volatile compounds are both known to decrease with increasing concentration but the latter has been reported to decrease more sharply (Thijssen 1975))
In order to make prediction of aroma retention during drying, Coumnans et al.(1994) state that it is necessary to have knowledge of the heat and mass transfer equations describing the simultaneous diffusion of water and aroma compounds. These equations must be coupled with the concentration dependence of the difusivities of water and aroma compounds.

This method is somewhat tedious, Bruin(1992) has suggested a method to simplify this approach by making a correlation between drying time and aroma retention. In this paper we wish to explore further the relation between the drying rate during the sublimation stage and the volatile retention.

Materials and Methods

The experimental method broadly involved 4 steps:
- Preparation of the coffee solution stripped of all volatiles
- Adding marker volatile compounds.
- Freeze-drying.
- Analysis of the marker compound.

Preparation of the Coffee Solution.
Coffee solutions were prepared using Nescafe granules. The granules were dissolved in deionized water and concentrated using a pilot plant climbing film evaporator under vacuum. The temperature of evaporation was maintained between 40 and 48 C. The solids content of the coffee solution was increased from 10% to 35%w/w.
In order to remove the maximum amount of volatile compounds, the 35 % solution was rediluted to 10% and concentrated once again. A comparison between the initial and final amount of volatiles can be seen figure 1.

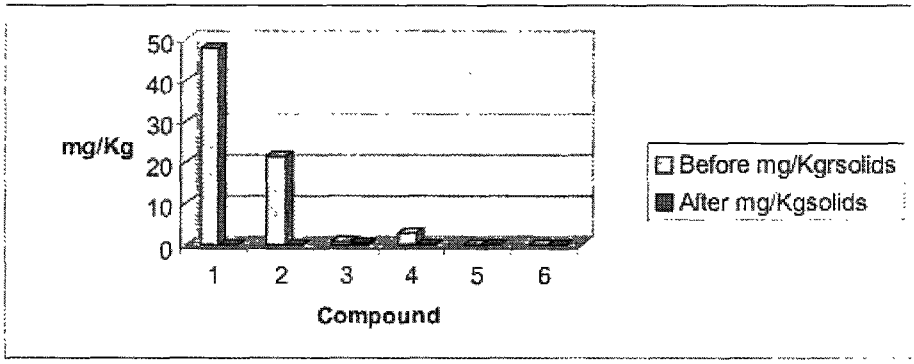

Figure 1. Levels of volatiles before and after distillation of Nescafe.

Addition of the Marker Volatile Compounds.
Six volatile compounds were selected and added to the stripped coffee solutions before freeze-drying. These were: Methylpyrazine, Dimethylpyrazine, Benzaldehyde, Methoxyphenol, 4 Ethylbenzaldehyde and 2 methoxy 4 methylphenol

A stock solution was made up, and the volume added to different coffee solutions was, in all the cases, calculated so as to maintain the ratio of the mass of volatile compounds to that of total solids, constant at 230mg/kg solids. This concentration was selected on the basis of the amount of Guaiacol present in Coffee, which has been described as an impact odourant in brews (Semmerloch P and Grosch W. 1996).

Freeze-drying.
Freeze-drying was carried out in aluminum trays having an area of 0.09 m^2. A pilot plant Stokes freeze dryer was used. Sample was heated by radiation with two plates above and below the tray. The pressure in the chamber was controlled manually by a needle valve. The weight of the sample was monitored using a mechanical balance and thermocouples were used to measure the temperature of the heating plates, the surface of the slab and the temperature at the center of the sample.

A statistical experimental design enabled the evaluation of the effects of different variables: linear, quadratic and interactive. A set of 30 experiments was planned and the variables were allowed to vary in the range described in table 1:

Table 1. Experimental Variables and Their Units.

Parameter	Symbol	[Range]/units
Initial solid contents	Xso	[0.1-0.3]/-
Thickness of the Slab	Th	[0.01-0.02]/mm
Pressure in drying chamber	P	[30-70]/Pa
Heating Temperature	T	[298.15-318.15]/K
Freezing rate	Fr	[3 levels slow,medium,fast]/*

*Slow = 18 hours at –18 C + 6 hours at –35; medium=6 hours at –18 C + 18 hours at –35 C; fast = 24 hours at –35C.

Analysis of Volatile Compounds
A sample of 100 ml was separated from every batch of liquid coffee before freeze-drying. These samples were stored at –35C until they were analyzed. 50 gr. of

freeze-dried samples was also stored at –35C. The volatile compounds were analyzed by using a Gas Chromatograph mass spectrometer. Details relating to the sample preparation for analysis are described by Shcultz et al. (1977).

RESULTS AND DISCUSSION

Genstat version 4.2 for Windows was used to analyze the effect of process variables- pressure, temperature, freezing rate, initial solids content and thickness of the slab- on the drying rate and the volatile retention of each compound. The result of the analysis was an expression similar to the equation 1, that included the effect of each parameter linearly, quadratic and interactive.

$$R = (\sum_{1}^{n} K_j.V_j) + (\sum_{1}^{n} L_j.V_j^2) + (\sum_{1}^{n} M_j.(X.Y)_j) \qquad (1)$$

Where R is the response variable (Drying rate or volatile or amount of volatile compound in the sample); K, L and M are constants Vj the process variable and $(X.Y)_j$ are pairs interactive variables.

The variables that were included in the equation were selected on the basis of the probability associated with the Student's t-value, P(t). The parameters included in the equation were those with a P (t) less than 0.05 (95% confidence interval).

Drying Rate

By applying equation 1 to the experimental data, the following equation was obtained for the drying rate (correlation coefficient = 0.87):

$$dr = 68.161 - 3.301P + 8.98T - 6.36Xso - 2.81Th + 4.74.Fr + 3.98PT + \\ -3.73.PS + 1.58TS - 5.48PTh + 1.32TTh - 6.27PFr + 2.95ThFr \qquad (2)$$

It is important to note that all the parameters were normalized so that their values lie in the range [-1,0,1]. The normalization was done using equation 3.

$$V = \frac{U - \overline{U}}{abs(U - \overline{U})} \qquad (3)$$

where V is the standardized variable, U is the variable to standardize and U is the mean value of the variable.

Equation (2) was used to generate surface and contour plots shown in the figures 2 to 4. Figures 2a and 2b show the adverse influence of the solid content on the drying rate. This could be due to a decrease in pore size formed when the solids content is high. A higher solid content would be expected to improve heat transfer, and therefore the drying rate. This, however, did not happen. It can therefore be concluded that the dominant factor in the process is the movement of vapour, which is hindered due to a decrease in pore size with increased solid content.

In the figures 3b, 3c, it can be seen that the influence of the thickness on the drying rate is very low. The change in drying rate at constant temperature is negligible. This may be due to the presence of a thin insulating layer formed between the tray and the bottom of the frozen sample, which makes the entry of heat through the bottom negligible in comparison with that entering from the top. This result is in agreement with Mellor (1979) and Liapis (1994). The freezing rate unexpectedly showed, slight influence on the drying rate (figure 4a, 4b), an increase in freezing rate increased the drying rate. Thijssen and Rulkens(1969) concluded that a decrease in freezing rate caused an increase in the drying rate It is possible that the differences between the three freezing levels were not high enough to detect any significant changes. The slight increasing trend could be attributed to experimental errors; more experiments are needed to observe the differences between freezing treatments. Pressure shows a big negative influence on the drying rate, this also shows that mass transfer is controlling the process.

Volatile retention

Given that the mass of volatiles added to each sample was exactly the same, the volatile retention between the various samples was determined by comparing the concentration of the compounds retained after drying. Due to the minute quantities of compounds used, it was difficult to distinguish the first two compounds (Methylpyrazine and dimethylpyrazine) from the background noise of the other volatile compounds that remained in the coffee, this can bee seen in figure 5

No significant difference was detected in the retention of Benzaldehyde between the different experimental runs. It is important to note that a small amount of Benzaldehyde remained in the coffee after stripping (figure 1); this shows that it is difficult to remove this compound completely. Further experiments with higher concentrations of Benzaldehyde are necessary in order to confirm this observation.

A Quadratic model was fitted using the data for the retention of 4-Ethylbnzaldehyde and 2-Methoxy-4-Methylphenol. The correlation factor R^2 was low for the Ethylbenzaldehyde (46%). In the case of 2-Methoxy-4-Methylphenol, the correlation factor was much greater at 83%; the equation relating the retention with process parameters is as follows:

$$Ret = 1.642 - 0.0145P + 0.1244T - 0.1306Xso + 0.0898Th - 0.004Fr - 0.1916P^2 + 0.202T^2 - 0.374Xso^2 +$$
$$+ 0.3227Th^2 + 0.1386Fr^2 - 0.2222PS + 0.4665TS - 0.0732TTh - 0.0771SFr - 0.1171ThFr \qquad (4)$$

The strong influence of the quadratic terms in the equation is evident. It can also be seen that the interactive terms containing freezing rate (Fr) affect volatile retention to a greater extern that the linear term in Fr. Regardless, it is evident from 6a, 6b, and 6c that the freezing rate has a weak influence on volatile retention. This, coupled with the earlier observation on drying rate, confirms that the variation between the three freezing levels was not significant enough to observe differences.

The plots in figures 7a, 7b and 7c show that highest retention is obtained under conditions of high T and P values. This suggests that the adverse influence of P on overall volatile retention, as indicated by the negative coefficients in equation 4, is offset by the strong positive influence of T. This contrasts with the earlier observation relating to drying rate where P had a strong adverse influence. From equation 4, it can

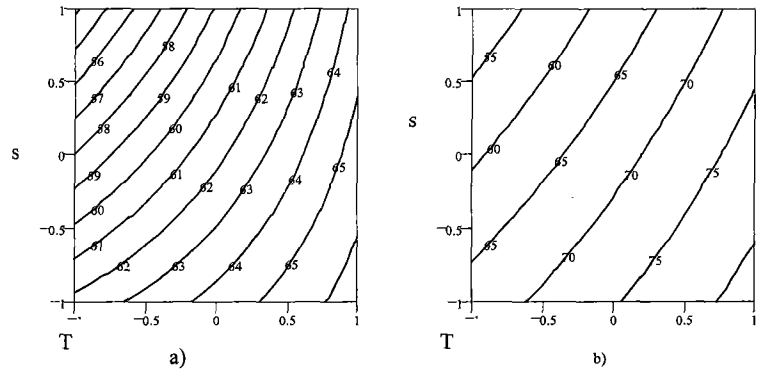

Fig. 2 . Influence of temperature and solid contents on drying rate at different levels of pressure (P), thickness(Th) and freezing rate (Fr). a) P, Th, Fr=-1 b) P, Th, Fr=0

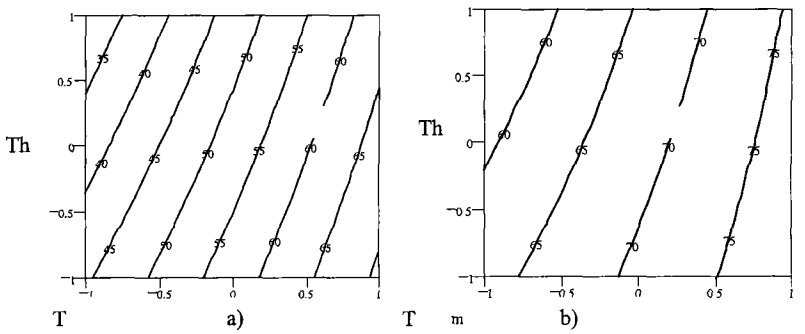

Fig. 3 Influence of temperature and thickness on drying rate at different levels of pressure (P), solids content (s) and freezing rate (Fr). a) P, s, Fr=-1 b) P, s, Fr=0

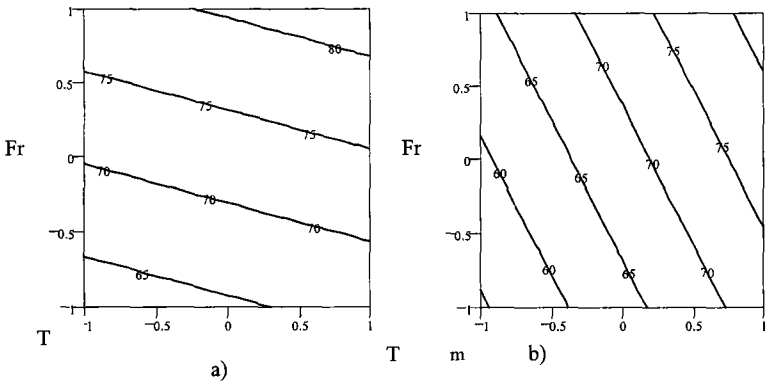

Fig .4 Influence of temperature and freezing rate on drying rate at different levels of pressure (P), thickness(Th) and solids content (s). a) P, Th, s=-1 b) P, Th, s=0

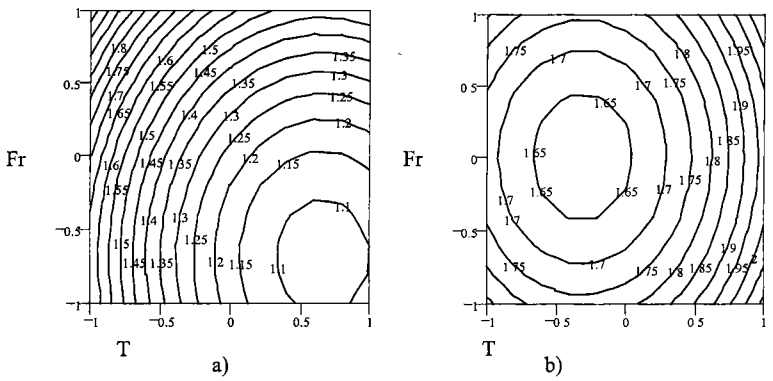

Fig. 6 Influence ot temperature and freezing rate on methyl guaiacol retention at different levels of pressure (P), thickness(Th) and solids contents (s). a) P, Th, s=-1 b) P, Th, s=0

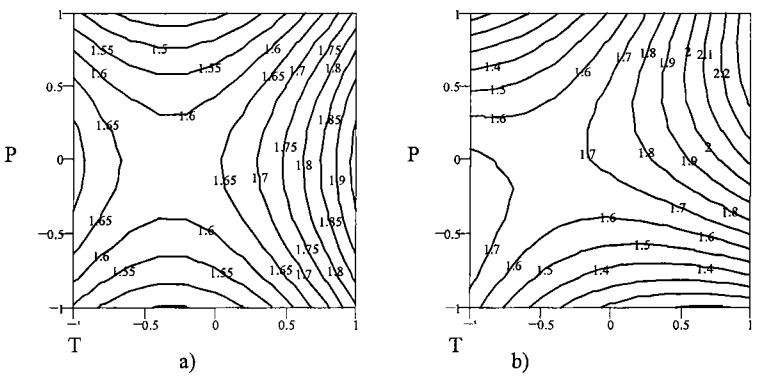

Fig. 7 Influence of temperature and pressure on methyl guaiacol at different levels of solids contents (s), thickness(Th) and freezing rate (Fr). a) s, Th, Fr=-1 b) s, Th, Fr=0

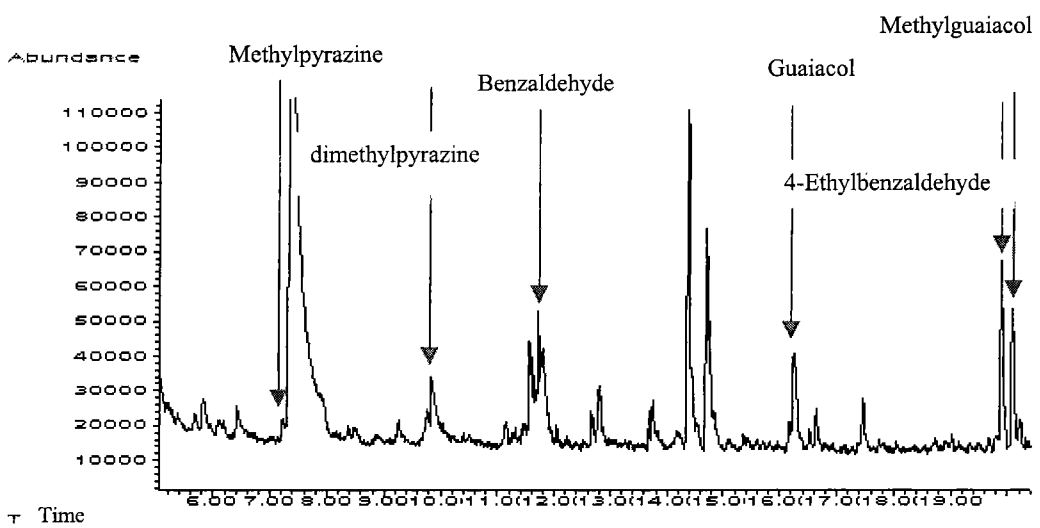

Figure 5.Typical Chromatogram of a Freeze dried sample

be seen that there is an inverse relationship between volatile retention and solid content. This trend is consistent with the relationship between solid content and drying rate reported earlier. Further, in equation 4, the thickness shows a high quadratic dependence. It is not clear, yet, how a greater thickness can increase volatile retention, since its influence on the drying rate is not significant.

Concluding Remarks

Temperature, pressure and initial solid content showed to have strong influence on the drying rate and in the retention of the marker, 2-Methoxy-4-Methylphenol. These three variables affect drying rate and volatile retention in the same way. The interactive effect of the variables on volatile retention is very strong, indeed stronger than their effect on drying rate. In fact, the second order non interactive terms were shown to be insignificant in influencing drying rates.

Freezing rate did not show a significant effect on the drying rate or on the retention of 2-Methoxy-4-Methylphenol under the range of conditions investigated. The possibility of using a wider range of freezing levels should be explored. Thickness did not show significant effect on the drying rate, but showed a strong favorable influence on volatile retention. It is important to find an explanation for this unexpected trend.

It would be desirable to add higher levels of volatile markers in order to confirm the trends observed.

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Kinetics of Coffee Infusion: Determination of the Geographical Origin of Medium Roasted Coffees using Extraction Kinetics

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INTRODUCTION

Coffee beans (Arabica and Robusta) contain a variety of different elements of which potassium represents the largest proportion. In addition other metallic elements found in reasonable quantity are magnesium and calcium while the non-metallic elements are mainly phosphorus and sulphur. The other mineral ions exist in trace amounts. Numerous workers (Ferreira *et al* 1971, Roffi *et al* 1971, Tserevitnov *et al* 1972, Quijano Rico and Spettel 1975) have found that the mineral content of coffee beans depends upon the country of origin, a factor which is associated with the soil conditions. The other reason is the method of processing the green coffee, whether subjected to the wet or the dry method (Ferreira *et al* 1971, Clarke and Walker 1974 and Clarke and Macrae 1985). Clarke and Walker (1975) have shown that soaking of coffee beans during the fermentation and washing stages causes potassium to diffuse out, resulting in a wide range of potassium contents in coffee beans. The mineral constituents in coffee beans are not lost during the roasting process except for sulphur and phosphorus to a small degree (Ferreira *et al* 1971). It is also believed that minerals act as catalysts during roasting and during the various biochemical transformations in the plant. Arabica cannot be distinguished from Robusta by determination of mineral ions as indicated by Kroplien's data (1963). However, recent work (Martin *et al.* 1998) has shown that the mineral content can be related to the origin of the green coffee and be used as a tool for characterising the coffee varieties.

The subject that has received very little attention until recently is the kinetics of extraction of mineral ions (Jaganyi *et al.* in Press). Even with caffeine, there is no comparative study to determine if the rate of extraction is dependent on the coffee species, the blend of coffee or country of origin. The present paper presents a comparative study of the extraction of caffeine and mineral ions from six different geographical regions. It was hoped that the kinetic data could be used to distinguish the origin of each coffee bean or shed some light on the differences or similarities of the beans.

EXPERIMENTAL

The coffee beans investigated were medium roasted Arabica namely; Kenyan Special, Sumatra Blue Mountain Java, Brazilian Santos, Ethiopian Mocha, Zimbabwean coffee and South African Grown. (Colombo Coffee and Tea Co., Durban). A Glen Creston mill equipped with agate mortar and pestle was used to grind the coffee beans which were later

sieved through a standard set of stainless-steel Endecotts sieves, using an Endecotts sieve shaker machine. The sieved fraction between 1.70 and 2.00 mm was selected for the study. All containers used were made of plastic and the water was demineralised Milli-Q-water (Milli-Pore). The cleaning process involved using Extran detergent (Merck) which is phosphate-free, then rinsing the apparatus with a 0.1M HCl (BDH Aristar) solution. This precaution was necessary because of the determination of mineral ions.

A plastic conical flask containing 200 ml of water equilibrated to 80°C in a thermostatically controlled waterbath was used in all the kinetic experiments. The lid of the flask had two holes, one for the thermometer and the second for the sampling tube made out of a thin plastic material. On the outside of the flask the sampling tube was attached to a 2 ml sabre syringe while on the inside, the end of the tube had a hollow plastic cone to which a Gilson filter (Anachem) was inserted so as to exclude coffee beans and particulate during sampling. It has been found (Spiro & Siddique, 1981) that simply dropping tea leaves into hot water tends to give irreproducible results. Because of this 4 g of coffee beans was quickly added to the 200 ml of water via a long wide-spout glass funnel. The stop-watch and the immersible magnetic stirrer attached to the waterbath were started as soon as the coffee was added.

Samples (1 ml) were withdrawn at half minute intervals for the first five minutes and the last equilibrium sample after 90 minutes. The samples taken were transferred into 20 ml plastic sample bottles containing 9 ml of water and mixed thoroughly. After each sampling, the sample tube was cleaned of any solution by attaching a clean syringe and injecting air through it and the filter. Corrections were made to the concentration of the solubles (Spiro & Jago, 1982; Jaganyi, 1992) for volume lost through sampling and evaporation by weighing the flask and the contents before and after each run.

Reverse phase high pressure liquid chromatography (HPLC) was used for the analyses of caffeine. The instrument employed was a Thermo Separation Products fitted with a Spectra System UV 3000 (Scanning) detector which was set at 275 nm. The column used was a Waters Nova-Pak® C18 60Å 4μM which was calibrated with known concentrations of caffeine (Fluka). The mobile phase used was similar to that of Jaganyi and Price (1998). The peak integration was performed automatically by the PC1000 software which operated the HPLC. The mineral ions were analysed using inductively coupled plasma-atomic emission spectroscopy (ICP-AES). The type of the instrument was a Varian Liberty AXISO Turbo. The ICP-AES was calibrated against known concentrations of the various ions and the instrument was set to accept calibration graphs with a correlation coefficient of 0.995 and over.

RESULTS AND DISCUSSION

The variation of caffeine and mineral ion concentration C, with time t, followed first-order behaviour according to the equation

$$\ln\left(\frac{C_\infty}{C_\infty - C}\right) = k_{obs}\, t + a \qquad (1)$$

which is predicted by the steady state theory of extraction (Spiro and Selwood 1984). Here C and C_∞, are the concentrations of the soluble components at any time t and at equilibrium, while k_{obs} and *a* represent the first-order rate constant and the intercept, respectively. Plots of the ln function against time were indeed linear with small intercepts for all the coffees.

The mean rate constants obtained from the slope of the graphs of equation (1) and the half-life values $t_{1/2}$ of infusion determined from equation (2)

$$t_{1/2} = (\ln 2 - a) / k_{obs} \qquad (2)$$

are tabulated in Table 1. Each result is based on at least three independent experiments whose results were found to be reproducible. This is indicated by the standard deviations indicated in parentheses in Table 1. These were calculated using MINITAB a statistical software.

Kinetic data

Statistical analysis (One-way Analysis of Variance) was performed on the equilibrium concentrations as well as the rate constants, so as to determine which of the results were similar or significantly different. The analysis calculates P-factors at two levels, if the value is lower than 0.01 it means that there is a significant difference among the beans. The opposite is true if the factor is greater than 0.05.

The analysis of the present results were based on individual 95% confidence intervals. The caffeine equilibrium concentrations fell into two groups, Kenya and Brazil with a P-factor of 0.28 and Sumatra, Zimbabwe, Ethiopia and S. Africa with a P-factor of 0.31. Combining the two groups together clearly indicated that there was a significant deference between them with a P-factor of 0.00. The concentrations of phosphorous and magnesium showed no significant difference having a P-factor of 0.08 and 0.04 respectively. The concentrations of manganese from Ethiopian beans was very different from all the other beans investigated which had a P-factor of 0.21. The equilibrium concentrations which were found to be dependent on the origin of coffee were those of potassium whose P-factor was zero.

Comparing the equilibrium concentrations shown in Table 1, the amount of potassium leached from the beans was the highest, followed by caffeine. The values for phosphorus and magnesium were next with similar magnitude while manganese had the least. This trend was the same in all the coffees investigated apart from the fact that the potassium concentration of the Zimbabwean coffee whose value (190.6 ppm) was less than that of caffeine. It was also 49% smaller than the highest equilibrium concentration of potassium. The difference between the potassium and the caffeine concentration of the South African Grown coffee was only 4% as compared to ~24% in all the other coffees with the exception of Mocha with 11%. This difference in equilibrium concentration is also observed between the special Kenyan reported here and the Kenyan A (Jaganyi *et al* 1999). The reasons for this difference may be twofold:

Table 1 Mean kinetic data for the infusion of caffeine and mineral ions from different types of coffee beans (1.70-2.00 mm) into Milli-Q-Water at 80°C

Type of Coffee (Country)	Species	C_∞ (ppm)	C_∞ (mM)	k_{obs} ($10^{-3}s^{-1}$)	Mean intercept a	$t_{1/2}$ (s)
Special Kenya (Arabica)	Caffeine	269.8(±21.5)	1.39	2.71(±0.10)	0.11	215
	K^+	365.8(±10.3)	9.36	3.50(±0.09)	0.14	176
	P as ($H_2PO_4^-$)	25.1(±3.8)	0.81	1.89(±0.16)	0.14	293
	Mg^{2+}	28.6(±0.9)	1.18	1.16(±0.10)	0.14	477
	Mn^{2+}	0.39(±0.02)	0.0071	0.98(±0.08)	0.09	615
Santos (Brazil)	Caffeine	253.0(±9.5)	1.30	3.59(±0.14)	0.08	171
	K^+	318.2(±13.7)	8.14	3.95(±0.12)	0.11	135
	P as ($H_2PO_4^-$)	25.9(±2.6)	0.84	2.42(±0.18)	0.13	233
	Mg^{2+}	24.3(±3.1)	1.00	1.55(±0.14)	0.12	370
	Mn^{2+}	0.34(±0.06)	0.0062	0.98(±0.09)	0.16	544
Blue Mountain Java (Sumatra)	Caffeine	292.2(±16.4)	1.50	3.37(±0.12)	0.11	173
	K^+	376.0(±17.8)	9.62	4.23(±0.13)	0.06	150
	P as ($H_2PO_4^-$)	25.2(±1.6)	0.81	2.13(±0.15)	0.13	264
	Mg^{2+}	27.4(±1.2)	1.13	1.25(±0.12)	0.16	427
	Mn^{2+}	0.36(±0.04)	0.0066	0.93(±0.09)	0.20	530
Zimbabwe (Zimbabwe)	Caffeine	295.7(±12.9)	1.52	3.42(±0.13)	0.14	162
	K^+	190.6(±10.5)	4.87	5.66(±0.20)	0.25	78
	P as ($H_2PO_4^-$)	22.3(±1.2)	0.72	2.23(±0.21)	0.17	235
	Mg^{2+}	25.5(±1.0)	1.05	1.24(±0.09)	0.91	406
	Mn^{2+}	0.42(±0.02)	0.0076	0.95(±0.07)	0.13	593
Mocha (Ethiopia)	Caffeine	306.9(±8.1)	1.58	2.72(±0.12)	0.12	211
	K^+	345.4(±12.9)	8.83	3.08(±0.10)	0.13	183
	P as ($H_2PO_4^-$)	27.5(±1.5)	0.89	1.96(±0.19)	0.13	287
	Mg^{2+}	27.4(±1.5)	1.13	1.34(±0.12)	0.09	450
	Mn^{2+}	0.27(±0.01)	0.0049	1.04(±0.10)	0.14	532
S. A. Grown (South Africa)	Caffeine	287.6(±9.2)	1.48	3.06(±0.11)	0.13	184
	K^+	300.2(±14.6)	7.68	4.48(±0.15)	0.13	126
	P as ($H_2PO_4^-$)	22.6(±0.4)	0.73	1.87(±0.13)	0.16	285
	Mg^{2+}	23.5(±2.4)	0.97	1.14(±0.08)	0.16	468
	Mn^{2+}	0.38(±0.03)	0.0069	0.75(±0.05)	0.14	738

First is that the total potassium content in the coffees investigated were naturally very low. Secondly and the most probable explanation as to the low level of potassium is the handling of the green coffee, whether it underwent the wet or the dry process (Ferreira *et al* 1971, Clark and Walker 1974, and Menshu 1967).

Inspection of the results in Table 1 shows that the order of infusion is K > caffeine > P (as $H_2PO_4^-$) > Mg^{2+} > Mn^{2+}. This trend was found to be true for all the coffees investigated. Since kinetic study of caffeine infusion from Kenyan coffee has been reported in the literature, the rate constant obtained with the Special-Kenyan beans will be examined. The k_{obs} of 2.71×10^{-3} s^{-1} obtained, is comparable with $2.67 \times 10^{-3} s^{-1}$ reported by Jaganyi *et al* (in Press) using Kenyan A. Spiro and Selwood (1984) have reported a value of 9.0×10^{-3} s^{-1} for particle size 0.85-1.18 mm at 84.1°C. Since the rate of infusion varies inversely with the square of the particle radius (Spiro and Selwood, 1984: Spiro 1988) and $\ln k_{obs}$ is inversely related to temperature in the Arrhenius equation, the reported value is equivalent to a rate constant of 2.68×10^{-3} s^{-1} using the present experimental conditions of particle size and temperature. The agreement with the results tabulated in Table 1 is very good considering the fact that food products do vary from year to year.

The statistical analysis of rate constants for different species, revealed that the rate constants of P as ($H_2PO_4^-$) were not significantly different (P = 0.12). In case of Mg^{2+}, the values for Brazilian coffee was significantly different from the rest which had a P-factor of 0.21. The analysis also showed that the values for Mn^{2+} were similar for all the coffees (P = 0.61) with the exception of the South African grown. Because of the similarities observed with the rate constants of these three species, they cannot be used for identification of the coffee origin. But the values of caffeine can be used by grouping the different coffees. The statistical analysis for caffeine showed that the rate constants fell into three groups: Kenya and Ethiopia, Brazil,Sumatra and Zimbabwe, with South Africa lying in between as can be seen in Figure 1. This grouping was confirmed by performing a cluster analysis of the rate constants and the dendrograms is shown in Figure 2. The rate constants of K^+ produced a P-factor of 0.00 and showed no clustering, an indication that the individual values were significantly different. This is illastrated in Figures 1 and 2. Therefore, even though Brazil, Sumatra and Zimbabwe coffee beans have been grouped together in caffeine analysis, these can be differentiated by using the rate constants for K^+ which are different. This is also true for the Kenyan and Ethiopian coffees. It can be argued that the rate constants are likely to change from one year's crop to another but if one looks at the rate constant for caffeine from Kenyan coffee which has been studied for many years (Spiro and Selwood, 1984, Jaganyi *et al* 1999) it is clear that the change is very small.

Analysis of Variance for Caffeine(Using Rate Constant)

Source	DF	SS	MS	F	P
Factor	5	2.1040	0.4208	28.89	0.000
Error	12	0.1748	0.0146		
Total	17	2.2788			

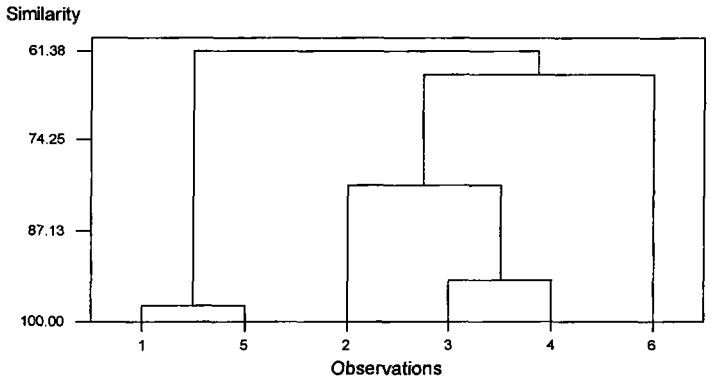
Level	N	Mean	StDev	Individual 95% CIs For Mean Based on Pooled StDev
Kenya	3	2.7100	0.1000	(---*----)
Brazil	3	3.5900	0.1400	(----*---)
Sumatra	3	3.3700	0.1200	(---*----)
Zimbabwe	3	3.4200	0.1300	(----*---)
Ethiopia	3	2.7200	0.1200	(----*---)
S.Africa	3	3.0600	0.1100	(---*----)
Pooled StDev =		0.1207		2.80 3.15 3.50

Analysis of Variance for Potassium (Using Rate Constant)

Source	DF	SS	MS	F	P
Factor	5	11.9359	2.3872	171.40	0.000
Error	12	0.1671	0.0139		
Total	17	12.1030			

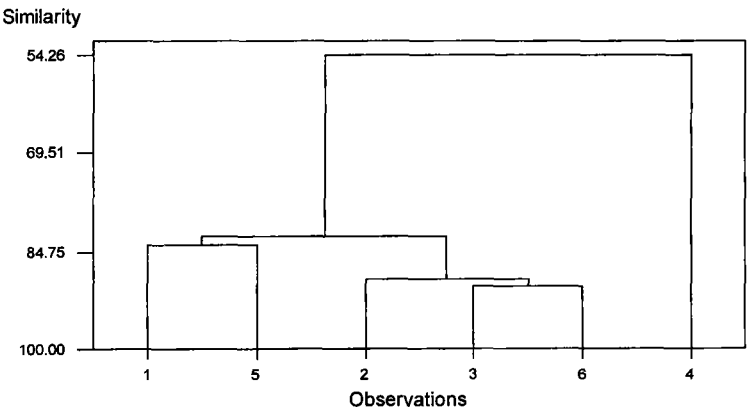
Level	N	Mean	StDev	Individual 95% CIs For Mean Based on Pooled StDev
Kenya	3	3.5000	0.0900	(-*-)
Brazil	3	3.9500	0.1200	(*-)
Sumatra	3	4.2300	0.1300	(-*-)
Zimbabwe	3	5.6400	0.1709	(-*)
Ethiopia	3	3.0533	0.0643	(-*-)
S.Africa	3	4.4467	0.1041	(-*)
Pooled StDev =		0.1180		3.20 4.00 4.80 5.60

Figure 1 One-way Analysis of Variance for caffeine and potassium



Dendrogram for caffeine

1= Kenya; 2 = Brazil; 3 = Sumatra; 4 = Zimbabwe; 5 = Ethiopia; 6 = South Africa



Dendrogram for potassium

Figure 2: Hierarchical Cluster Analysis of Observations using Minitab.

Summary:

Arabica and Robusta are the most important commercial varieties of coffee. Arabicas are considered to be of higher quality and therefore more expensive. Several papers have been published on how to differentiate the two varieties, and the origin of Arabica coffee beans. In search of a reliable method to determine the geographical origin of coffee beans, the present paper evaluates kinetic data on the extraction of caffeine, P as ($H_2PO_4^-$) K, Mg and Mn. The determination of equilibrium concentrations and rate constants of extraction of these species from Medium roasted coffees of particle size range 1.70-2.00 mm from six different geographical regions (Kenya, Brazil, Sumatra-Dutch East Indies, Ethiopia, Zimbabwe and South African) have been examined.

High performance liquid chromatography was used for the analyses of caffeine and inductively coupled plasma atomic emission spectrometry for the chosen elements. The equilibrium concentrations of all the species and the trend were found to be independent of the various coffee beans. The order of the rate of infusion was found to be K^+ > Caffeine > P as ($H_2PO_4^-$) > Mg^{2+} > Mn^{2+}. Statistical analysis (One-way Analysis of Variance) was performed on the equilibrium concentrations as well as the rate constants, so as to determine which of the results were similar or significantly different. The results clearly indicated that the kinetic data for P as ($H_2PO_4^-$), Mg^{2+} and Mn^{2+} couldn't be used for the identification of the coffee origin. But the values for caffeine and K^+ can be used for the identification.

Key words: coffee, caffeine, mineral ions, extraction kinetics.

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Decaffeination of Non-aqueous Solvents using Caffeic Acid

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INTRODUCTION

The ability to use caffeic acid (CA) to decaffeinate aqueous caffeine solutions and coffee extracts was reported at the 17th ASIC Conference in Nairobi [1]. It was demonstrated that addition of excess CA to such solutions results in formation of a nearly insoluble 1:1 molar physical complex with caffeine which is easily crystallized from solution to provide effective decaffeination [2]. This technique has also been employed to decaffeinate non-aqueous solvents utilized in direct decaffeination of coffee beans or extracts [3,4]. This paper summarizes the results of preliminary experiments which demonstrate that CA can be used to significantly increase the decaffeination efficiency of ethyl acetate (EtAc), methylene chloride ($MeCl_2$), and soybean oil (SBO). Solubility data for caffeine, CA, and caffeine-CA in a variety of solvents are also provided.

MATERIALS

All materials used were reagent grade. CA (3,4-dihdroxycinnamic acid; 97% purity; predominantly *trans*) was sourced from Aldrich and recrystallized from water. Caffeine was sourced from Sigma. All solvents except 200-proof ethanol (Aaper Alcohol and Chemical) were sourced from Fisher Scientific. Wesson® SBO was grocery sourced. EtAc and SBO used in decaffeination were saturated with caffeine and water as described below. $MeCl_2$ coffee extracts were prepared by countercurrent extraction of a concentrated 25% wt/wt aqueous extract of roasted coffee at 180°C using 4.5 volumes of $MeCl_2$. The resulting 0.25-0.30% wt/wt extract was vacuum distilled at 35°C to 40-50X concentration to yielded 7-8% caffeine in 10-12% wt/wt total coffee solids. Caffeine-, CA-, and caffeine-CA-saturated solvents were prepared by adding excess crystals to anhydrous solvents and equilibrating overnight in stoppered flasks at 25°C with continuous stirring.

EXPERIMENTAL

Decaffeination Overview

Decaffeination of caffeine- and water-saturated EtAc and SBO was conducted by adding CA crystals and varying amounts of excess water. Decaffeination of $MeCl_2$ samples spanning a range of caffeine concentration was conducted by adding CA crystals and two volumes of water. Decaffeination of $MeCl_2$ coffee extract was conducted by adding CA crystals and 1-2 volumes of water. Decaffeinated $MeCl_2$ coffee extracts were further concentrated by evaporation, and decaffeination repeated by subsequent exposure to CA until >97% decaffeination was achieved. All systems were stirred at 25°C and allowed to reach reaction equilibrium prior to analysis. In all systems except SBO, it was possible to observe gradual dissolution of CA crystals and growth of caffeine-CA crystals.

Analysis

Crystals were removed from decaffeinated solvents by paper filtering through a Buchner funnel under vacuum. Supernatants were sampled and analyzed by HPLC using the method of Zeller and Saleeb [1]. Solubility of caffeine, CA, and caffeine-CA in non-aqueous solvents was determined by evaporating saturated solutions to dryness and weighing the residue. Caffeine-CA residues were dissolved in water and analyzed by HPLC to determine the relative amounts of caffeine and CA.

RESULTS

EtAc Decaffeination

2L EtAc was contacted with 75g water for 1hr while stirring. 66g water dissolved in the EtAc and the remaining aqueous phase was removed. 35g crystalline caffeine was added to the water-saturated EtAc and allowed to stand for 18hrs, after which the remaining undissolved caffeine was filtered out. HPLC analysis indicated that 25g caffeine dissolved to yield a 1.3% wt/wt solution. 25g CA was added to 250mL of the saturated EtAc solution to provide a slight molar excess of CA (1.1 moles CA per mole caffeine). The suspension was stirred for 3hrs to allow sufficient time for conversion of CA to caffeine-CA to reach equilibrium. After standing for 1hr to allow crystals to settle, the solution was sampled and analyzed by HPLC. It was found that 67% decaffeination had been achieved.

Similar experiments were performed both with and without CA addition, as well as with and without addition of varying amounts of water. Results are summarized in Figure 1. It was found that although caffeine-CA will form in water-saturated EtAc, addition of a discrete water phase speeds decaffeination in addition to increasing the extent of decaffeination due to increased partitioning of caffeine into the water phase. Adding increasing amounts of water should cause the two curves in Figure 1 to merge when sufficient water is added to render the incremental effect of CA irrelevant.

$MeCl_2$ Decaffeination

A series of 500mL $MeCl_2$ caffeine solutions were prepared by dissolving caffeine in $MeCl_2$ at 25°C. Each solution was divided into 250mL aliquots. One of each was contacted with 500mL water to serve as controls, while the others were contacted with 500mL water and sufficient CA crystals to provide a 2:1 molar ratio of CA:caffeine. Because the density of $MeCl_2$ is greater than water and the two liquids are immiscible, a discrete phase layer forms above the $MeCl_2$. Rapid growth of caffeine-CA in the aqueous phase at the $MeCl_2$ interface occurs and is complete after 15-30 minutes. Use of smaller amounts of water can result in solidification of the entire system due to

formation of a large mass of intertwined caffeine-CA crystals. After standing for 1hr to allow crystals to settle, the solutions were sampled and analyzed by HPLC. It was found that much greater decaffeination levels had been achieved in samples treated with CA. Results are summarized in Figure 2. A discrete water phase was required for decaffeination to occur since conversion of CA to caffeine-CA did not occur in the $MeCl_2$ phase.

$MeCl_2$ Coffee Extract Decaffeination

Water and CA were added to a variety of concentrated $MeCl_2$ coffee extracts, similar in appearance to red wine, in the manner used to decaffeinate pure $MeCl_2$. Both CA and caffeine-CA crystals have density intermediate between that of the two liquid phases, causing crystals to accumulate at the bottom of the water phase. Because concentrated $MeCl_2$ coffee extracts contain high levels of caffeine, somewhat greater than 90% decaffeination was typically obtained. However, a minimum of 97% decaffeination is desirable if the decaffeinated $MeCl_2$ is to be recontacted with coffee extract to promote transfer and recovery of residual coffee flavor. Partial decaffeination of $MeCl_2$ coffee extracts allows further concentration, typically 3-5X, by vacuum distillation to increase caffeine to levels which provide extensive decaffeination, typically 70-80%, during a second treatment with CA. At this point, the 97-99% decaffeinated $MeCl_2$ coffee extract has been concentrated to at least 120-150X and contains nearly pure non-caffeine solids at >10% wt/wt concentration. This fraction can be added back to the decaffeinated aqueous coffee extract to improve its flavor and the $MeCl_2$ removed by distillation.

SBO Decaffeination

500mL SBO was contacted with 100mL water and 5g caffeine while stirring for 1hr. The aqueous phase was removed and the saturated SBO filtered to remove undissolved caffeine crystals. Analysis indicated that the SBO contained 0.11% caffeine and ~ 0.2% water. A 200mL aliquot of the saturated SBO was contacted with 25mL water to serve as a control. Another 200mL aliquot was contacted with 25mL water and 0.5g CA to provide 2.5 moles CA per mole of caffeine. Both systems were stirred for 18hrs and analyzed as above. It was found that the control was 57% decaffeinated while the sample treated with CA was 96% decaffeinated. Microscopic analysis of the crystals filtered from the variant verified that approximately half of the CA had been converted to caffeine-CA, as evident from their different crystalline structures.

100mL aliquots from each of the decaffeinated systems were contacted with 12.5mL of fresh water and allowed to equilibrate for 48hrs. Again, 57% of the remaining caffeine in the control partitioned into the water to provide 81% overall decaffeination, while the extent of decaffeination of the variant advanced to 98%. Repeating this process for the control using the same volume ratio of water-saturated SBO to water (8:1) does not achieve 96% decaffeination until the fourth contact with water. It was found that caffeine-CA would not form in water-saturated SBO without addition of sufficient excess water to form a discrete aqueous phase. Results are summarized in Figure 3. Figure 4 was reproduced from reference 1 to supplement interpretation of solvent decaffeination results.

Solubility Data

Solubility data for caffeine, CA, and caffeine-CA are summarized in the table. It is evident that the halogenated solvents are good solvents for caffeine but not for CA-caffeine. Such a combination favors effective decaffeination with addition of CA and water. Because these solvents are also able to break the caffeine-CA complex, they can be used to reflux caffeine-CA crystals to reclaim pure caffeine and CA [1]. The other solvents evaluated do not have this ability, as can be seen from approximately equimolar caffeine:CA ratios.

Figure 1: Extent of Decaffeination of EtAc by Water and Water + CA

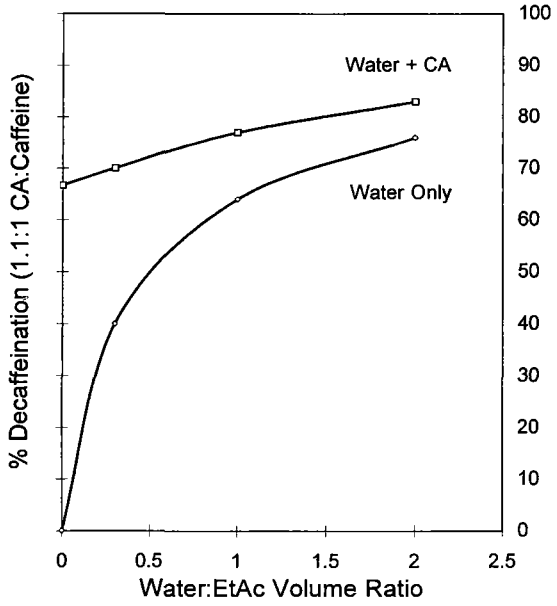


Figure 2: Extent of Decaffeination of $MeCl_2$ by Water and Water + CA

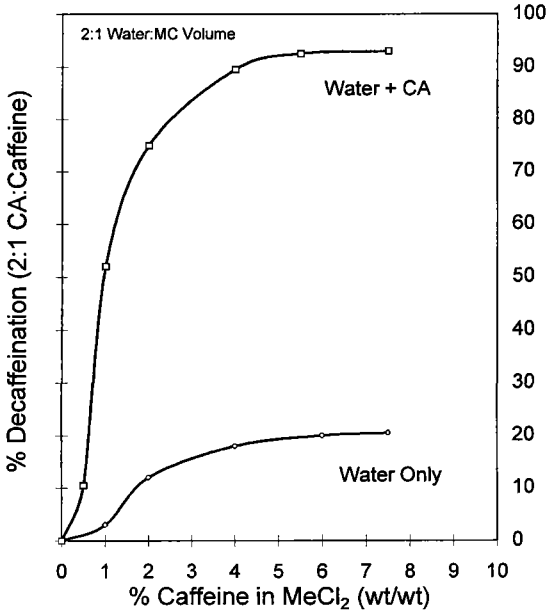


Figure 3: Extent of Decaffeination of SBO by Water and Water + CA

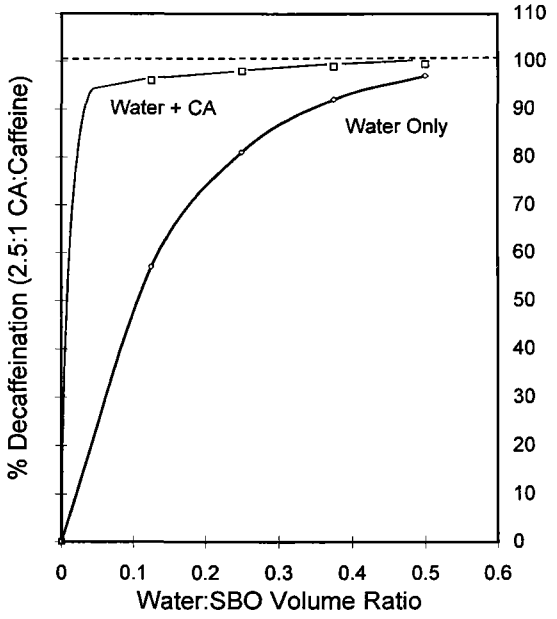
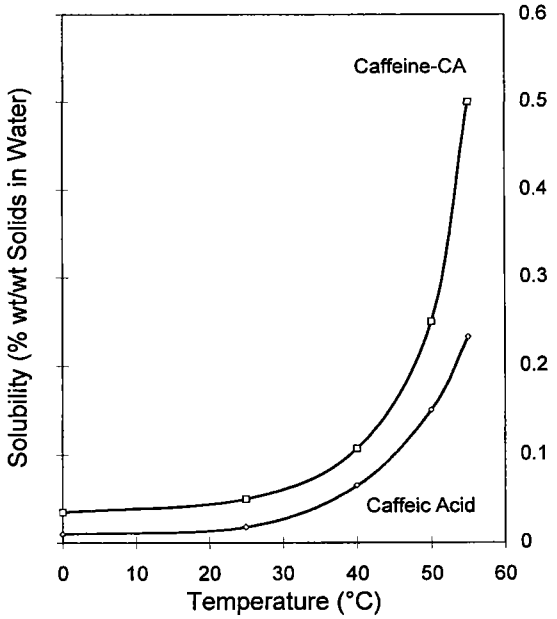


Figure 4: Solubility of Caffeine-CA and CA in Water vs Temperature



Caffeine, CA, and Caffeine-CA Solubility at 25°C (wt/wt in solvent)

Solvent	Caffeine	CA	Caffeine-CA	Molar Ratio Caffeine:CA[1]
Chloroform	13.23	< 0.03	0.52	87:1
Methylene Chloride	7.76	< 0.03	0.46	71:1
Ethyl Acetate	0.81	0.27	0.72	1.1:1
Ethanol	0.41	3.13	1.78	1.0:1
Acetone	1.39	2.34	3.04	1.0:1
Isopropanol	0.41	0.97	1.31	0.7:1
Methanol	1.19	8.74	3.45	n/a
Water[2]	2.12	0.02	0.05	1.0:1

[1] molar ratio of caffeine to CA in solvent to which caffeine-CA crystals were added
[2] previously reported by Zeller and Saleeb [1]

DISCUSSION

As in decaffeination of aqueous solvents, high solvent caffeine levels and high levels of CA and water addition increase the extent of decaffeination obtained in non-aqueous solvents. However, the benefit of using CA to decaffeinate non-aqueous solvents decreases in proportion to the amount of water used. The high solubility of caffeine and low solubility of CA and caffeine-CA in $MeCl_2$, relative to EtAc and SBO, along with its ability to be easily concentrated, would make $MeCl_2$ an effective choice for use in decaffeinating aqueous coffee extracts when it is to be subsequently decaffeinated using CA. The ability to decaffeinate EtAc without addition of excess water may also provide processing advantages. The high selectivity of CA for caffeine allows it to be used to decaffeinate non-aqueous coffee extracts with minimal loss of coffee solids. Use of CA, along with excess water if required, can be employed as an effective technique to increase the batch decaffeination efficiency of non-aqueous solvents.

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SUMMARY

Caffeic acid was used to significantly increase the batch decaffeination efficiency of methylene chloride, ethyl acetate, and soybean oil in lab-scale experiments. Contacting these non-aqueous solvents with a suspension of caffeic acid crystals in water caused rapid crystallization of caffeine-caffeic acid complex at room temperature. The extent of decaffeination obtained was much greater than when the same solvents were contacted with water only. Preliminary results indicate that this technique holds the potential to reduce water use, increase caffeine selectivity, and perhaps reduce coffee flavor loss in downstream solvent decaffeination operations used to recover caffeine. Quantitative decaffeination results are presented for methylene chloride, ethyl acetate, and soybean oil. In addition, solubility data are reported for caffeine, caffeic acid, and caffeine-caffeic acid in a variety of organic solvents.

Computer Simulation as a Tool to Model Coffee Brewing Cellular Automata for Percolation Processes; 2D and 3D Techniques for Fluid-dynamic Simulations

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INTRODUCTION

The understanding of fluid dynamic processes which take place within a coffee pod (consisting of a filtered paper covering 6.9 grams of ground and tamped illy blend) while brewing a cup of espresso, are of great strategic importance for achieving a better product.
Therefore the features connected to percolation have been take into consideration, at least from a physical point of view. Percolation is in fact highly connected to the quality in the cup[1]: brewing a cup of espresso means percolating a certain quantity of water through a bed of ground coffee particles. There are basically four parameters that influence mostly the flow rate through this bed: temperature, pressure, particle size distribution (coming from the grinding process) and void ratio, i.e. the tamping degree.
The target is the discovery of how these parameters affect the velocity of water and therefore the extraction of substances. While temperature and pressure have been modelled by a traditional experiment, the granulometric distribution obtained with a standard grinder, which shows a three-modal distribution of the particle surface exposed to the water (Fig. 1), is almost constant when the distance between the grinder blades is changed. It is not known whether this particle-size distribution is the one that optimises the percolation, in terms of maximum soluble extraction and minimum percolation time. A random change of the grinder is far from being an effective solution to the problem. The solution can be found by means of computer simulation of the flow during the percolation process. Different tamping degrees and different particle size distributions can be simulated with Cellular Automata (CA) [2] by changing the initial conditions.

MATERIAL AND METHODS

Experimental percolation

The percolation flow is a very complex non-linear function of time (t), which depends on several parameters: water temperature (T), pressure (P), particle size distribution (s), and porosity of the bed (e)

$$\phi = \phi(t;P,T,e,s) \qquad (1)$$

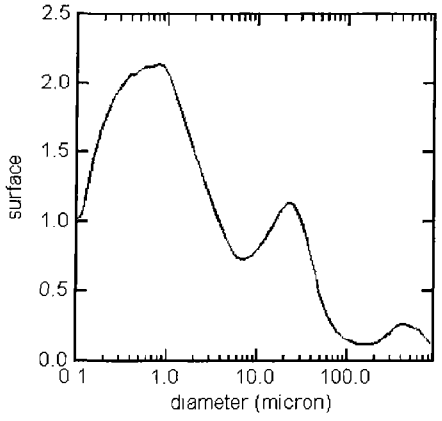

Fig. 1. Particle size distribution. A three-modal distribution of the particle surface exposed to the water can be appreciated. The first peak is at 1 μm, the second at 30 μm, and the third at 450 μm.

The profile of the flow shows two distinctive trends: an initial transitory peak, with a fast increase of the flow, corresponding to the invasion of water into coffee bed, and then a decreasing front (the modification of the topology of the bed [3]), and a quasi-asymptotic behaviour (Fig. 4).
The influence of temperature and pressure has been studied in detail [4]. Tests have been run with pressure ranging form 1 to 14 bar, and temperature ranging from 4°C to 94°C, with "standard" pods, i.e. fixed e and s, leading to the following equation:

$$\varphi(P,T;t) = (a(t) + b(t)P + c(t)P^2)T^{-d(t)} \tag{2}$$

Parameters a, b, c, d are quadratic functions of time.

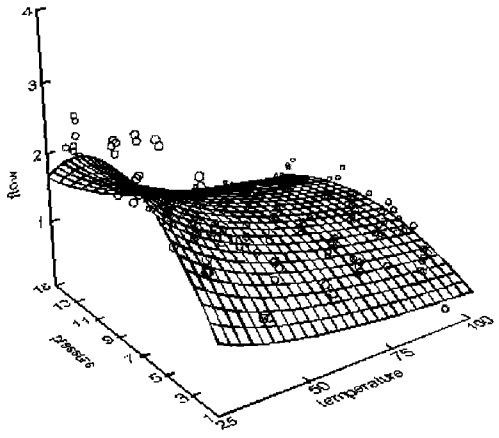

Fig. 2: Response surface for 25°C<T<100°C and 1 bar<P<15bar at time t_1

At a given time, the response surface of flow, temperature and pressure is a saddle (Fig. 2), which drifts towards smaller values of the flow, and it changes its curvature with time.

Lattice Gas Automata

Equation (2), found by fitting experimental values, lacks the dependence on particle size distribution (s), and porosity of the bed (e) which was pointed out in equation (1). While temperature and pressure can be monitored by means of an experiment, the design of an experiment which involves granulometry and compaction can be cumbersome and time-wasting. The interest in CA is therefore due to its ability of simulating the fluid dynamics inside a coffee bed. In the last years there has been an increasing interest on Lattice Gas Automata (LGA) [5] model as an alternative to the traditional computational fluid-dynamic approach for solving fluid problems.
CA can be implemented on a parallel platform (like CINECA's Cray T3E, with 160 parallel processors) thus reducing the computational time of several orders of magnitude, from 15 hours on a PC to 10 minutes [6].
The model used to simulate coffee percolation is the FHP-N model [7] (Fig. 3). It is an extension of the classical FHP model [8], implemented by the *fislab* at the University of Milan. It is based on the physics occurring during the process and it reproduces Navier-Stokes differential equations which rule the processes occurring during percolation. FHP-like CA cannot reproduce the physical-chemical extraction processes within the pod: however, since the extraction is connected to the surface exposed to water, the modelling of the latter allows an indirect knowledge of the first. The FHP-N code allows to calculate the flow rate, the surface exposed to water, and the distribution of the module of the momentum (i.e. the velocity of water within the pod). The latter is a good indicator of the presence of high velocity channels, which have to be minimised to enhance the extraction processes.

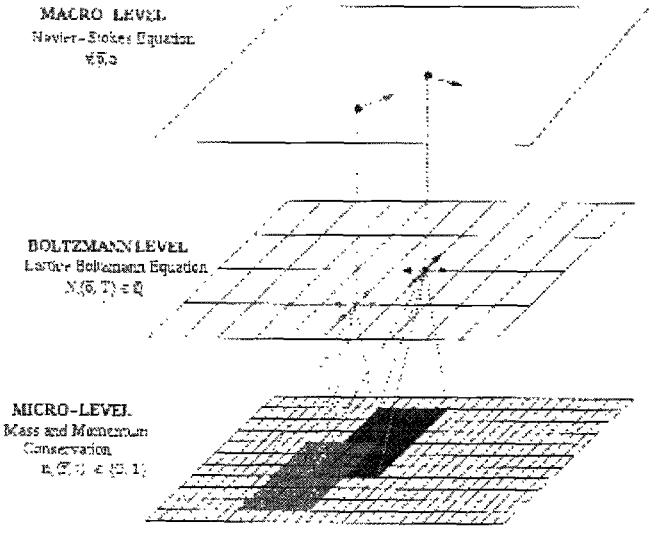

Fig. 3: The FHP-N model derives the Navier-Stokes equation on a hexagonal lattice of boolean cells by imposing conservation of mass and momentum. An averaging procedure over space and time allows the required physical quantities to be calculated.

For the experimental data to be efficiently compared to the simulations, these have been run with the initial conditions as close to the experimental ones as possible. For this reason the percolation bed has been built reproducing the porosity of the pod (i.e. the void ratio), and the shape of the percolation channels, which are all but regular [9]. Moreover, since the physical process is three-dimensional, two-dimensional modelling of one plane is not enough to reproduce reality. Therefore the adjacent planes have been taken into consideration, as well. For this reason a coffee pod has been cut with a microtome into several slices, and digital images of the slices have been acquired. The superposition of the images have given a three dimensional image.
The pod has been soaked with a paraffin-like substance, which fills the pores substituting the air, without interacting with the coffee. In this way, the pod has become rigid enough to be cut in slices. The thickness of these slices is very small and can be approximated to a plane.

Glass slides have been then recorded with a digital camera placed on a microscope, and processed with a graphic software. A gradient of the compaction can be easily detected, along the direction top down. A threshold has been set to discriminate void and obstacles. The retouched image represents the boundary conditions for the CA.

RESULTS AND DISCUSSION

Simulation results

The flow rate is the actual physical quantity which can be monitored and compared with real data. The comparison is however qualitative since no law has been found yet, which links the void ratio in a 2D simulation bed to the pressure applied by the pod-making device during tamping, and therefore to the tamping degree.
Two experimental flows are reported as a function of the pressure (in bar) applied in the pod-making process (Fig. 4 left). The equivalent simulations, on the other hand, use the void ratio within the percolation bed (i.e. the percentage of space not occupied by particles) as a parameter (Fig. 4 right).

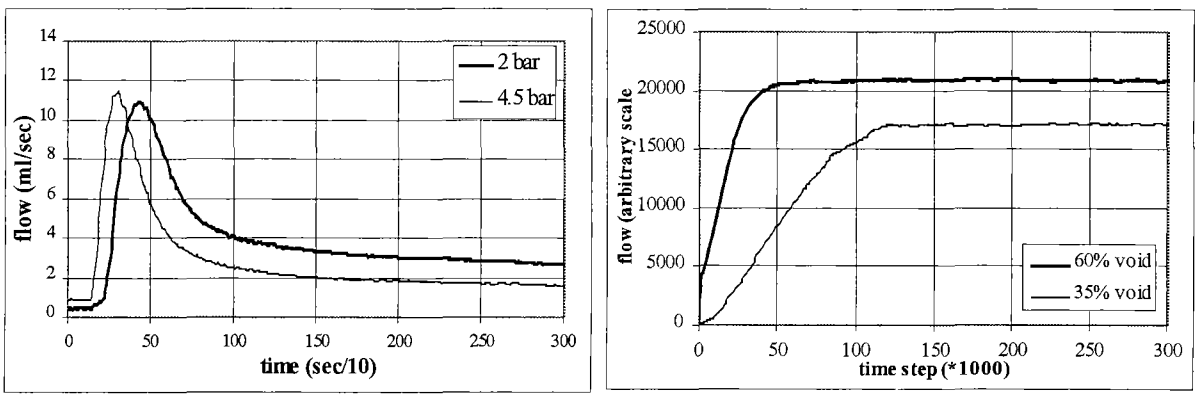

Fig. 4.: Left: experimental flow rate with different tamping degree (2 and 4.5 bar applied by the pod-making device). An initial transitory part is followed by an asymptotic part. Right: simulated flow rate with 2 different void ratios. The invasion percolation is not modelled and therefore the transitory peak cannot be appreciated.

The lack of the transitory peak in the simulation plots is due to the fact that the rearrangement of the particles, which occurs in the "real" percolation, is not yet performed by the FHP-N model, which deals with a fixed topology.
The agreement of the simulations and experimental results, in terms of flow rate, confirms the validity of the model, which is therefore an effective tool for experimenting "in silicon" situations out of the experimental range.
As a case study, two simulations have been chosen which point out the effect of particle size distribution and tamping (i.e. void ratio). One represents a non-compacted percolation bed made up by coarse particles, while the other one fairly well reproduces the real situation. The initial conditions are given in table 1.

	NON-TAMPED POD	TAMPED POD
r1/r2 ratio	1:8	1:4
n1/n2 ratio	10:90	90:10
void ratio	60%	35%
contact surface	46402	185453

table 1: boundary conditions for the case study. On the left parameters for non-compacted percolation bed made up by coarse particles; on the right tamped pod with a major percentage of fine particles.

The effect of tamping and of granulometry is clear. The small particles increase the surface exposed to water by four times, letting the extraction work more efficiently. The presence of small particles and the tamping provide for the elimination of the high velocity channels that cause the water to flow without extracting any substances. The histograms of the distribution of the modulus of the momentum (Fig. 5) give a further explanation. In the case

of tamped pod (Fig. 5 right) the tail of the distribution is much shorter, and in fact no high-velocity channel can be detected.

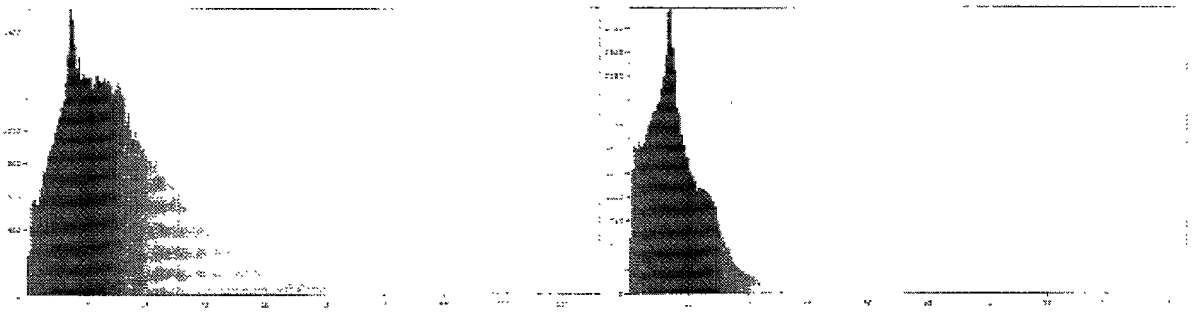

Fig. 5.: Left: Histogram of the module of the momentum for a non tamped pod. Right: tamped pod. The tail is much shorter. There are not high velocity channels.

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ABSTRACTS

In this paper we describe how computer simulation can be used for understanding the fluid dynamics of the coffee percolation process, which is of great importance in designing coffee-based products and brewers. Four parameters basically influence the flow rate: water pressure, water temperature, particle size distribution and void ratio within the coffee bed. While the first two parameters can be studied by a traditional experiment, the latter two are very difficult to be monitored: a computer simulation is therefore necessary to get a deeper knowledge of what happens during the percolation process. The goal of these simulations is to understand how particle size distribution and void ratio affect water velocity and therefore the extraction of soluble substances, which provide for the body and aroma of the espresso coffee. The model does not take directly into account the chemical

processes, which occur during percolation. Nevertheless the water velocity is available and can be used as an index of the extraction processes. An experimental model of the flow as a function of time, pressure and temperature has been developed while computer simulations have put in evidence the presence of high velocity channels in particular conditions of coarse grinding and low tamping. The agreement of simulations and experimental results, in terms of flow rate, confirms the validity of the model, which is therefore an effective tool for experimenting "in silicon" situations out of the experimental range.

Sur ce document, nous expliquons comment une simulation sur ordinateur peut être utilisée pour comprendre la dynamique d'écoulement de l'eau lors du processus de percolation du café, qui est d'une importance primordiale afin d'étudier les produits et boissons à base de café. Quatre paramètres de base influencent cet écoulement: la pression de l'eau, la température de l'eau, la répartition de la taille des particules, le pourcentage de vide.
Alors que les deux premiers paramètres peuvent être étudiés à l'aide d'experience traditionnelles, les deux derniers sont très difficiles à controller : une simulation informatisée est donc nécessaire pour avoir une connaissance plus précise de ce qu'il advient lors de la percolation. Le but de ces simulations est de comprendre comment la répartition de la taille des particules de café et le pourcentage de vide influencent la vitesse d'écoulement de l'eau et par conséquent, l'extraction des substances solubles qui a un role prépondérant sur le corps et l'arome de l'espresso. Ce modèle informatique ne prend pas directement en compte les processus chimiquent qui interviennent durant la percolation. Néanmoins, la vitesse d'écoulement de l'eau est disponible et peut être utilisée comme référence dans le processus d'extraction. Un modèle expérimental du flux utilisant des fonction de temps, de pression et de température a été développé tandis que des simulations informatisées ont mis en évidence la présence de canaux à haute vitesse, apparaissant dans des conditions particulières d'utilisation de café moulu en grosse particules et faiblement pressé. La compatibilité des simulatons et des résultats expérimentaux en terme de vitesse d'écoulement confirme la validitè du modèle informatique, qui est donc un instrument efficace pour expérimenter "in silicone" des situations hors-domaine expérimental.

Mapping of Brazilian Coffee Quality

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INTRODUCTION

Brazil has to take advantage of being a producer of coffee of good quality or even better quality than other producing countries due to its great territorial extension, climate and variable soil conditions, which enables it to produce various types of coffee and to attend the different requirements of consumers.

The quality of the coffee may be affected by many factors. The weather conditions of the region, or the type of soil where the coffee is grown, the harvesting techniques employed, the method by which the coffee is processed in the field, the storage conditions, the way in which it is shipped from producing areas to industrial processing areas, the type of industrial processing itself, the blend prepared, the brewing techniques, the quality factors and /or ingredients involved during brewing all affect the flavour of the beverage(ICO, Report no 4, 1991).

Descriptive flavour profiles of coffees from different producing areas could be a way to make consumers more aware of the diversity of coffee flavours.

The objectives of the present investigation were to generate coffee flavour descriptions and to notice those attributes that make some coffees extremely distinctive or similar to others resulting in a mapping showing a good separation of the Brazilian regions studied.

MATERIAL AND METHODS

Samples

Coffees provided by 3 producing areas of Brazil were used to carry out the first part of this investigation, they are identified in Table 1. The coffee samples were prepared for sensory evaluation under strictly controlled conditons, as listed in Table 2.

TABLE 1
SAMPLES OF COFFEES PROFILED

COFFEE ORIGIN AND REGION	VARIETY AND PROCESS	TYPE	BEAN SIZE SCREEN >15(%)
1-Cooperativa de São Manuel- São Manuel-SP	Arabica Unwashed	3-15	99,7
2-Sítio Córrego da Onça- Franca- SP	Arabica Unwashed	3-25	78,1
3-Fazenda da Lagoa- Pedregulho- SP	Arabica Unwashed	4-10	71,1
4-Fazenda São Sebastião- Jeriquara- SP	Arabica Unwashed	2	80,8
5-Sítio Sào Sebastião- Espírito Santo do Pinhal-SP	Arabica Unwashed	4-40	85,5
6-Fazenda São Pedro- Espírito Santo do Pinhal-SP	Arabica Unwashed	7-15	85,1
7-Sitio Olho d'água- Espírito Santo do Pinhal-SP	Arabica Unwashed	5-20	80,0
8-Fazenda Prata- Águas da Prata-SP	Arabica Unwashed	3-10	99,5
9-Sítio São José- Santo Antônio do Jardim-SP	Arabica Unwashed	5-5	83,1
10-Sítio São Benedito- Espírito Santo do Pinhal-SP	Arabica Unwashed	3-35	83,4
11-Sítio Bacaúva – Mogi Guaçu-SP	Arabica Unwashed	5-5	79,2
12-Fazenda Floresta IV- Espírito Santo do Pinhal-SP	Arabica Unwashed	4-40	80,9
13-Sítio Santa Barbara- Santo Antônio do Jardim-SP	Arabica Unwashed	6-10	83,5
14-Fazenda Lagoa do Morro- Brejões- BA	Arabica Washed	2	100,0
15-Fazenda Macaúba de Minas- Patrocíno- MG	Arabica Unwashed	4-5	98,6
16-Fazenda Chapada dos Rodrigues- Paracatú – MG	Arabica Unwashed	3-5	99,8

TABLE 2
SAMPLE PREPARATION CONDITIONS FOR THE PROFILING EXPERIMENT.

METHOD	EXPERIMENTAL CONDITIONS
Roasting	Conventional drum roaster
• Temperature	185-210°C
• Time	13 – 15 minutes
• Degree	Medium light roast
Grinding	Fine grind
Brewing	
• Method	Filter paper
• Formula	55 grammes of coffee per 1 liter of spring water
• Time	4 – 6 minutes

Size and shape of the beans: size is identified with a 14 to 19 sieve number, mokka (peaberry) and pan fraction(broken bean) sizes.
Defects: using defect reference chart of Brazilian classification system.
Roasting of Coffees Samples for Sensory Evaluation Experiments
Around 5 kg of green coffee beans were roasted until medium roasted in a conventional drum roaster at 185°C to 210°C for 13 15 minutes with a weight loss of 16% - 17%.

A colour measurement of the roasted and ground coffee was recorded to indicate the degree of roast at which the coffees were evaluated.

To achieve the degree of roast desired, moisture content and bean size of the green coffee beans were to be taken into account. The roasted sample was separated into batches which was packed and kept in a refrigerator over a period of one or two weeks.

Sensory Evaluation
The presence of immature beans is one of the most damaging defects in coffee and was used for panel performance evaluation. Having tested the reliability and consistency of the sensory evaluation panel, as a whole and individually, and having standardized the procedures for preparing and serving the samples, it was appropriate to begin the sensory evaluation of coffee beverage.

Quantitative descriptive flavour profiling of the samples were carried out following the ICO coffee sensory evaluation method. The method uses a coffee vocabulary created by consumers and a trained panel to select all sensory characteristics of each individual coffees. To profile a coffee, the intensity of each aroma, flavour and mouthfeel are attributed to ICO coffee vocabulary in order of perception the intensity of selected sensory terms is assessed on a line scale. Panel members entered scores directly into a computerized sensory data collector system Compusense with statistical software for immediate analysis.

A complete randomized design was selected for the assessment of the different coffees. A total of 16 sensory evaluation sessions were required in order to assess each of 16 coffees in duplicate.

Panelists received only two samples per session and each was assessed individually. The tasting temperature was between 60-70°C and an unstructured 10 cm scale was used to rate the intensity of each of the sensory characteristics in a blind test.

Statistical Analysis
The statistical analysis applied to the collected sensory evaluation data for this profiling experiment were principal component analysis (PCA) and three way analysis of variance.

Chemical analysis
The chemical composition of the green and roasted coffee was determined as complementary information.

The physico-chemical constituents determined were moisture, protein, lipids, total and reducing sugars, fiber, tannin, total acidity and ash by using the AOAC and ISO standard methods.

The analysis of mineral elements and trace elements in green coffee was carried out by using Inductively Coupled Plasma Atomic Emission Spectrometry(ICP-AES). The average content of main constituents could be obtained by coffee from different origins.

RESULTS

Panel performance was tested and proved to be highly acceptable, with only a few cases of interaction of tasters by replicate for each attribute.

Sensory analysis of unwashed immature and mature (cherries dried on the trees) coffees and cherries washed and artificially dried coffee (almost 70 hours) resulted in:

- Highly statistically significant differences were always detected between coffees harvested when immature and when mature and artificially dried coffee.
- The different profiles of mature and immature and artificially dried samples of Arabica coffees are presented in Figure 1.
- Immature coffees have green/grass, chemical, animal, rancid/rotten, mould and astringency flavour characteristics and vary like their mature and artificially dried counterparts that were more cereal/malty/toasted bread, chocolate and caramel aroma and full bodied.

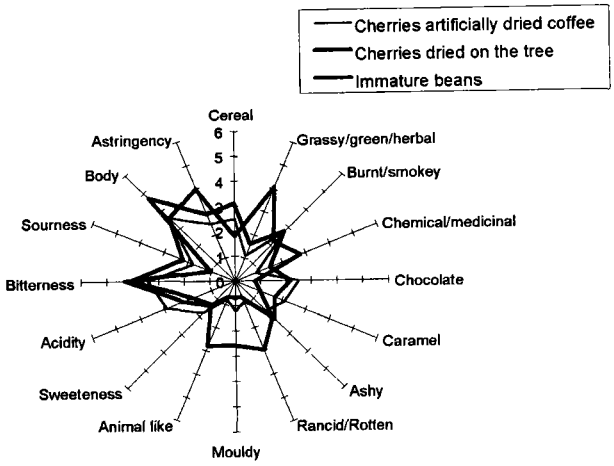


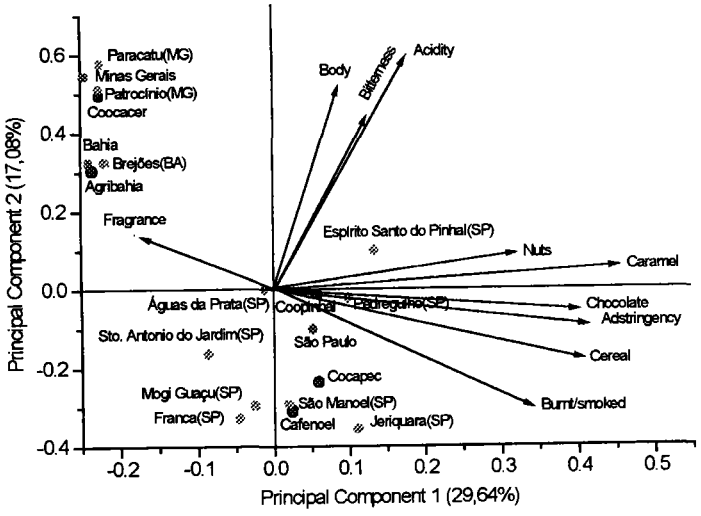
FIGURE 1. Graphic presentation of profiles of mature and immature unwashed Arabica coffees from Brazil.

For the sensory evaluation of the 16 coffees seven trained panelists profiled in a replicate blind test and showed consistency.

Descriptors not used for any of the 16 coffees were discarded from the analysis.

A comparison of samples is shown in Figure 2.

FIGURE 2. Principal component analysis of 16 brewed coffees.



Mapping of the results of this study produced a good separation of all the coffees (Figure 2). The samples from different origins clearly grouped from left to right on the horizontal axis which accounted for 29,64% of the total variance of the experiment. The vertical axis accounted for another 17,08% of the total variance and is responsible for the perceived differences on body, bitterness and acidity attributes .

Each of the Arabicas coffees presented an individual flavour description with some identified differences and similarities to the rest of the coffees.

Aroma, taste and mouthfeel found present in all coffees samples were:
Cereal, burnt, astringency, chocolate, caramel and nutty were perceived in samples from São Paulo State.

Samples from Minas Gerais and Bahia States were characterized as having moderate acidity and bitterness, medium body and high fragrance.

The chemical analysis of the samples showed slightly high values for protein, lipid, total sugars than values found in the Food Composition Table. The moisture contents of green coffee were approximately 11% and the moisture level of roasted coffee did not exceeded 1,0% w/w (Tables 3, 4).

An examination of the original variables of the mineral content that are grouped in the principal components may give meaningful insight into the type of variation being explained by each of the principal components. A total of 51% of all the variances between the samples are represented in two dimensions. In a Figure 3 these groupings of variables can be graphically presented to show product separation in two dimensional space that can be visualized.

TABLE 3
CHEMICAL COMPOSITION OF 16 GREEN COFFEES*

	Coffee sample origins	Moisture %	Ash %	Proteins %	Lipids %	Raw Fiber %	Sugars Total %	Sugars Reducing %	Tannin %
1	Coop. Zona de S. Manuel	11.0 (0.2)	3.7 (0.2)	13.4 (0.1)	7.7 (0.0)	12.7 (0.3)	9.1 (0.2)	0.3 (0.0)	3.7 (0.1)
2	Sítio Córrego da Onça	10.4 (0.0)	3.6 (0.1)	13.9 (0.1)	9.1 (0.0)	11.4 (0.3)	8.5 (0.1)	0.5 (0.0)	4.4 (0.1)
3	Fazenda da Lagoa	10.3 (0.1)	3.5 (0.1)	13.1 (0.1)	8.5 (0.3)	13.3 (0.0)	7.7 (0.5)	0.3 (0.0)	4.1 (0.2)
4	Fazenda São Sebastião	9.8 (0.0)	3.6 (0.1)	14.3 (0.2)	8.9 (0.0)	12.8 (0.0)	8.9 (0.2)	0.5 (0.0)	4.1 (0.1)
5	Sítio São Sebastião	10.1 (0.0)	3.5 (0.0)	13.3 (0.1)	11.4 (0.2)	11.7 (0.2)	6.5 (0.1)	0.5 (0.5)	4.5 (0.0)
6	Fazenda São Pedro	10.3 (0.2)	3.7 (0.0)	13.7 (0.0)	13.1 (0.2)	11.7 (0.3)	9.6 (0.3)	0.5 (0.0)	4.4 (0.1)
7	Sítio Olho D'Água	10.1 (0.1)	3.7 (0.0)	14.1 (0.1)	10.5 (0.0)	11.1 (0.0)	7.9 (0.1)	0.5 (0.1)	4.2 (0.1)
8	Fazenda da Prata	10.6 (0.0)	3.5 (0.0)	12.5 (0.0)	14.4 (0.0)	11.2 (0.1)	9.1 (0.2)	0.2 (0.0)	4.4 (0.1)
9	Sítio São José	10.4 (0.2)	3.6 (0.0)	13.5 (0.0)	10.8 (0.1)	11.8 (0.3)	8.6 (0.4)	0.7 (0.1)	3.8 (0.0)
10	Sítio São Benedito	10.0 (0.1)	3.5 (0.0)	13.2 (0.1)	12.9 (0.2)	12.2 (0.1)	9.5 (0.1)	0.6 (0.0)	4.3 (0.1)
11	Sítio Bacaúva	10.5 (0.0)	3.6 (0.0)	14.1 (0.1)	12.8 (0.2)	11.4 (0.1)	9.6 (0.4)	0.2 (0.1)	3.8 (0.1)
12	Fazenda Floresta IV	9.5 (0.0)	3.6 (0.1)	13.5 (0.0)	12.2 (0.3)	13.6 (0.1)	10.9 (0.4)	0.6 (0.1)	4.0 (0.0)
13	Sítio Santa Bárbara	10.0 (0.0)	3.4 (0.1)	13.4 (0.1)	13.9 (0.0)	12.6 (0.1)	9.0 (0.2)	0.4 (0.0)	3.7 (0.0)
14	Fazenda Lagoa do Morro	9.7 (0.0)	3.4 (0.0)	14.5 (0.1)	12.6 (0.4)	12.2 (0.3)	10.4 (0.1)	0.3 (0.0)	3.2 (0.2)
15	Fazenda Macaúbas de Cima	11.0 (0.1)	3.5 (0.0)	14.5 (0.1)	12.7 (0.3)	11.7 (0.5)	9.2 (0.0)	0.9 (0.0)	3.4 (0.1)
16	Fazenda Chapada dos Rodrigues	11.1 (0.2)	3.7 (0.0)	12.6 (0.1)	12.9 (0.1)	12.8 (0.2)	8.9 (0.2)	0.5 (0.0)	3.5 (0.1)

*Average±standard error

TABLE 4
CHEMICAL COMPOSITION OF 16 ROASTED COFFEES

	Coffee samples origins	Moisture %	Ash %	Proteins %	Lipids %	Raw Fiber %	Sugars Total	Sugars Reducing %	Acidity (ml NaOH N/100g)	Tannin %
1	Coop. Zona de S. Manuel	0.7 (0.0)	4.5 (0.0)	15.8 (0.1)	8.8 (0.0)	14.4 (0.4)	2.7 (0.4)	0.8 (0.1)	11.8 (0.2)	3.1 (0.1)
2	Sítio Córrego da Onça	1.1 (0.0)	4.0 (0.0)	15.7 (0.2)	15.6 (0.0)	13.1 (0.5)	2.4 (0.1)	0.9 (0.2)	15.8 (0.3)	4.0 (0.0)
3	Fazenda da Lagoa	1.0 (0.0)	4.3 (0.1)	15.2 (0.0)	16.0 (0.1)	13.0 (0.4)	1.4 (0.1)	0.6 (0.0)	15.3 (0.0)	4.0 (0.0)
4	Fazenda São Sebastião	1.2 (0.0)	4.4 (0.1)	16.3 (0.2)	15.7 (0.1)	12.0 (0.1)	2.0 (0.0)	0.8 (0.0)	15.2 (0.1)	4.2 (0.1)
5	Sítio São Sebastião	1.0 (0.0)	4.4 (0.1)	15.0 (0.0)	16.0 (0.0)	14.2 (0.1)	1.8 (0.1)	0.9 (0.2)	13.1 (0.1)	3.3 (0.0)
6	Fazenda São Pedro	0.9 (0.0)	4.3 (0.1)	15.5 (0.0)	15.7 (0.0)	12.9 (0.5)	1.0 (0.2)	0.8 (0.0)	13.7 (0.3)	3.1 (0.2)
7	Sítio Olho D'Água	0.9 (0.0)	4.4 (0.0)	16.1 (0.1)	14.9 (0.1)	18.6 (0.2)	2.1 (0.4)	0.8 (0.0)	12.4 (0.0)	3.0 (0.1)
8	Fazenda da Prata	0.6 (0.0)	4.3 (0.0)	15.1 (0.2)	16.5 (0.0)	15.0 (0.2)	1.4 (0.5)	0.7 (0.1)	11.9 (0.0)	3.3 (0.1)
9	Sítio São José	1.2 (0.0)	4.9 (0.3)	15.2 (0.0)	13.7 (0.4)	14.3 (0.0)	1.5 (0.1)	0.8 (0.0)	20.9 (0.1)	2.4 (0.0)
10	Sítio São Benedito	1.0 (0.0)	4.2 (0.1)	14.4 (0.1)	13.7 (0.2)	14.2 (0.1)	1.6 (0.0)	0.6(0.0)	21.8 (0.2)	3.3 (0.1)
11	Sítio Bacaúva	0.9 (0.0)	4.6 (0.0)	15.6 (0.1)	12.8 (0.0)	14.4 (0.7)	1.2 (0.1)	0.6(0.0)	20.5 (0.7)	2.9 (0.1)
12	Fazenda Floresta IV	0.9 (0.0)	4.2 (0.0)	15.6 (0.2)	16.9 (0.1)	14.8 (0.2)	0.8 (0.1)	0.3(0.0)	16.1 (0.2)	3.1 (0.1)
13	Sítio Santa Bárbara	1.0 (0.0)	4.1 (0.1)	14.9 (0.2)	13.3 (0.0)	12.6 (0.3)	1.3 (0.2)	0.6(0.0)	19.4 (0.2)	3.5 (0.2)
14	Fazenda Lagoa do Morro	1.2 (0.1)	4.0 (0.1)	15.0 (0.0)	16.1 (0.2)	12.5 (0.6)	1.0 (0.0)	0.9 (0.0)	15.7 (0.4)	2.5 (0.3)
15	Fazenda Macaúbas de Cima	1.2 (0.0)	4.2 (0.1)	16.5 (0.0)	13.2 (0.0)	13.7 (0.5)	1.8 (0.1)	1.2 (0.0)	18.4 (0.5)	3.0 (0.1)
16	Fazenda Chapada dos Rodrigues	1.3 (0.0)	4.3 (0.0)	14.8 (0.0)	14.7 (0.3)	13.6 (0.0)	2.2 (0.0)	1.0 (0.0)	18.2 (0.4)	2.2 (0.1)

*Average±standard error

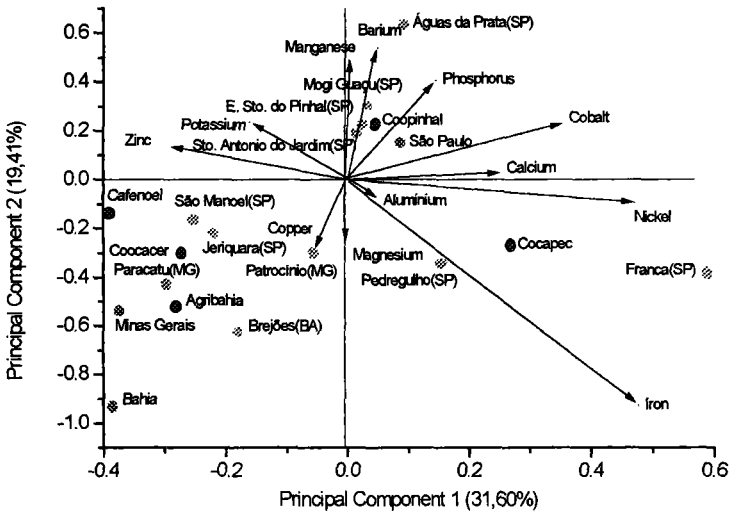


FIGURE 3. Principal component analysis of mineral content of 16 green coffees.

CONCLUSIONS

Vector plots of sensory variables and mineral content variables can explain in a simplified way the differences between all of the samples analysed, and confirmed the unique characteristics of each producing coffee area.

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A NEW PHYSIOLOGICAL METHOD TO EVALUATE GASTRIC IRRITATION OF DIFFERENT COFFEES

Prof. Dr. med. Peter W. Lücker

Due to its psycho-physio-stimulating effect, coffee is one of the most popular drinks in the civilised world. However, from the medical point of view, coffee causes several adverse events besides the wanted effect.

In relation to the sensitivity of the individual person the following groups of symptoms were reported [Fig 1]

1. Adrenerg symptoms related to the central-nervous-system expressed as sleeplessness, nervousness, and anxiety.
2. Positive inotropic symptoms expressed as tachycardia or as hypertension.
3. Gastrointestinal symptoms expressed as pyrosis, (or heartburn), stomach pressure, sensation of fullness, abdominal discomforts, nausea, and vomiting.

Positive inotropic symptoms and the stimulating effect are related to the concentrations of the methylxanthines caffeine, theophylline, and theobromine in the coffee beans. The gastrointestinal disorders are actually dose dependent in relation to the concentrations of the irritating substances, e.g. phenols pyrogallol, hydrochinone and pyrocatechin, or brenzcatechin. These phenols are derivatives of the chlorogenic acid, produced during the roasting of raw coffee beans. They belong the substances which may be the cause for the stomach irritating characteristics. These symptoms are not due to methylxanthines.

My topic of today concerns a new physiological method of determining the irritation potency of defined substances which could be drugs, beverages as coffee for example, or food compounds. Today's question is: should it be possible to show by a method, developed in our group and which I would like to talk about, that treated coffee reveals a less gastric irritation potential than the untreated one, a result which then would implicate a better digestibility of the treated coffee.

Coffee may produce mucosal irritations of the stomach, depending upon the individual's gastric sensivity and the stomach irritating quality of the respective coffee.

Intact mucosa of the human stomach is able to maintain a pH-gradient of 1 in the lumen of the stomach versus 7.4 in the blood stream. Certain chemical substances including alcohol and aspirin as well as the above named phenols, can cause damage to the gastric mucosa, followed by an increase in permeability of the cell-membrane. Electrolytes will shift, especially the H^+-rediffusion into the cell, causing a change in the transmural potential difference. This leads to an intercellular acidosis which can be the starting point for mucosa diseases.

This shift of H^+ in conjunction with the net shift of Na^+ ions from the mucosal cells into the lumen, can be measured as a decrease in potential difference quantified in millivolts. The normal electric tension between the membranes of a mucosa cell is –90 mV. The tension falls, when the mucosal barrier is damaged. Measurement of this difference is considered in a simple, reliable, and reproducible method for assessing the integrity of the gastric mucosal barrier. The gastric potential difference equipment consists of a nasogastric probe to pick up gastric potential differences in the stomach [Fig 2]. The positive reference electrode is fixed onto the skin of the forearm. Both probe and skin electrode are connected to a data-logger continuously recording the tension at the cell membrane. The next figure shows an idealised gastric potential difference profile after administration of a mucosa irritating agent [Fig 3].

Parameters for statistical evaluation of gastric potential difference measurements are the so-called Reiz-Index, the area under the baseline (AUB), mean time, mean drop, maximal potential difference (Pd_{max}), and total time (t_{tot}).

The parameters are defined as follows [Fig 4, 5].

In our institute in recent years several studies were performed measuring the gastric irritation potential of different drugs, for example aspirin or non-stereoidal-antirheumatics. One of the aims of those studies was to develop a galenic formulation with a less irritation potential than the active ingredients of the tablets or capsules itself.

To apply our method to differently pre-treated coffees, we planned and carried out two studies sponsored by Idee Kaffee Darboven, Hamburg, to prove that the treatment of unroasted coffee beans with the newly developed and patented Darboven improvement procedure resulted in a better digestibility and a less irritation potential of coffee.

Preceeding these studies, a chemical analysis and an in vitro experimental pilot study were performed.

In the chemical analysis, conducted by Meyer, Menthe, and Neumann-Hensel, in 1997, the concentrations of pyrocatechin, hydrochinone and pyrogallol of coffee, treated with the newly developed Darboven procedure, were compared with the concentrations of these substances in non-treated coffees. As shown in the table [Fig 6], the concentrations of the phenols are markedly reduced, especially those of Brenzcatechin, which decreased about more than one third of the initial concentration.

In the bacterial toxicity test, also conducted by Meyer, Menthe, and Neumann-Hensel, in 1997, the model organism pseudomonas putida – as a representative for heterotrophic micro-organisms – has been cultivated over some generations under defined conditions. During this cultivation phase, it has been investigated to which extent the water extracts of the test

substances can inhibit the growth of pseudomonas putida, in this case preparations of treated and non-treated coffee beans,. The chronic influence of the test samples to the bacteria were examined after an application time of 16 hours.

The results of the pseudomonas putida growth inhibition test show markedly less growth inhibition for the treated coffee (about 15% in comparison of 45% for the untreated coffee).

As both investigations, the chemical analysis and the experimental study, gave first hints of a probably better digestibility of the coffee, treated with the Darboven improvement procedure, we investigated the stomach irritation potential of treated coffee samples compared to untreated coffee samples. With our model, the continuous documentation of the transmural gastric potential difference is evaluated by a suitable and non-invasive method to quantify objectively the gastric tolerability and irritation of coffee samples.

In both studies the subjects swallowed a stomach probe, model LOT 440-M3 from Ingold-Mettler, Toledo, which monitored the potential difference during the test interval; as reference a skin electrode, model EK 56, was fixed at the forearm.

The tip of the probe was placed in the fundus, about 50 cm away from the teeth. The subjects were placed on a bed. The initial potential difference was plotted to receive the baseline. After a stable baseline was reached, the subjects received the differently treated coffee samples in a cross-over manner with a washout phase of 48 hours. The potential difference was monitored until the baseline values were reached again.

The main parameter measured were the gastric potential difference after intake of the different coffee samples. Additionally, some specific parameters of clinical chemistry as well as the safety parameters were assessed. For safety parameters, clinical laboratory with clinical chemistry and haematology were investigated as well as the subjects were asked with open, non-leading questions for possible adverse events which they may have experienced during the study.

The studies were designed as open, single dose, randomised, three-factorial two-treatment two-period Latin square with a wash-out period of 48 h. The volunteers were hospitalised during the study.

The first study served as a pilot study which included 4 volunteers. The second study included 18 healthy male volunteers, aged 21-45 years, divided evenly in two groups. Group b1 were given treated coffee, group b2 were given untreated coffee. Within the two treatment groups, 9 volunteers with a Helicobacter pylori-(Hp-)positive finding and 9 volunteers with a Helicobacter pylori- (Hp-)negative finding were defined as subgroups.

On study days 1 and 3, GPD were measured as described and in a reclining position over 120 min after intake of the coffee samples. GPD was started at 8 a.m. with volunteers in fasting state. The last snack was served at 11 p.m. the night before. When a stable baseline of GPD had been reached, treatment was administered.

For the treatment, 14 g of coffee powder were freshly brewed with 200 ml boiling water: after a brewing period of 5 min, the coffee samples were decanted and put in a water bath of 45°C. 150 ml processed (coffee sample b2) or non-processed blended coffee samples (coffee sample b1) with neither milk nor sugar were drunk by the subjects according to the randomisation plan.

Smoking was prohibited on days 1 and 3, standardised meals were served 5 and 10 h after administration.

Blood samples for the determination of clinical chemistry parameters were taken on study days 1 and 3 just before administration (-5 to 0 min), as well as 5, 10, 20, 30 min and 1, 1.5, 2, 3, 4, and 5 h after administration. Blood samples for safety evaluation were taken prior to enrolment and at final check.

For statistical analysis, single GPD values were given as profiles. Primary and secondary parameters of GPD values were listed individually and described statistically by geometric means, dispersion factor, arithmetic means, standard deviation, coefficient of variation, median, minimum and maximum values. 90% confidence interval ratios were calculated. The parameters were tested using an analysis of variance model (ANOVA) for the Latin square design after log-transformation. Safety parameters were listed individually and described statistically.

The study was carried out in accordance with GLP-Guidelines and all concerned international standards and regulations for human experimentation.

The evaluation of gastric potential difference revealed the following results [Fig 7].

In the overall population, the evaluation of main parameters Reiz-index (RI), area under the baseline (AUB), maximum potential difference (Pd_{max}) and total time (t_{tot}) revealed marked differences between both coffee samples.

RI and AUB were statistically significantly lower ($p=0.0282$, $p=0.0136$) and t_{tot} shorter ($p=0.0286$) for the treated coffee samples than for the untreated ones. The differences for Pd_{max} were marked but not statistically significant.

Looking at the graphs of the medians of the individual GPD's, the difference between both coffee samples became obvious [Fig 8]. The graph of the untreated coffee sample is more pronounced than the graph of the treated coffee. It could also be seen, that in the group with

the treated coffee the baseline is reached earlier than in the group of the untreated coffee samples.

In both groups mean time and mean drop were lower for the untreated than for the treated coffee samples [Fig 9].

Overall, there are significant differences of the treated coffee sample compared to the untreated coffee expressed in a significantly smaller Reiz-index, a significantly smaller area under the baseline and a significantly shorter total time of irritation; furthermore the treated coffee sample revealed a maximum potential difference, as well as mean time and mean drop which were markedly less compared to those of the untreated coffee sample.

Concerning the Hp-subgroups, a difference between both groups could also be seen. In the Hp-positive group the differences were statistically significant between the two coffee samples in all GPD parameters. These exciting results show that Hp-positive persons, counting about 80 % of the European population, are very sensitive against untreated coffee samples [Fig 10].

Mean time and mean drop were lower for the untreated than for the treated coffee samples for both subgroups [Fig 9].

Out of all tested clinical chemistry parameters, total cholesterol, HDL-cholesterol and pancreatic peptides showed an increase of the arithmetic means in both coffee samples. In Hp-positive subjects coffee revealed a statistically significantly higher increase of the mentioned parameters than in Hp-negative.

[Fig 11] We could summarize, that with our method I have just presented, it is possible to validate, that the pre-treatment of unroasted coffee beans with the new Darboven improvement procedure is followed by a statistically significantly lower stomach irritation potential, than it is to be found in untreated coffee.

It can be concluded that the treated coffee has a better digestibility with a less stomach irritating potential than the untreated coffee.

[Fig 12] **(Gastric potential difference, summary)**

Literature by the author

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Fig. 1 Adverse events of coffee

- Adrenergic symptoms related to the central nervous system expressed as sleeplessness, nervousness, and anxiety
- Positive inotropic symptoms expressed as tachycardia or hypertension
- Gastronintestinal symptoms expressed as pyrosis (or heartburn), stomach pressure, sensation of fullness, abdominal discomforts, nausea, vomiting and last but not least gastritis.

Fig. 2 Measuring of gastric potential difference

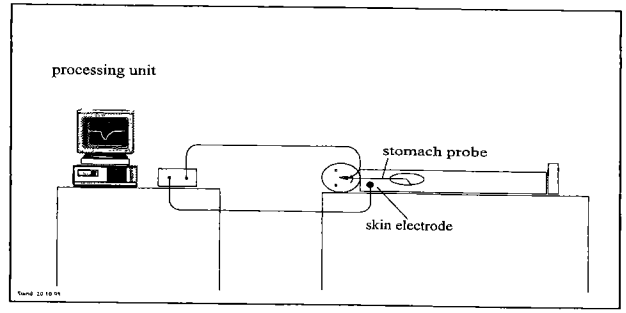


Fig. 3 Ideal Graph of Gastric Potential Difference

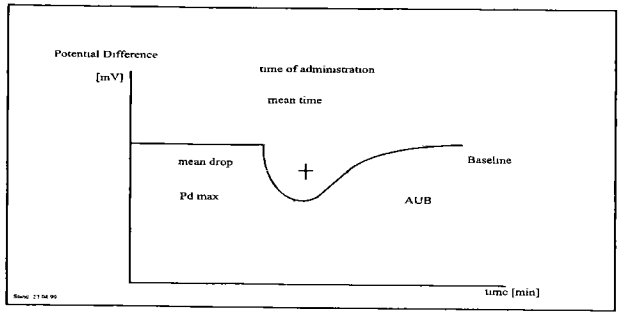


Fig. 4 Definition of geometric parameters (I)

BL — The baseline is a linear regression of the steady state values before and after reaction.

AUB — The area under the baseline is calculated by using the trapezoidal rule; $\Delta Pd/dT$ [min · mV]

Pd_{max} — The maximal decrease in GPD was originated by Caspary; in this model, Pd_{max} is used indirectly as a term in the Reiz-index (Reiz means irritation) calculation, [mV]

RI — The Reiz-index is an empirical parameter calculated as follows:

$$RI = \frac{Pd_{max} \cdot AUB}{1000} \quad [mV \cdot mV \cdot min]$$

Fig. 5 Definition of geometric parameters (II)

t_{tot} The total time is the time interval the potential remains below the baseline, [min]

MT axis The mean time is the first statistical moment around the Volt

$$MT = \frac{\int t\, Pd/dt}{AUB} \text{ [min]}$$

MD axis The mean drop is the first statistical moment around the time

$$MD = \frac{1/2 \int t\, Pd^2/dt}{AUB} \text{ [mV]}$$

Fig. 6 Concentration of Phenols in the aqueous Extract of treated and untreated Coffee Beans, as used in our study

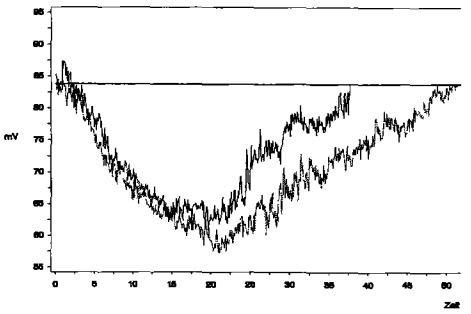
Phenols	Untreated Coffee mg/Kg	Treated Coffee Mg/Kg	Percentage
Brenzcatechine	118	72	61%
Hydrochinone	14	11	78%
Pyrogallol	38	32	84%

Fig. 7 Medians, Standard deviations, and p-values of the primary pharmacodynamic Parameter (n=17)

Parameter	Statistics	B1: Treated Coffee sample (reference)	B2: Untreated Coffee sample (test)	p-value Treatment efficacy
RI [$mV^2 \cdot min$]	Median SD	13.73 10.36	23.34 12.67	p=0.0282 ●
AUB [$mV \cdot min$]	Median SD	455.98 249.14)	662.83 323.25	p=0.0136 ●
Pd_{max} [mV]	Median SD	28.75 5.29	31.71 5.46	p=0.2253
t_{tot} [min]	Median SD	36.88 12.94	46.67 14.26	p=0.0286 ●

RI=Reiz-index, AUB = Area under the baseline, Pd_{max} = maximum potential difference, t_{tot} = total time, ● = significant treatment difference ($p < 0.03$)

Fig. 8 Gastric Potential Difference of untreated and treated Coffee samples



Blue: treated coffee sample, red: untreated coffee sample

Fig. 9 Mean time and Mean drop overall and in both subgroups

Mean Time Median	SD	Mean Drop Median	SD	
Overall				
23.01	6.92	10.08	9.74	untreated coffee samples
18.59	6.91	8.64	1.87	treated coffee samples
Hp-negative volunteers				
24.16	6.94	9.07	1.92	untreated coffee samples
21.22	7.43	8.74	2.22	treated coffee samples
Hp-positive volunteers				
21.84	7.17	10.48	1.53	untreated coffee samples
18.48	5.75	8.64	1.60	treated coffee samples

SD = Standard deviation, Hp = Helocibacter pylori

Fig. 10 p-Values of the primary pharmacodynamic Parameters with Respect to the Subgroups

Parameter	p-value Treatment efficacy Hp-negative	Hp-positive
RI [mV²*min]	p=0.6813	p=0.0198 •
AUB [mV*min]	p=0.4338	p=0.0184 •
Pd_{max} [mV]	p=0.4819	p=0.0395
t_{tot} [min]	p=0.4430	p=0.0148 •

RI = Reizindex, AUB = Area under the baseline,
Pd_{max} = maximum potential difference, t_{tot} = total time,
Hp = Helicobacter pylori,
•: Significant treatment difference ($p < 0.05$)

Fig. 11 Conclusion

- The pretreatment of unroasted coffee beans with the new Darboven improvement procedure revealed a statistically significant lower stomach irritation potential compared to the untreated coffee beans, proven by the measurement of gastric potential difference.
- It can be concluded that the treated coffee has a better digestibility with a less stomach irritating potential than the untreated coffee.
- Especially the Helicobacter pylori-positive volunteers (80% of the European population are Hp-positive) showed significantly better results with treated coffee compared to the Hp- negative volunteers.

Fig. 12 Gastric Potential Difference (Summary)

- Validated Model in Human Clinical Pharmacology
- Non-invasive Method
- Continuous measuring of the transmural Potential
  ⇒ Electric Tension between Surface of gastric Mucosa and the Skin
- Assessment of the Integrity of the gastric Mucosa
- Quantification (Extend and Time) of the Mucosa-Irritation-Potential of several Substances (Aspirin, Coffee, NSAIDs)
- Validated Modification of the classical Method
  - Skin Standard Probe as Reference instead of probe in the venous Blood
  - Stomach Probe (LOT 440-M3, Ingold/Mettler, Toledo) instead of KCl-Agarose-bridges; Datalogger DL 79, Standard Instruments Karlsruhe, with connected Processor

BIOTRANSFORMATION AND LACK OF MUTAGENICITY OF OCHRATOXIN A USING COMBINATIONS OF MAMMALIAN BIOTRANSFORMATION ENZYMES

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Abstract
The genotoxicity of ochratoxin A was studied in many *in vitro* systems, mostly negative or inconclusive results were obtained. However, in recent studies, a mutagenic response was observed in mammalian cells transfected with certain human cytochrome P450 enzymes. Moreover, an involvement of glutathione in this mutagenic response is indicated by a reduced mutagenicity after glutathione depletion. Ochratoxin A was incubated with cytochrome P450 containing protein fractions for varying times. Formation of metabolites was assessed by HPLC with UV- and fluorescence detection. Enzymatic activity of specific P450 enzymes in the fractions was determined using established marker substrates. For some incubations, subcellular fractions with glutathione S-transferase activity and glutathione were added to the incubations. Using rat liver cytochromes P450, biotransformation of ochratoxin A to metabolites was observed by cytochrome P450 1A1/2 and 3A. Cytochrome P450 3A likely results in hydroxylation of the isocoumarin ring, P450 1A1/2 forms a ochratoxin A metabolite in low yields which has not been characterized. Addition of subcellular fractions with glutathione S-transferase activity and glutathione did not modify the products formed based on HPLC retention and UV- or fluorescence spectra. Incubations with genetically expressed human P450 3A4, 1A1/2 , 2C9 and ochratoxin A resulted in product formation in very low yields despite a high activity of the preparation with established marker substrates. Ochratoxin A mutagenicity was studied in the presence of human cytochromes P450 and purified glutathione S-transferases. No mutagenic response was observed. The experimental results obtained to date indicate that ochratoxin A is a poor substrate for cytochromes P450s and glutathione dependent biotransformation reactions are unlikely to occur due to the lack of formation of electrophilic intermediates. Based on this results, formation of DNA-binding of intermediates in ochratoxin A metabolism seems unlikely.

Introduction

Human exposure to the mycotoxin ochratoxin A varies widely and may reach up to a few µg/day depending on dietary habits. Calculations on the exposure of humans to ochratoxin A suggest daily doses of 20 ng ochratoxin A through coffee consumption. In experimental animals, ochratoxin A is nephrotoxic and induces tumors, primarily in the kidney, but also in the urinary bladder, liver and mammary gland of rodents. In humans, exposure to high levels of ochratoxin A in diet has been linked with chronic renal disease (Balkan endemic nephropathy, interstitial nephritis) and an increased incidence of urinary tract tumors (renal pelvis, ureter, and urinary bladder). The observed organotropism can be explained partly by toxicokinetics, but the mechanistic basis for the tumorigenicity of ochratoxin A is not well understood.
Although a genotoxic potential of ochratoxin A has been demonstrated *in vitro* and *in vivo*, the nature of DNA-damage and/or mutations caused by ochratoxin A deserves further investigations.
The mechanisms of ochratoxin A tumor induction in the urinary tract are not well defined, both genotoxic and non-genotoxic mechanisms may contribute to tumor formation. The formation of spots interpreted as ochratoxin A derived DNA-adducts was observed in the target tissues of ochratoxin A toxicity in rodents by the use of

the very sensitive ^{32}P-postlabeling assay. In only one study, a mutagenic response was observed in mammalian cells transfected with certain human cytochrome P450 enzymes. However, since the structures of the possible adducts have not been defined, definite conclusions for the assessment of the human tumor risks associated with ochratoxin A exposure may not be drawn from these observations. These observations suggest an involvement of DNA-damage and genotoxicity in the tumorigenicity of ochratoxin A.

Table 1: Mechanistic data on ochratoxin induced tumorigenicity

Support for direct genotoxicity	Support for non-genotoxic mechanisms
Unscheduled DNA-synthesis in target cells	No mutagenicity in the Ames-test
DNA-modifications by ^{32}P-postlabeling (spots on TLC-plates)	No DNA-adduct structures
Mutagenicity in mammalian cells transfected with human P450 enzymes	No electrophilic metabolites identified so far

Support for non-genotoxic mechanisms of ochratoxin A renal carcinogenicity can be derived from studies on the mutagenicity of ochratoxin A. The genotoxicity of ochratoxin A was studied in many *in vitro* systems, mostly negative or inconclusive results were obtained (Table 1).
Exposure to ochratoxin A must be minimized in order to protect public health. However, based on the available scientific information, it is currently difficult to accurately establish a safe level of ochratoxin A exposure for humans. The elucidation of the mechanism by which ochratoxin A causes DNA damage in kidney tissue may greatly help to select the most relevant risk assessment procedure based on which, appropriate limits for food products could be established. Overall, this project will ensure that efforts and actions devoted to keep human ochratoxin A exposure as low as reasonably possible are appropriate and based on a strong scientific basis.

Because of the lack of relevant human data, the health risk resulting from ochratoxin A-exposure has been assessed based on experimental animal data. According to the mechanism of ochratoxin A tumorigenicity postulated, two approaches have been envisaged for human health risk assessment (Fig. 1).

- *Ochratoxin A acts through a direct genotoxic mechanism (covalent binding to DNA).* Therefore, the Fisher 344 rat carcinogenicity bioassay is used as the critical study for a model-based risk assessment. Applying this approach, virtually *safe dose estimates* as low as 1.5 ng/kg bw/d have been derived.
- *Ochratoxin A causes DNA damage through an indirect mechanism such as oxidative stress and/or cytotoxicity.* Considering this hypothesis, JECFA applied a safety factor-based approach for the risk assessment of ochratoxin A and established a Provisional Tolerable Weekly Intake of 100 ng/kg bw (Provisional Tolerable Daily Intake of 15 ng/kg bw).

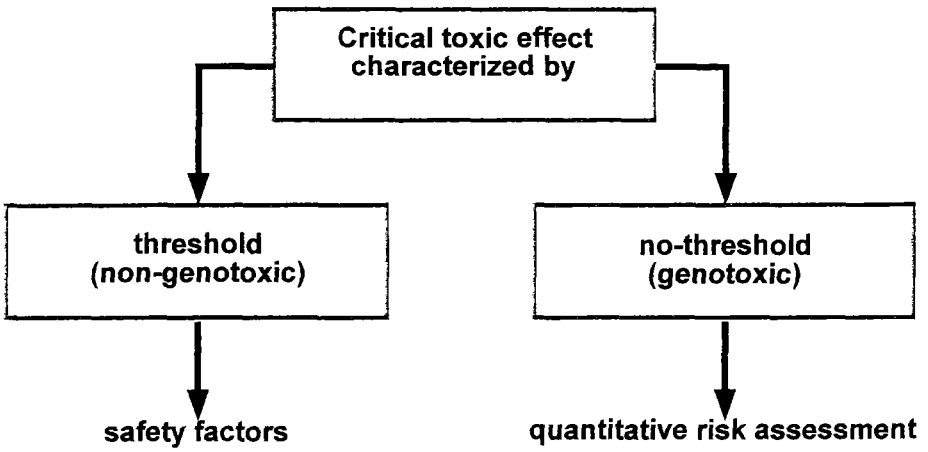

Fig. 1: Human risk assessment process based on different mechanisms of action.

In this study, we have investigated the capacity of subcellular fractions and recombinant or purified enzymes to transform ochratoxin A to devise an optimal activation system for mutagenicty testing. The obtained results suggest only a very low turnover of ochratoxin A to metabolites and do not indicate the formation of reactive intermediates.

Results

The biotransformation of ochratoxin A was studied in microsomal fractions from liver and kidney of mice and rats. Formation of 4-hydroxyochratoxin A could only be observed in liver microsomes from both species. In kidney microsomes rates of ochratoxin A oxidation were below the limit of detection despite activity of the microsomes with marker substrates. When liver microsomes from rats pretreated with inducers were used to study ochratoxin A biotransformation, large increases in the rats of oxidation were seen with phenobarbital, methyl cholanthren and dexamethasone as inducers

Biotransformation of ochratoxin A by human cytochromes P450. Oxidation of ochratoxin A by human cytochrome P450 was investigated using microsomes from cells selectively expressing specific human P450 enzymes and with human liver microsomes. Low rates of formation of 4-hydroxyochratoxin were observed with human cytochrome P450 3A4, the rates of formation with cytochromes P450 1A2 and 2C9 were very low and at the limit of quantitation.

Table 2: Ochratoxin A biotransformation in subcellular fractions and with isolated enzymes

Enzyme or subcellular fraction	Product formation	Mutagenicity
Rat kidney microsomes or cytosol	No metabolites	-
Rat liver cytosol	No metabolites	-
Rat liver GSH S-transferases semipurified	No metabolites	-
Rat liver microsomes from induced animal + GSH	4-Hydroxyochratoxin A	-
Human CYP 3A4 Supersomes™	4-Hydroxyochratoxin A < 100 fmoles/pmol P450/mM	-
Human CYP 1A2 Supersomes™	No metabolites	-
Human liver microsomes (n = 6)	No metabolites	n.a.
Horseradish peroxidase + H_2O_2	No metabolites	-

Biotransformation of ochratoxin A by isolated enzymes. A variety of other enzyme systems has been implicated in the biotransformation of ochratoxin A to potentially reactive intermediates based on indirect observations. In a series of experiments, the capacity of enzymes with model activity (peroxidases) or semipurified enzymes were assessed. Incubation of ochratoxin A with both horseradish peroxidase and soybean lipoxigenases did not result in the formation of metabolites when analyzed by HPLC with fluorescence detection or UV-detection. Moreover, glutathione S-transferase, combinations of semipurified glutathione S-transferase with liver microsomes from dexamethasone pretreated rats or liver 9 000 g supernatant from dexamethasone pretreated rats fortified with NADPH and glutathione (5 mM) did not result in formation of ochratoxin A metabolites (Table 2).

Mutagenicity of ochratoxin A. The mutagenicity of ochratoxin A was investigated using a variety of different conditions for bioactivation in the Ames-Test preincubation system refering to the various possible pathways of ochratoxin A bioactiviation suggested in the literature. Even in the presence of high concentrations of ochratoxin A (up to 1 mg/plate), an increase in the rates of revertants was not observed under the conditions tested in TA 100 amd TA 2638 (Table 2).

Summary

In rat liver, ochratoxin A is oxidized by cytochromes P450 3A 1/2 and 1A2 to 4-hydroxy-ochratoxin A. With human cytochromes P450 (supersomes™) only cytochrome P450 3A4 catalyses ochratoxin A oxidation to 4-hydroxyochratoxin A at very low rates. Oxidation is not observed with P450 1A2 supersomes or in human liver microsomes.
These data suggest that biotransformation of ochratoxin A to reactive metabolites capable of binding to DNA constituents is unlikely and does not account for the tumorigenicity of this mycotoxin to the kidney.

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WORKSHOP REPORT : EFFECTS OF COFFEE ON BRAIN AND BEHAVIOR. WHAT DOES COFFEE CONSUMPTION BRING IN DAILY LIFE ?

Chairperson : Dr Astrid NEHLIG, Strasbourg, France
Participants : Pr Andrew P. SMITH, Bristol, UK ; **Dr Jan SNEL,** Amsterdam, The Netherlands ; **Dr Martin van BOXTEL,** Maastricht, The Netherlands ; **Pr Bertil FREDHOLM ,** Stockholm, Sweden ; **Pr Edwin ZVARTAU,** St-Petersburg, Russia ; **Pr Gaetano DI CHIARA,** Cagliari, Italy

SUMMARY OF THE WORKSHOP

'Effects of Low Doses of Caffeinated coffee on mood and performance' (Pr Andrew Smith)

The aim of the present research was to review the effects of caffeine on behaviour of adult humans. Many of the generalisations about the behavioural effects of caffeine are based on studies which have used very large doses of caffeine, used students as subjects and tested them in the early morning only. A. Smith conducted research to demonstrate that multiple smaller doses of caffeine throughout the day resulted in the same beneficial effect as one large dose. The participants were given 60 mg caffeine and asked to carry out a number of different tests. The results showed that even this dose of caffeine led to improved mood and enhanced encoding of new information.

The published literature on the effects of caffeine on behaviour show that it increases alertness, reduces fatigue and leads to improved performance of vigilance tasks and simple tasks requiring sustained response. The researchers demonstrated that these effects were most apparent when alertness is reduced by other factors (e.g. sleep deprivation, working at night, prolonged work, consumption of lunch and minor illness). The positive effects of caffeine can be observed in very low/non-consumers which suggests that withdrawal doesn't underlie the effects.

Negative effects of caffeine have been found with extremely high levels of consumption and in certain individuals. These effects must, however, be distinguished from the behavioural changes observed when caffeine is consumed in moderation by the majority of the population. Generally, consumption of caffeine is well controlled and the pattern of consumption suggests that individuals ingest caffeine to help restore levels of reduced alertness to a more optimum level. Similarly, consumption is reduced when high alertness is undesirable, such as when the person is trying to sleep.

'Effects of coffee and caffeine on selective attention and information processing' (Dr Jan Snel)

The aim of this research was to assess the effect of moderate amounts of caffeine, the equivalent to about 2 cups of coffee, on information processing in healthy students of about 22 years old. The participants in the study were all regular coffee consumers, drinking between 3 – 7 cups of coffee per day. For some of the testing scalp electrodes were used to record brain activity during performance of cognitive tasks.

In this current study different amounts (1.0, 3.0 and 7.5mg/kg bodyweight) of caffeine were used in a dual task setting consisting of two choice reaction time tasks, with one of those tasks being presented at 2 levels of difficulty. The 7.0 mg dose shortened reaction time on both the primary and the secondary task. Overall there was a dose

dependent decline in reaction time for both tasks and indications of increased quantity of processed information. There was no effect of dose on the quality of task performance (number of errors). There were no dose-related differences in the reported effort needed to perform the dual task. In opposite to previous work by this group, the effect of caffeine was found only on the output stage, in view of the shortened reaction times. Caffeine yielded a higher overall arousal level, more profound processing of both attended and unattended information and faster motor processes. Further recent research has evaluated whether 250 mg of caffeine would have an effect on the processing of spatial and non-spatial (colour) information, and which sites of the brain were affected. Caffeine did not affect the quite early components of the selection potentials as found before, suggesting that caffeine does not modulate spatial attention alone.

It was concluded by these researchers that caffeine may widen attention for initial processing thereby enlarging the amount of information that can be processed, while later on in the information processing stream its influence on frontal control mechanism may narrow attention to increase attentional selectivity.

'Effects of coffee and caffeine on memory and aging' (Dr Martin Van Boxtel)

Acute effects of caffeine on cognitive performance have been found in areas of vigilance, selective attention and speed of information processing. The evidence to date for a positive effect of caffeine on memory function has been less unequivocal. In recently conducted studies in which the effect of caffeine on 2 models of age-related cognitive decline were investigated, beneficial effects of caffeine restoring some aspects of verbal memory performance were observed.

On average, memory function decreases from the age of 30 – 35 years onwards, along with other aspects of cognitive function, as a result of the usual aging process. This change in memory performance can for a large part be explained in terms of reduced speed of information processing. Self-administered caffeine in commonly consumed food and drinks may alleviate the decline in age-related performance to some effect. The subgroup of elderly people could especially benefit from the effects of caffeine on memory, possibly by adjusting their caffeine intake at a more appropriate level.

These researchers suggest further studies are needed to unravel the complex relationship between caffeine intake, cognitive performance and aspects of quality of life, as studies of this kind are easily confounded by socio-economic and health related variables.

'Neurochemical effects of caffeine and behavioral tolerance to caffeine' (Pr Bertil Fredholm)

Caffeine is known to produce biphasic behavioural effects – lower doses being stimulatory, higher doses being inhibitory. Tolerance rapidly develops to these effects. Of all the potential biochemical targets for the actions of caffeine the only one to be activated at the levels of caffeine during normal consumption of caffeine is antagonism of the actions of the endogenous adenosine at A1 and A2a receptors, the latter ones being more sensitive to low doses of caffeine than the former ones. A1 receptors are abundantly expressed – especially at the terminals of excitatory nerve endings. When the actions of adenosine are blocked at these sites transmitter release, especially glutamate release, is increased. This will affect the activity of neurones in the striatum known to regulate motor behaviour and motivational aspects of behaviour. The latter structure contains both A1 and A2a receptors. Adenosine A2a receptors are virtually confined to the striatal GABAergic neurones where they are colocalized with dopamine D2 receptors. In the same structure adenosine A1 receptors are colocalized with dopamine D1 receptors. The blockade of A2a adenosine receptors by caffeine appears to be crucial for the stimulatory effects of caffeine.

Locomotor tolerance to caffeine in rats is not reflecting an increased metabolism of the methylxanthine. Although the mechanisms responsible for this tolerance do not develop to a pure adenosine A2a antagonist and remain to be further explored, tolerance to the motor stimulant effects of caffeine is accompanied by a decreased expression of adenosine A2a receptors in the anterior parts of the striatum and an increased expression of the adenosine A1 receptor gene in the amygdala.

'Reinforcing effects of caffeine' (Pr Edwin Zvartau)

The experiments carried out by this group, on experimentally naive mice, were designed to determine if caffeine possesses reinforcing effects with the aid of a one-session initiation of intravenous drug self-administration method. Their data suggest a reinforcing action of caffeine and show the utility of one session self-administration for the study of reinforcing properties of drugs with unstable addictive potential. The reinforcing action of caffeine appears to be mediated by calcium channels agonists and antagonists and by adenosine A1 receptors.

A further study employed place conditioning technique to compare the rewarding potential of caffeine with that of cocaine, the classical narcotic euphoriant, and ethanol, widely abused non-narcotic substance with equivocal rewarding potential in animal experiments. Data showed that attractiveness of cocaine-paired cues was 100% higher than those for caffeine, whereas reinforcing actions of caffeine and ethanol seemed to be equal. Moreover, low doses of caffeine (3 mg/kg) are able to prevent the extinction of the cocaine seeking pattern of the mice while high doses of either caffeine (30 mg/kg) or cocaine (20 mg/kg) are ineffective. The effects of caffeine on cocaine-seeking behaviour might be due to the interaction with adenosine A1 but not A2a receptors.

'Caffeine and dependence: dopamine release' (Pr Gaetano Di Chiara)

In the last decade, several studies have shown that dopamine seems to play a critical role in the central actions of drugs of abuse. These drugs share the property of preferentially stimulating dopamine release in the shell of the nucleus accumbens as compared to the core of the nucleus accumbens and the medial prefrontal cortex. By using microdialysis, the present study shows that intravenous caffeine at doses which elicit behavioural stimulation (0.5 – 5 mg/kg), does not increase dopamine transmission *in vivo* in the shell and the core of the nucleus accumbens. Thus, the present results substantiate that caffeine's low addictive potential is due to its failure to increase dopamine transmission in the shell of the nucleus accumbens. However, simultaneously, the same low doses of caffeine (0.5 - 5 mg/kg) stimulate dose-dependently the release of dopamine and acetylcholine in the medial prefrontal cortex. Thus, caffeine differs from the classical drugs of dependence by its lack of capacity to stimulate dopamine transmission in the shell of the nucleus accumbens. Conversely, the activated release of dopamine in the prefrontal cortex is consistent with psychostimulant and reinforcing properties of caffeine and the enhanced release of cortical acetylcholine might reflect the cognitive enhancing properties of caffeine. The difference between the abuse potential of the other dopaminergic drugs like cocaine and amphetamine, and caffeine may relate to their different mechanism of action at the level of dopamine. Indeed, both amphetamine and cocaine act by increasing the brain level of dopamine which will then be free to bind to either dopamine D1 or D2 receptors. Conversely, caffeine acts preferentially at the level of adenosine A2a receptors which are colocalized with dopamine D2 receptors in the striatum. It remains to be demonstrated whether or not the binding of dopamine at the level of the D2 receptors can be considered as the critical mechanism of action underlying drug dependence.

'Caffeine does not activate the brain structures involved in drug addiction' (Dr Astrid Nehlig)

The purpose of the study was to examine the changes in cerebral functional activity measured as rates of glucose utilisation after the administration of increasing doses of caffeine ranging from 1 to 10 mg/kg that are relevant to the daily human consumption. Indeed the mean daily intake of caffeine is 1–2 mg/kg/day for the general population, increases to 2.4-4.0 mg/kg/day in the USA and the UK and reaches even 7.0 mg/kg/day in Scandinavia. It is well known that caffeine stimulates locomotor activity, disturbs sleep and influences mood. With the technique used, the authors were able to confirm the high sensitivity of the caudate nucleus which mediates locomotor activity and of the raphe nuclei and locus coeruleus which mediate sleep and mood to very low doses of caffeine (1 mg/kg). These data are in line with the high sensitivity of the latter functions to caffeine.

The second part of the talk was devoted to dependence. Caffeine has been hypothesized as a potential drug of abuse for over a decade. However since caffeine is not fulfilling enough criteria, it was not considered as a drug of dependence according to the 1992 APA classification. In 1994, Strain et al. found 16 out of 99 subjects that were considered as dependent on caffeine, but, most of them had a previous history of drug abuse and a higher incidence of psychiatric illnesses than in the general population. Thus, the possible dependence on caffeine became of great concern among consumers. Here, the authors showed that at doses ranging from 1 to 5 mg/kg/day, caffeine does not activate functional activity in the shell of the nucleus accumbens which is the key structure for addiction and reward. The activation of this area occurs only at high doses of caffeine (10 mg/kg) which increase glucose utilisation also in the core of the nucleus accumbens as well as in most other brain regions. This widespread unspecific response is likely to reflect the numerous adverse side effects that occur after the consumption of high doses of caffeine. Likewise, human studies performed by the same group show that 250 mg caffeine given in a cup of decaffeinated coffee do not induce any change in functional activity in the nucleus accumbens while nicotine which has a high addictive potential increases blood flow in that region of the human brain.Thus the present data confirm the well-described sensitivity of motor activity, sleep and mood to caffeine while they do not confirm the addictive potential of caffeine reported in the study by Strain et al. (1994).

Conclusions

From the present workshop it appears that most individuals manage to keep their daily intake of coffee or caffeine at a level which allows to trigger the desired positive effects on mood, alertness and relaxation. Caffeine at low doses appears indeed to be efficient in daily life situations, especially in low arousal states. This effect may be achieved by widening attention for initial processing hence enlarging the amount of information that can be processed and later on by focusing attention to increase selectivity. The subgroup of elderly people could especially benefit from the effects of caffeine on memory, possibly by adjusting their caffeine intake at a more appropriate level.

While the reinforcing effects of caffeine have been now clearly established both by behavioural and biochemical approaches, it appears that caffeine does not share with the common drugs of abuse the ability to increase functional activity and dopamine release in the area of the brain mediating dependence which is in line with its lack of addictive potential. This is true also for high consumers of caffeine like the Nordic countries. Indeed in humans, coffee or caffeine intake is fractioned over the day while in animal experiments caffeine is given as a single dose and only the doses ranging from less than 1 to 5 mg/kg at the most can be considered as relevant to the human situation.

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REPORTS OF THE PARTICIPANTS TO THE WORKSHOP:

Behavioral effects of caffeine in coffee

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An Overview of the Published Literature

Smith (*1*) has reviewed the published literature on caffeine and behavior. This review has been sub-divided into a number of different sections and the conclusions about each section are summarized below. The aim of the article was to review the effects of caffeine on the behavior of adult humans. The main areas covered were effects on mood, the efficiency of mental performance and sleep. In all areas it is important to make a distinction between effects of amounts of caffeine that are normally consumed from food and drinks and the very different effects observed with excessive amounts or in very sensitive individuals. Unlike other areas of research (e.g. studies of health effects) most studies of the behavioral effects of caffeine have examined acute changes following a single dose. Less is known about effects of regular consumption although there is now enough data on this topic to draw tentative conclusions. In addition to studying effects of caffeine consumption the research has also considered possible changes in behavior as a function of caffeine withdrawal. This issue is usually considered in the context of whether caffeine leads to dependence and this topic is not reviewed here. Rather a critical appraisal of claims that caffeine withdrawal influences performance and mood is provided.

Overall, the literature suggests that the following effects may occur when individuals consume moderate amounts of caffeine:

1. Caffeine increases alertness and reduces fatigue. This may be especially important in low arousal situations (e.g. working at night).
2. Caffeine improves performance on vigilance tasks and simple tasks which require sustained response. Again, these effects are often clearest when alertness is reduced although there is evidence that benefits may still occur when the person is unimpaired.
3. Effects on more complex tasks (e.g. memory tasks) are difficult to assess and probably involve interactions between the caffeine and other variables which increase alertness (e.g. personality and time of day).
4. In contrast to the effects of caffeine consumption, withdrawal of caffeine has few effects on performance. There is often an increase in negative mood following withdrawal of caffeine but such effects may largely reflect the expectancies of the volunteers and the failure to conduct "blind" studies.
5. Regular caffeine usage appears to be beneficial, with higher users having better mental functioning.
6. Most people are very good at controlling their caffeine consumption to maximise the above positive effects. For example, the pattern of consumption over the day shows that caffeine is often consumed to increase alertness.

Indeed, many people do not consume much caffeine later in the day as it is important not to be alert when you want to go to sleep.

In contrast to effects found with normal caffeine intake, there are studies which have demonstrated negative effects when very large amounts are given or sensitive groups (e.g. patients with anxiety disorders) studied. In this context caffeine has been shown to increase anxiety and impair sleep. There is also some evidence that fine motor control may be impaired as a function of the increase in anxiety.

Overall, the global picture that emerges depends on whether one focuses on effects that are likely to be present when caffeine is consumed in moderation by the majority of the population or on the effects found in extreme conditions. The evidence clearly shows that levels of caffeine consumed by most people have largely positive effects on behavior. Like most things excessive consumption will lead to problems and there are also some individuals who are more sensitive than others.

Effects of Low Doses of Caffeine on Mood and Performance

Many of the generalizations about the behavioral effects of caffeine are based on studies which have used very large doses of caffeine, used students as subjects and tested them in the early morning only. It is clearly desirable to have further information on the effects of the lower doses of caffeine which are more typically consumed, to examine effects in a representative sample of the population at several times of day and to consider the possible modifying effects of habitual caffeine consumption and individual differences.

Smith et al. (*2*) carried out a study to examine the above issues and comparing 60 mg caffeine with placebo. The results showed that even this dose of caffeine led to improved mood and enhanced encoding of new information. Interactions between caffeine and other factors were observed although these were usually specific to particular tasks. In other words, the general effects of caffeine were not influenced by demographic factors, time of testing, personality or habitual caffeine usage.

Importance of the Mode of Administering the Caffeine

It is important to consider whether effects of caffeine are modified by the vehicle in which it is administered. Smith et al. (*3*) examined this issue in a study with the following features. An experiment was carried out to examine the effects of 40 mg of caffeine given in different drinks (coffee, water, tea, cola) on mood and performance. One hundred and forty four volunteers were randomly assigned to one of the groups formed by combining the caffeine/placebo and drinks conditions. Following a baseline session measuring mood and different aspects of performance the volunteers were given their drink and then carried out another test session one hour later. Administration of the caffeine/placebo was double blind. The results showed that those given caffeine reported greater alertness and anxiety at the end of the test session as well as improved performance on choice reaction time tasks involving focused attention and categoric search, a semantic memory task and a delayed recognition memory task. The effect of the caffeine was not modified by the nature of the drink in which it was given. Overall, these results show that a dose of caffeine typical of the level found in commercial products can improve alertness and performance efficiency.

Effects of Regular Consumption Levels

About 25% of the participants in the above study consumed virtually no caffeine on a day to day basis. These volunteers showed similar beneficial effects of caffeine to those who regularly consumed greater amounts. This finding has been replicated in another as yet unpublished study and the presence of beneficial effects of caffeine in very low/non-consumers argues against the beneficial effects of caffeine merely reflecting removal of the negative effects of caffeine withdrawal. These findings also support the view that regular levels of caffeine consumption have little effect on the caffeine-induced behavioral changes (*4*).

Caffeine and caffeine withdrawal

In the majority of studies of caffeine the volunteers have caffeine withdrawn (usually over night) prior to testing. Positive effects of caffeine could, therefore, reflect removal of impairments produced by withdrawal. However, several studies (*4, 5, 6*) have demonstrated that one obtains similar effects of caffeine when volunteers have had caffeine withdrawn for at least 8 hours and when they have been free to consume caffeine prior to the experiment. Such results cause problems for an explanation based solely on the effects of caffeine withdrawal.

Consumption Regime

Most studies of the effects of caffeine have administered a single large dose, often equivalent to the person's total daily consumption level. Caffeine is usually ingested in a number of smaller doses and it is unclear whether effects observed after a single large dose are the same as those produced by an identical level produced by consuming several caffeine containing drinks over a longer time period. Brice and Smith (unpublished) examined this issue and found that the improved mood and enhanced performance found after a single dose of 200 mg were also observed following 4 doses of 65 mg given at hourly intervals (which resulted in an identical final level to the single 200 mg dose).

Metabolism of Caffeine

Most of the beneficial effects of caffeine show a linear dose-response relationship up to about 300 mg and this is then followed by either a flattening of the curve or, sometimes, impaired performance at higher doses. If one looks at the relationship between metabolism of a fixed dose of caffeine (as indicated by saliva levels) and mood and performance changes one finds that there is no strong association between the two. This is not too surprising in that it is not caffeine levels in the periphery per se which produce the behavioral changes but secondary CNS mechanisms. The individual differences in the metabolism of the caffeine may be very different from the individual differences in the CNS mechanisms which, plausibly, accounts for the lack of a strong association between plasma (or saliva levels) and behavioral changes.

Effects of Caffeine on Real-life Performance

The previous sections have shown that doses of caffeine typically consumed in real-life, and presented in commercial products, can improve mood and aspects of performance. The majority of studies have been laboratory experiments using artificial tasks. It is now important to ask whether similar effects are observed in simulations of real-life activities (e.g. driving).

Caffeine and Driving

A number of studies (e.g. *8*) show that caffeine can improve driving performance of fatigued drivers. In a recent as yet unpublished study caffeine was found to improve steering accuracy in drivers carrying out a one hour drive. Measures of mood and performance of artificial tasks were also taken during the last study and these also showed benefits of consuming caffeine. This suggests that changes in the laboratory may reflect a general benefit of caffeine that is also observed in real-life situations.

Changes Over the Working Day

Another method of assessing the effects of caffeine involves monitoring changes over the course of the working day. Indeed, if performance is measured before starting work and then again at the end of the working day, then the difference between the two times reflects performance efficiency over the course of the day. Smith et al. (*9*) examined both the effects of controlled consumption and free choice of caffeinated drinks over the course of the day. The results showed that caffeine consumption was greatest in the morning and that similar diurnal trends were seen in free living and free choice conditions. Consumption of decaffeinated drinks led to a reduction in alertness over the day and also slowed response times. Indeed, regular monitoring of alertness showed that decaffeinated drinks were associated with reduced alertness from 10.00 to 19.00. In the free living condition high caffeine consumption was associated with faster reaction times in the evening. Furthermore, there was evidence to suggest that it was the subjects with lower levels of alertness who subsequently drank more caffeine. Overall, these results demonstrate that caffeine consumption maximizes alertness over the working day and is often consumed to produce this effect.

Mechanisms Underlying the Effects of Caffeine on Mood and Performance

It is highly likely that many different CNS mechanisms underlie the effects of caffeine on behavior. Caffeine's major effect is as an antagonist of the adenosine receptors which in turn affects the release of a variety of neurotransmitters (e.g. noradrenaline, acetylcholine, dopamine and the GABA/benzodiazepine system). It is important to link specific behavioral changes with CNS mechanisms and also to develop profiles of the stages of information processing involved in and the energetics underlying the effects of caffeine.

Mechanisms can also be considered at the level of the cognitive processes and energetical mechanisms influenced by caffeine. Smith et al. (*10*) conducted a study to elucidate the stages of processing underlying enhanced performance by caffeine of choice reaction time tasks. In addition, they attempted to identify the

energetics of the mood and cardiovascular effects produced by caffeine. Ingestion of caffeine had no effect on initial mood but it did improve the encoding of new information and counteracted the fatigue that developed over the test session, resulting in greater subjective alertness at the end of the session in the caffeine condition.

Another approach to understanding the CNS mechanisms underlying the effects of caffeine has been to combine pharmacological challenges with administration of caffeine. If has been demonstrated that caffeine is especially beneficial when alertness is reduced. Alertness can be reduced by changing a number of the neurotransmitter systems. For example, by using clonidine, a drug which reduces the turnover of central noradrenaline, it is possible to mimic sleep deprivation in a period of a few hours. Smith et al. (unpublished) conducted a study combining caffeine/placebo and clonidine/placebo conditions. Caffeine was found to reverse the effects of clonidine but produce few effects when the volunteers were alert. However, some effects of caffeine (e.g. the beneficial effect on encoding of new information; the cardiovascular effects) were not related to changes in the noradrenergic system. Indeed, Rusted and Smith (unpublished) have shown that the encoding of new information reflects the cholinergic system and there is evidence from other studies that caffeine also influences this neurotransmitter.

Conclusions

The published literature on the effects of caffeine on behavior shows that it increases alertness, reduces fatigue and leads to improved performance of vigilance tasks and simple tasks requiring sustained response. Our research shows that these effects are most apparent when alertness is reduced by other factors (e.g. sleep deprivation, working at night, prolonged work, consumption of lunch and minor illness). Our recent research suggests that these effects can be obtained with realistic doses of caffeine (and normal consumption regimes) and that the performance improvements can be observed using simulations of real-life activities (e.g. driving) and by assessing changes over the working day. The positive effects of caffeine can be observed in very low/non-consumers which suggests that withdrawal does not underlie the effects. This conclusion is supported by studies which have shown similar effects of caffeine in withdrawn and non-withdrawn volunteers. The mechanisms underlying these effects are now being identified. Caffeine improves the encoding of new stimuli and this may reflect cholinergic changes. In addition, it improves performance when central noradrenaline is reduced which may be the mechanism underlying the large effects of caffeine in low alertness situations.

Effects on more complex tasks are less clear and probably depend on complex interactions between caffeine and other variables (e.g. personality and time of day). Indeed, the combined effects of caffeine and other variables is clearly an area which requires further study. Negative effects of caffeine have been found with extremely high levels of consumption and in certain sensitive individuals (e.g. patients with anxiety disorders). These effects must, however, be distinguished from the behavioral changes observed when caffeine is consumed in moderation by the majority of the population. Generally, consumption of caffeine is well controlled and the pattern of consumption suggests that individuals ingest caffeine to help restore levels of reduced alertness to a more optimum level. Similarly, consumption is reduced when high alertness is undesirable, such as when the person is trying to sleep.

Acknowledgements

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Summary

The present paper will give a review of 10 years of our research on the effects of caffeine on cognition and mood. Research has demonstrated that caffeine has beneficial effects on performance of tasks requiring sustained attention and that these effects are readily observed in low alertness situations (after lunch; at night; when a person has a cold). Although many of the studies of caffeine have used very large single doses, recent studies have demonstrated that beneficial effects can be observed with more realistic doses and drinking regimes that equate to real-life intake. Furthermore, the improved performance can be demonstrated using simulations of real-life tasks and in the context of a normal working day.

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Effects of caffeine on attention

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The aim of our research on caffeine is to assess the effect of moderate amounts of caffeine, the content of about 2 cups of coffee, on information processing in healthy, regular coffee drinkers (3-7cups/day); students of about 22 years old. The main methodology we use is recording of brain activity by scalp electrodes during performing cognitive tasks.

- Using a selective visual attention task (Lorist, 1995) with targets of different orientation and spatial frequency, with 3mg caffeine/kg BW it was found that task performance improved. No effect was found on selection potentials (ERPs). An early differential positivity was found indicative of increased sensitivity, although no effect on sensory discrimination was found. In this task caffeine effects suggested faster central or peripheral motor processes.
- In a visual focused selective search task, caffeine enhanced the quite early ERP components, the selection of relevant information was improved, but search negativity not. The results suggest that caffeine has specific rather than general effects on information processing.
- In a focused and divided attention conditions of a selective search task with different display loads, subjects had to locate a target item. The P3b peak latency, indicating the time needed for stimulus evaluation, decreased after 3 mg caffeine/kg BW in the low display load condition and in the focused attention condition. Apparently, the effects of caffeine are dependent on the number of relevant display items, and not on the total number of display items displayed. Search processes were unaffected.
- In our recent study (Ruijter et al., 1999a) doses of 1.0, 3.0 and 7.5 mg/kg BW were used in a dual task setting consisting of two choice reaction time tasks, one task was presented at two levels of difficulty. The 7.0 mg dose shortened RT on both the primary and secondary task. Overall there was a dose-dependent decline in RT for both tasks and increasing P3 amplitude, indicating an increased quantity of processed information. There was no effect of dose on the quality of task performance (errors). There were no dose-related differences in the reported effort needed to perform the dual task. In opposite to our previous work, an effect of caffeine was found only on the output (motor processes) stage), in view of the shortened RTs.
- Under 250 mg caffeine (Ruijter et al, 1999b) subjects were instructed to attend to stimuli with a specified color (red or blue) in order to react to the occurrence of such stimulus within the attended category. Caffeine revealed a more positive frontal P2 and an interaction between attention and treatment indicative of selective processing of information (N2b). Caffeine yielded a higher overall arousal level, more profound processing of both attended and unattended information and faster motor processes.
- The next question was whether 250 mg caffeine would have a differential effect on the processing of spatial and non-spatial information (colour) and which sites in the brain were affected (Ruijter et al., 1999c). As for the spatial selection task, caffeine did not affect the quite early ERP components as found before, suggesting that caffeine does not modulate spatial attention alone. Current Source density maps did not show an influence of caffeine, except for a trend to a slightly more active frontal localized source.

We conclude that caffeine may widen attention for initial processing thereby enlarging the amount of information that can be processed, while later on in the information processing stream its influence on frontal control mechanism may narrow attention to increase attentional selectivity

Drinking coffee is more than taking caffeine: Coffee drinking is for many a pleasant and regular ritual. The pleasure of it and the ritualized setting in which coffee drinking takes place, may form together the reason why people drink coffee and affect their behaviour. Kole et al. (1998) studied with our usual methodology the effects of coffee odour and found that just as done with caffeine alone, in the presence of coffee odour as a background stimulus the frequency of the EEG moved from alpha to beta, indicative of increased arousal. In the ERPs, parietally, enlarged N1 and P1 amplitudes were found, indicative of intensified attention. Also increased stimulus evaluation (enlarged P3) has been reported.
In conclusion, we plea for studying the effects of coffee rather than caffeine on mood, performance, and brain activity (in particular 'pleasure areas') in 'ecologically valid' situations, that is in non-abstained coffee drinkers, in a natural setting.

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Effects of coffee and caffeine on memory and aging

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Caffeine in coffee and other beverages is the most widely used psychostimulant compound today. Its vast popularity may in part be explained by its cognition-enhancing properties (1). It is assumed that these effects are related to the adenosine-A_1 and A_{2A} antagonism of caffeine in the brain which result in the increased release and turnover of several central neurotransmitters, including acetylcholine and noradrenaline (2, 3). Adenosine receptors are particularly abundant in the hippocampus, a brain structure which is critical for memory formation. Acute effects of caffeine intake on cognitive performance in humans have typically been found in the domains of vigilance, selective attention and speed of information processing (4).The evidence to date for a positive effect of caffeine on memory function has been less unequivocal. In recent experiments in which our group studied the effect of caffeine in two models of age-related cognitive decline (scopolamine- and exercise-induced cognitive impairment) we found in young healthy individuals a beneficial effect of low doses of caffeine in restoring some aspects of verbal memory performance (5, 6). In addition, in the Maastricht Aging Study (MAAS), a population-based longitudinal study into the determinants of cognitive aging (N=2,000) that is based at our institute, verbal memory performance was positively associated with the estimated habitual intake of caffeine when potential confounding variables were controlled for (7). These results corroborate earlier findings of Jarvis in another population study (8). In this much larger sample of adult individuals (N=9,000) the positive effect of habitual caffeine intake on (incidental) memory function followed a dose-response trend and was specifically attributed to the oldest age group (55 and over). The

overall scientific evidence to date suggests that caffeine primarily modulates the actual level of arousal, thereby mildly facilitating some aspects of cognitive functioning, including memory.

Cognitive aging

On average, memory function decreases objectively from the age of 30-35 years onward, as a result of the usual (non-pathological) aging process. The decline in memory performance is however more gradual than that of cognitive skills in which attentional or speed-related processes are implicated (9) (Fig.1). This age-related change in memory performance can to some extent be explained in terms of a reduced speed of information-processing. It has even been suggested that a single common 'speed' factor lies at the basis of all age-associated cognitive impairment (resource limitation theory, cf. Salthouse). Another striking observation is that the variability in cognitive performance between individuals of the same age seems to increase, as a function of age-group membership: people seem to differ with respect to the speed of decline. One important goal of the research into cognitive aging is to identify the risk factors (or protective factors) that mediate these individual differences. Examples of mediating variables which have been identified to date are educational achievement (protective) and an unfavorable cardiovascular risk profile (risk factors, such as increased blood pressure and diabetes) (10). Other socio-demographic, health- and lifestyle-related variables are currently under study as potential mediators of cognitive aging (9).

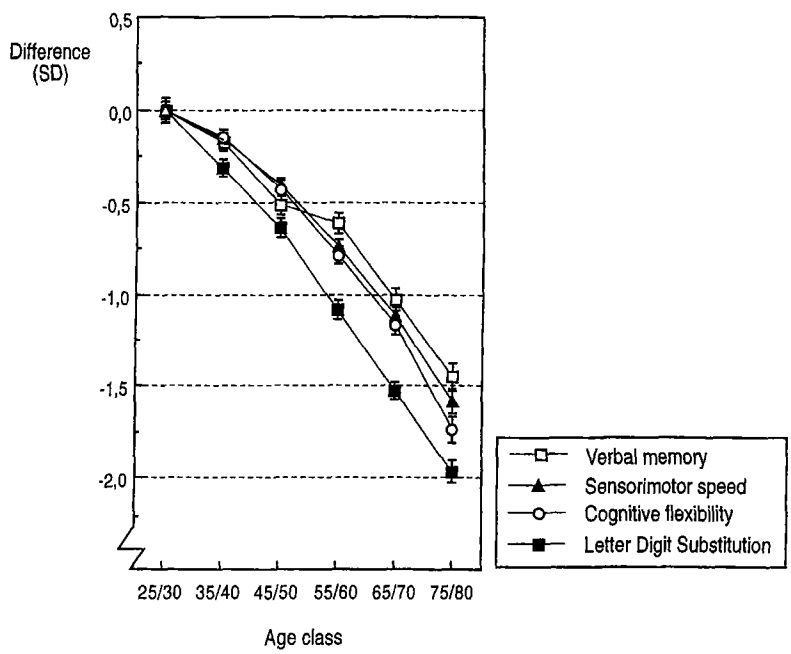

Figure 1. Age-related decline in cognitive performance measures in the Maastricht Aging Study (baseline measurement; N = 1,400): population-based Z-transformed scores, using the youngest group (25-30 years) as reference point. Thus, for example, memory performance in the 75-80 year old group is 1.5 standard deviations (SD) below the mean performance in the 25-30 year old age group. Mean values of verbal memory, sensorimotor speed, and cognitive flexibility are compound variables, based on raw scores from different tests.

Compensation of age-related cognitive decline by habitual caffeine use?

In the light of what is now known of the psychopharmacological effects of caffeine it seems tempting to hypothesize that the self-administered caffeine in food and drinks may help to compensate in part for the age-related

performance deficit. The stimulating effects of caffeine on cognitive behavior seem particularly outspoken in suboptimal conditions, such as fatigue or exhaustion by increasing the energetic resources for basic cognitive processes. In terms of the 'resource limitation model' the central activating properties of caffeine may thus temporarily boost the capacity to process information. Older individuals may take more advantage of caffeine consumption than younger individuals as the former group already may have experienced a reduction in this central processing capacity (4). This hypothesis was studied and partially confirmed by Hogervorst and others in a small experiment (N=60) (11). They compared the acute effects of a 225 mg dose of caffeine with placebo, in a parallel group design with young, middle-aged and old individuals. Positive effects of caffeine were apparent on the first trial in a word recall paradigm in the middle-aged, but not in the old subjects. In the former group the habitual caffeine intake was twice that in both other groups which made the authors to conclude that '...the habitual use of caffeine by middle-aged subjects may be a means to overcome the age-related decrease in cognitive functioning...'. Data from the Maastricht Aging Study show that the amount of coffee intake follows a inverted U-shaped curve whereas tea consumption remains relatively constant as a function of age (7, 9) (Fig. 2). These figures indicate that the habitual coffee intake in this study increased steeply in the life episode in which the first age-related cognitive deficits become apparent. It remains however largely speculative which factors mediate the habitual intake of caffeine. Do older individuals moderate caffeine intake to compensate for their increased sensitivity to the compound, or perhaps because of experienced or presumed adverse effects, such as reduced sleep quality? It is uncertain if the consumption profile of older people is optimal in order to experience a net effect of caffeine that is beneficial in daily life. These questions need to be explored further before definite conclusions can be drawn about the net effect of caffeine use in the process of cognitive aging.

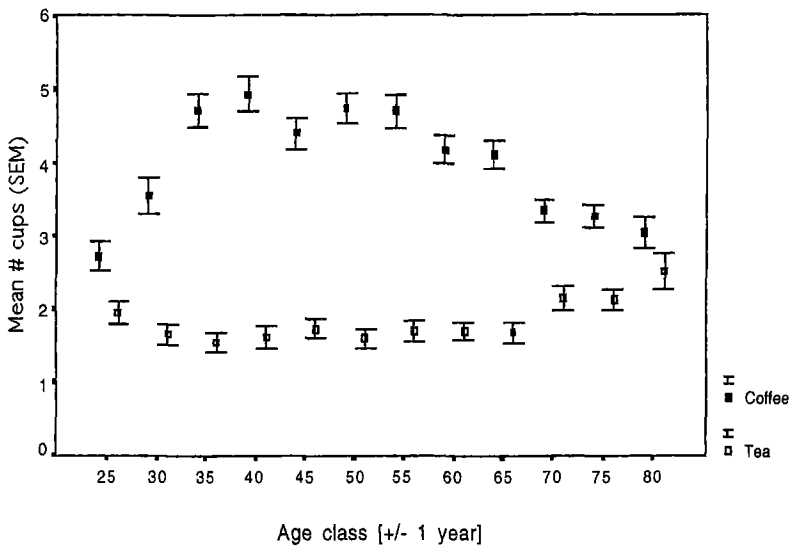

Figure 2. Habitual coffee and tea consumption (number of cups/day) as a function of age in the Maastricht Aging Study (N = 1,900). SEM indicates the standard error of the mean.

Future studies on caffeine, aging and cognition at the Maastricht Brain & Behavior Institute

Prospective studies are the only tool to unravel the extremely complex relationship between habitual caffeine intake, cognitive performance change over time and aspects of quality-of-life, as studies of this kind are easily confounded by socio-economic or health-related variables. The Maastricht Aging Study (MAAS) can provide such framework, as it is focussed on cognitive development in adulthood in relation to a broad range of life-style and health-related variables (9, 10). MAAS consists of a community-dwelling population sample, stratified for age (24 to 81 at baseline), sex and educational level. All participants are screened on a 3 (age 50 years or older) or 6 (below 50 years) for at least a period of 12 years in an extensive cognitive and biomedical test battery. Additional information

on e.g. sociodemographical status, comorbidity, and life-styles (including caffeine intake) are obtained by questionnaire on each test occasion. The study is now finalizing its first follow-up wave after 3 years. The available data will enable the analysis of cognitive change as a function of caffeine intake. Longitudinal evidence of a caffeine effect on cognitive change is far more substantial than the relevant cross-sectional studies published so far (7, 8) in that important potential sources of error are controlled for (e.g. selection bias and cohort effects). In addition to the already available data on caffeine consumption behavior we hope to learn more about specific aspects of caffeine use, such as the source, the intake related to the time-of-day, the experienced effects of caffeine use and the possible reasons behind a change in caffeine intake. Based on such epidemiological data it may be possible to design dedicated intervention studies to test if specific consumer profiles may cognitively improve from habitual caffeine consumption.

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Neurochemical effects of caffeine and behavioral tolerance

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Caffeine is known to produce biphasic behavioral effects - lower doses being stimulatory, higher doses inhibitory. Furthermore, tolerance rapidly develops to these effects. Of all the potential biochemical targets for the actions of caffeine the only one known to be activated at the levels of caffeine reached during normal consumption of caffeine is antagonism of the actions of endogenous adenosine at A_1 and A_{2A} receptors. A_1 receptors are abundantly expressed - especially at the terminals of excitatory nerve endings. When the actions of adenosine are blocked at these sites transmitter release - especially glutamate release is increased. This will i.e. affect the activity of neurons in the basal ganglia known to regulate motor behavior and motivations aspects of behavior. A_{2A} receptors are virtually confined to the striatopallidal GABAergic neurons in the same brain region. When these receptors are

blocked by caffeine the activity in these neurons goes down, which leads to a disinhibition of activity in the globus pallidus. This further translates into increased motor activity. It is important to remember that this effect is similar to the effect of a dopamine D_2 receptor agonist, but different from that of a D_1 agonist. There is, however, synergy between A_{2a} antagonism and D_1 stimulation.
Tolerance to caffeine cannot be explained by increased metabolism - even though that occurs - but may be related to a decreased number of adenosine A_{2A} receptors. Interestingly, tolerance does not develop to a pure A_{2A} receptor antagonist and hence the mechanisms responsible remain to be elucidated.

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Reinforcing effect of caffeine

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This report presents an overview of our recent studies of behavioral pharmacology of caffeine with special reference to its reinforcing effects in animal models of drug reward. The study was focused on: 1) Developing reliable and sensitive animal models which are able to reveal caffeine reward as well as to discriminate it from that of prototype major addictive stimulants; 2) Assessment of caffeine-cocaine interaction addressed to caffeine effect on initiation of cocaine self-administration, priming properties of caffeine in reinstatement of cocaine-seeking behavior and contribution of A1 vs A2a adenosine receptors antagonism of caffeine priming; 3) Involvement of intracellular calcium flow via voltage and receptor gated calcium channels in reinforcing effects of caffeine.

It must first be reminded that a positive reinforcer is an event which increases the probability of a response upon which it is contingent, e.g. drug infusions maintaining lever pressing, alcohol ingestion maintaining licking. Possession of incentive motivational properties, i.e. ability to serve as a stimulus having intrinsic appetitive properties that elicits approach behavior (positive incentive) or withdrawal behavior (negative incentive). A conditioned incentive acquires such properties via Pavlovian conditioning. Incentives and conditioned incentives may also function as reinforcers and conditioned reinforcers, respectively, depending on environmental contingencies. So, these are two sides of the coin.

Primary reinforcing effect of caffeine

The present experiments were designed to determine if caffeine possesses reinforcing effects in experimentally naive mice with the aid of original modification of one-session initiation of i.v. drug self-administration method. Nose-poke response of the "active" mouse resulted in a response contingent infusion of caffeine solution (0.05-0.1-0.2-0.4% concentrations) into the lateral tail veins of both the "active" and "passive" (yoked control) mice. Gradual criteria of i.v. self-administration, *D* and *R* (*D*ifference and *R*atio of operant responses of active vs. passive mice, respectively) were statistically significant at 0.1 and 0.2% concentrations of caffeine (unit doses 0.08 and 0.16 mg/kg, respectively). It was also the case with quantal *N-plus* criterion (percentage of mice with *R* above upper confidence limit of saline control), suggesting a reinforcing action of caffeine. EC50 of caffeine was 0.062 (0.038-0.1) %. These findings demonstrate for the first time the utility of one-session mice self-administration paradigm which could be applied in the wider studies of reinforcing properties of drugs with unstable addictive potential.

Secondary reinforcing properties of caffeine-associated cues and concurrent appetitive properties of drugs' cues (concurrent place conditioning technique)

The present study employed place conditioning technique to compare rewarding potential of caffeine with that of cocaine, the classical narcotic euphoriant, and ethanol, widely abused non-narcotic substance with equivocal rewarding potential in animal experiments. When estimated independently, caffeine (1,5 mg/kg, i.p.) cocaine (5 mg/kg, i.p.) and ethanol (1.5 g/kg, i.g.) produced comparable in magnitude secondary reinforcing effects in place conditioning. When the animals had the opportunity "to compare" rewarding effects of two drugs, data showed that attractiveness of cocaine-paired cues was absolutely (100%) higher than those of caffeine, whereas reinforcing actions of caffeine and ethanol seemed to be equal. Thus, caffeine possesses ambivalent motivational effects in place

conditioning test depending upon the dose: rewarding at low dose and aversive at high doses. The rewarding potential of caffeine is incomparable to that of cocaine and comparable to "minor" reinforcing substances.

Prevention of extinction of cocaine-seeking behavior: involvement of adenosine A1 receptors

Here we will consider what we call incentive stimulus properties (which are different from simple discriminative stimulus properties) of caffeine and their generalization to those of cocaine in mice i.v. self-administration and rat place conditioning paradigms. Drug-naive DBA/2 mice were trained to self-administer cocaine i.v. by nose-poking. The number of nose-poke responses was higher in mice receiving response-contingent injections of cocaine (active group) than in yoked controls and animals receiving response-contingent saline injections. Twenty-four hours after the training session (cocaine or saline self-administration) mice were injected i.p. with saline, cocaine, caffeine, an adenosine A_1 receptor selective antagonist [1,3-dipropyl-8-cyclopentyl xanthine (DPCPX) or 8-cyclopentyl theophylline (8-CPT)], an adenosine A_{2A} receptor selective antagonist (SCH 58261) or the non-selective, non-xanthine adenosine receptor antagonist (CGS 15943) and placed again in exactly the same operant boxes as during the training session but without response-contingent i.v. infusions. In saline treated animals, cocaine seeking behavior was rapidly eliminated. Administration of either caffeine or cocaine in the highest dose (30 and 20 mg/kg, respectively) also led to the extinction of the cocaine-oriented behavior. At low doses, however, cocaine (5 mg/kg) and caffeine (3 mg/kg) prevented extinction of the cocaine seeking pattern. The A_1 antagonists DPCPX and 8-CPT, as well as the non-selective antagonist CGS 15943, but not the A_{2A} antagonist SCH 58261, partially reproduced the effect of a low dose of caffeine on the cocaine-associated behavior in a dose-dependent manner.

It is thus confirmed that a drug from a different pharmacological class (adenosine receptor antagonist) can mimic the effects of the original self-administered drug (indirect dopamine receptor agonist). The data also suggest that the effects of caffeine on cocaine-seeking behavior might be due to interaction with adenosine A_1 but not A_{2A} receptors and that adenosine receptors involved in reinforcement (D1 for cocaine and A2a for caffeine) are different from those involved in incentive stimulus properties (D2 for cocaine and A1 for caffeine).

Cocaine-caffeine interactions

CPP priming: Caffeine reinstates appetitive features of extinguished cocaine-paired cues. It means that there are some common components of incentive stimulus properties in cocaine and caffeine as well as in the effect of addictive substances. It appears from these experiments that the acute administration of caffeine could prevent extinction or facilitate reinstatement of cocaine-seeking behavior pattern. It means that there is a generalization of caffeine incentive stimulus properties to those of cocaine following a *single dose of caffeine*.

Chronic effect of caffeine: The acute i.p. administration of caffeine increased cocaine intake in parallel to the increase in nose-poke activity, however the criteria of cocaine reward was not changed. After 10 days caffeine consumption (drinking, daily dose 150 mg/kg) the caffeine intake was reduced and mice became tolerant to non-specific operant activated effect. Thus the effects of acute and chronic caffeine are different. Behavioral influences were mirrored by alteration of NGFI-A mRNA used as a marker of neuronal activation.

Caffeine and DHP-calcium channel positive and negative modulators

Initiation of caffeine i.v. self-administration in drug naive mice is facilitated by the calcium agonist Bay K 8644 and attenuated by the calcium channel blocker nimodipine. The effects of caffeine on the release of intracellular calcium has been mainly shown in vitro at very high doses that cannot account for the physiological effects of caffeine. Adenosine acting on A1 receptors decreases calcium entry via N-channels in neurons (Mogul et al., 1993; Umemiya and Berger, 1994). Activation of A1 receptors inhibits the cyclase and N and Q voltage-dependent calcium channels (Fredholm et al., 1994). Thus, if the action of DHP modulators via calcium channels is assumed, one can suppose that caffeine leads to an increase of calcium entry into the cells of reward pathways.

Caffeine reward and NMDA receptors

The initiation of mice i.v. self-administration of caffeine was prevented by pretreatment with the NMDA receptor competitive antagonist CPP-ene and the low affinity channel blocker memantine. These data suggest that glutamatergic transmission via NMDA receptors contributes to the reinforcing potential of caffeine.

One can speculate that caffeine-NMDA antagonist interaction may be mediated as follows:

1) *Via adenosine A1 receptors*: caffeine block of presynaptic A1 receptors results in glutamate release whose postsynaptic effect is counterbalanced by NMDA antagonists.

2) *Via adenosine A2a receptors*: A2a receptors appear to negatively modulate NMDA receptor channel conductance via the phospholipase C/inositoltriphosphate/Ca^{2+} pathway rather than the adenylate cyclase/PKA pathway (Norenberg et al., 1998). Caffeine blocking A2a receptors can increase NMDA receptor channel conductance.
3) *Facilitation of postsynaptic NMDA receptors by dopamine D1 receptor stimulation*: Activation of postsynaptic D1 receptors enhances the synaptic activation of NMDA receptors in the nucleus accumbens neurons, thereby promoting a transsynaptic feedback inhibition of glutamatergic synaptic transmission via the release of adenosine. This process may contribute to the locomotor stimulant action of dopaminergic agents in the nucleus accumbens (Harvey and Lacey, 1997).
4) *Reduction by NMDA receptor antagonists of the caffeine-induced expression of c-fos and NGFI-A mRNA in the medial part of the caudate putamen of rats*: Glutamatergic transmission via NMDA receptors contributes to the expression of immediate early genes in the striatum.

In conclusion, caffeine possesses weak reinforcing properties not comparable with prototypic drugs of abuse. Thus caffeine cannot be classified as a model or potential drug of dependence. A1 adenosine receptors might be involved in the priming effect (appetitive stimulus properties) of caffeine. Calcium influx, both via voltage-gated and receptor-gated channels, contributes to the reinforcing effect of caffeine.

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Caffeine does not activate the brain structures involved in drug addiction

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Caffeine is the most widely used psychoactive substance in the world. The most notable behavioural effects of caffeine occur after low to moderate doses (50-300 mg) and are increased alertness, energy and ability to concentrate. Moderate caffeine consumption leads very rarely to health risks (1). Higher doses of caffeine rather induce negative effects such as anxiety, restlessness, insomnia and tachychardia, primarily in a small subset of caffeine sensitive individuals. On the other hand, caffeine has been considered in one study as a potential drug of abuse (2) and more recently described as "a model drug of abuse" (3) and the possibility that caffeine withdrawal should be added to diagnostic manuals has been considered in the USA (4). Here we will review the available data on caffeine consumption, the known effects of caffeine on locomotion, mood and sleep and present new data on caffeine dependence that show in which respect caffeine differs from the classical drugs of abuse.

Caffeine consumption

Caffeine is present in many dietary sources, i.e. tea, coffee, cocoa beverages, candy bars, and soft drinks. The content of caffeine of these food items ranges from 70-220 mg/150 ml for coffee to 30-50 mg/150 ml for tea, 32-70 mg/330 ml for cola and 4 mg/150 ml for cocoa (5). Caffeine consumption from all sources can be estimated as 76 mg/person/day worldwide but reaches 210-240 mg/day in the USA and Canada and more than 400 mg/person/day in Sweden and Finland where 80-100% of the caffeine intake comes only from coffee (5,6). In the UK the consumption is as high as in the latter two countries but 72% are represented by tea. In children, the soft drinks represent 55%, chocolate foods and beverages 35-40% and tea 6-10% of the total caffeine intake (7).

Caffeine and Cerebral Energy Metabolism

The effects of caffeine on cerebral metabolic functional activity can be explored by a quantitative autoradiographic imaging technique (8). Which allows the quantification of local cerebral metabolic rates for glucose (LCMRglcs) simultaneously in all areas of the brain of conscious animals or humans. This technique permits the identification of neuronal pathways affected by a pharmacological agent and is very useful for relating behavioural effects to the central action of a drug. In the present study, we measured LCMRglcs after the administration of increasing doses of caffeine (1-10 mg/kg) reflecting the general human consumption. The present study was performed in adult male rats. The purpose of our study was first to control whether we could confirm the known effects of caffeine on locomotion and sleep and then to study the effects of this methylxanthine on the brain structures involved in addiction and reward.

Caffeine, Locomotion and Sleep

The caudate nucleus which is involved in the control of locomotion appears to be very sensitive to the effects of caffeine since LCMRglc is already significantly increased in this structure after the administration of the lowest dose of caffeine, 1 mg/kg to adult male rats (Figure 1) and increases further after 2.5-10 mg/kg of caffeine. There is

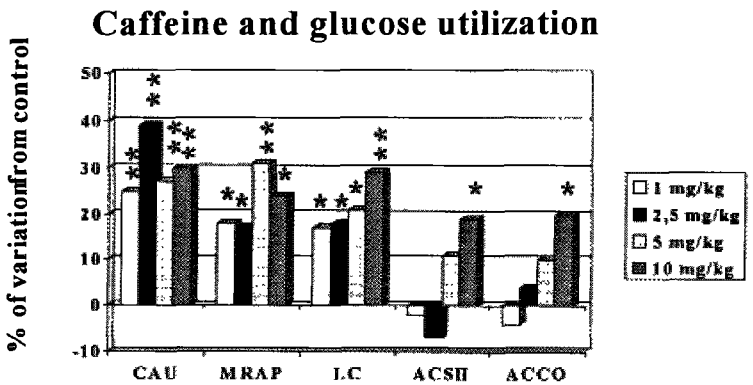

Figure 1: Effects of the acute administration of increasing doses of caffeine on LCMRglcs in various regions of the brain. Data are presented as percent of variation from control values. The animals received an acute i.v. injection of 1-10 mg/kg caffeine at 15 min before the onset of the [^{14}C]2-deoxyglucose procedure.
Abbreviations: DMCAU: dorsomedial caudate nucleus, MRAP: medial raphe, LC: locus coeruleus, ACSH, nucleus accumbens, shell, ACCO: nucleus accumbens, core.
** $p<0.05$, ** $p<0.01$, statistically significant differences from controls (Dunnett's t-test for multiple comparisons).*

a good correlation between caffeine-induced functional activation of this structure and the well-known stimulant effects of caffeine on locomotion which occur at doses as low as 1.5 mg/kg which is in accodance with thedata of the present study (for review, see 9).

The serotoninergic cell groupings, the raphe nuclei as well as the noradrenergic cell grouping, the locus coeruleus are very sensitive to caffeine. These structures are involved in the control of sleep, mood and well-being. In these structures, LCMRglcs are already activated after 1 mg/kg and remain increased at the higher doses of caffeine (Figure 1). These data correlate well with the known sensitivity of sleep and mood to caffeine (9).

Caffeine and addiction

The molecular mechanisms underlying reinforcement and drug dependence were recently reviewed (12) and the critical role of the mesolimbic dopaminergic system, especially the nucleus accumbens, emphasized. Rats self-administer amphetamine and dopamine directly into the nucleus accumbens (12). The latter nucleus that plays a central role in the mechanism of drug dependence is functionally and morphologically divided into a core and a shell part. The medioventral shell part is assumed to play a role in emotional, motivational and reward functions, whereas the laterodorsal core part regulates somatomotor functions (13). The specificity of cocaine, amphetamine, morphine, alcohol and nicotine is to selectively activate dopaminergic neurotransmission in the shell of the nucleus accumbens (14,15), a property that has been related to the strong addictive properties of these drugs (12). Conversely to the drugs of abuse, caffeine increases dopamine release in the caudate nucleus (16) which relates to the stimulatory properties of caffeine on locomotor activity (9,10) but does not induce any release of dopamine in the shell of the nucleus accumbens when injected at doses ranging from 0.5 to 5.0 mg/kg (17). This data is consistent with the low addictive potential of caffeine. Conversely, at the latter doses, caffeine stimulates the release of dopamine in the prefrontal cortex which is consistent with its reinforcing properties (17).

Our data on the effects of caffeine on cerebral energy metabolism show that the increase in LCMRglcs recorded in the nucleus accumbens are of lower amplitude than those recorded in the other brain regions studied (Figure 1). Moreover the significant increase of functional activity in that structure appears only after 10 mg/kg caffeine. These data show that the activation of functional activity in the shell of the nucleus accumbens occurs only at high doses of caffeine (10 mg/kg, i.e., about 4-5 times the average daily human consumption) at which the methylxanthine induces widespread non specific metabolic increases in a majority of brain regions (for review, see 9). These widespread effects of high doses of caffeine on brain functional activity are likely to reflect the numerous adverse side effects of the ingestion of large amounts of caffeine. Conversely the effects of amphetamine, cocaine and nicotine on the neural substrates underlying addiction are rather specific and occur at doses that do not usually lead to the activation of many other brain regions (for review, see 10).

The difference in the functional consequences of the psychostimulants, cocaine and amphetamine compared to caffeine could relate to their respective mechanism of action. Amphetamine and cocaine induce a release or inhibit the uptake of dopamine which will bind to both D1 and D2 dopamine receptors in the striatum. At low doses caffeine acts preferentially at the level of adenosine A2a receptors (18) that are mainly found in the striatum where they colocalize with dopamine D2 receptors (19). When the circulating levels of caffeine increase, the methylxanthine binds also to adenosine A1 receptors (18) which are among other regions located in the striatum where they colocalize with D1 dopamine receptors (19). In the present study, it appears that caffeine mimics the effects of amphetamine and cocaine only at rather high doses (10 mg/kg) when the binding to A1 adenosine receptors is likely to occur.

Conclusion

The areas controlling locomotor activity and the sleep-wake cycle appear to be highly sensitive to low concentrations of caffeine while the structures involved in addiction and reward are only activated after high doses of caffeine. These doses activate also numerous brain regions and are likely to induce also the adverse effects occurring after the ingestion of large doses of caffeine (9). Our data are rather in accordance with the reported observation that the effects of caffeine are used consciously or unconsciously to manage the mood state and to alleviate the adverse effects of caffeine deprivation (20,21). Moreover, it must be reminded that human caffeine consumption is fractioned over the day while the doses given in the present study were injected as an i.v. bolus. Thus, the present data are rather in favor of caffeine acting as a positive reinforcer at doses reflecting the general

human consumption and do not support the participation of the brain circuitry of addiction and reward in the dependence on caffeine reported even at low doses in a recent human study (11).

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Report on the Workshop 'Enhancement of Coffee Quality by Reduction of Mould Growth' Thursday 5th, August ASIC Helsinki, 1999

Dr Gerrit van der Stegen, as Chairman of the first session, introduced the members of the panel to the audience. The panel was made up of, Dr M Blanc, Dr M Frank, Dr F Vega, Dr J Frisvad, Dr Mrs M Taniwaki, Dr E Boutrif, Mr P Dubois, Dr R Viani, Dr R Naidu and Dr H Ngabirano

Dr Van der Stegen outlined the objectives of the workshop as being to

- Exchange experiences
- To inform a larger audience
- To raise awareness of OTA throughout the whole coffee chain
- To focus on the quality element

Dr Maurice Blanc provided the audience with a summary of the Nairobi Workshop 1997. Following the Nairobi workshop documents were prepared, A 'Risk Assessment' by Professor Walker of the University of Surrey, and a list of Good Agricultural Practices (GAP) and Good Manufacturing Practices (GMP) which were to be implemented in producer countries with the objective of educating producers in practices which would ultimately result in a better quality commodity. The main aim was to minimise the time during which the raw material are kept under conditions known to be favourable to mould formation.

The learning's from this workshop went into the establishment of the 'Prevention Programme Pilot Study' which in turn played a major role in the larger 'Enhancement of Coffee Quality by the Prevention of Mould Formation Project'.

Dr Mick Frank reported on his activities in relation to the 'Pilot Study' and provided the audience with an overview of Hazard Analysis Critical Control Points (HACCP) and the role that these can play, together with the GAP/GMP mentioned earlier in this report, in improving the commodity that reaches the consumer.

Dr Jens Frisvad then reported to the group on his activities explaining in some detail the different species of moulds that can develop into mycotoxins. In addition Dr Frisvad also explained where the moulds were found on both unripe and ripe coffee's. Several species of fungi have been reported in the past as producers of Ochratoxin A. Dr Frisvad has examined 75 species in total, of which 10 have been confirmed as actual OTA producers. Dr Frisvad overall conclusion was that, in relation to coffee, the species *A.Ochraceus* was the most significant.

Dr F Vega provided the meeting with interesting data on the possible role played by insects in spreading *A.Ochraceus infection*, with particular reference to the coffee berry borer(CBB). Insects had been collected from coffee berries which had been damaged by the CBB, and were then examined to establish whether they were actually

carrying the contamination, which in many cases they were. Dr Vega concluded that insects could play a role in spreading infection and should therefore be considered as an important factor in the production of OTA in the field.

Dr Mrs Taniwaki reported on her work in Brazil. Dr Taniwaki worked with the co-operation of farmers to collect samples for analysis. Dr Taniwaki reported on sample collection and analysis of coffee for OTA, and on the materials and methods used. Different coffee producing areas had differing incidence of OTA, which could be in part attributed to climatic differences.
Dr Taniwaki concluded that *A.Ochraceus, A.Carbonarius* and possibly a third species were consistently found to be involved in OTA production. That the incidence of OTA increased in the drying yard, but importantly, that it was mainly only damaged/ badly handled coffee that actually contained OTA.

Questions were taken from members of the audience and answered by the panel at the end of this session.

Dr Maurice Blanc took the Chair for the second session of the workshop introducing Dr Ezzeddine Boutrif, of the FAO, as the first speaker.

Dr Ezzeddine Boutrif provided the group with an update on the International developments on mycotoxins in general. The main events to which Dr Boutrif referred were the 13th Session of Codex, held in March 1998, The International Conference on Mycotoxins held in 1999, and the 23rd Session of Codex held in July 1999.

Mr Pablo Dubois of the International Coffee Organisation reported on the 'Enhancement of Coffee Quality by the Prevention of Mould Formation Project', confirming that this project has the support of coffee producing and consuming nations across the world. Mr Dubois reported that the project had been approved for funding by the Common Fund for Commodities.

Dr Ezzeddine Boutrif reported that the project had been under discussion for several years and that both the consumer and producer countries would benefit from the programme. Dr Boutrif informed the meeting of the development objectives, and highlighted the economical, financial, social, environmental and health impact, which could have resulted had the project not gone ahead. Dr Boutrif advised that the project agreement had been under review by the FAO/CFC legal authorities, that finishing touches had been made and that the project would commence in September of this year.

Dr Rino Viani spoke about activities in Uganda where work focussed on dry processed Robusta. Dr Viani discussed how near is a HACCP system in dry Robusta coffee production. Dr Viani pointed out that work was still needed to establish critical limits, a system to monitor critical control points, procedures for verification, and for documentation and records.

Dr Naidu reported on the occurrence of OTA in coffee in India, and on its management to date. Dr Naidu presented, in detail, table to the audience with results of samples/testing that had been carried out. Dr Naidu also reported on the training

and transfer of technology necessary to create a wider awareness of the potential problem. Dr Naidu concluded from his data that if the commodity is looked after properly, with the implementation of both Good Agricultural Practices and Good Manufacturing Practices, OTA is not a problem.

Dr Henry Ngabirano shared with the group his experiences in Uganda as the first country in which work has started as part of the project. Dr Ngabirano provided background on coffee production in Uganda, and on the importance of the commodity in relation to foreign earnings, of which coffee represents some 65%. 3 million people depend on coffee, the majority of which are small scale farmers. Dr Ngabirano reported that the results seen so far were encouraging, however, the small farmers are concerned that they would need to spend more money on equipment in order to adhere to the GAP/GMP, when the actual margin would remain the same – or even be reduced.

The overall opinion of the workshop was that the 'Enhancement of Coffee Quality by the Prevention of Mould Formation Project' was necessary, and that the benefits of such actions were already being seen. The International Life Sciences Institute (ILSI) conference in 1996 concluded that prevention was the most effective method of controlling OTA. The clear message from this workshop supports that position, and the results to date endorse that position (Tables 1 and 2)

Dr Van der Stegen thanked the participants for their involvement and concluded by saying that there were still many countries to work with, that the train has now started and is running, and that favourable preliminary results are already being seen.

Table 1: Mean OTA Contents in R. & G. Coffees

in ppb (number of samples)

Publication Year	Denmark	Finland	Germany	Spain	U.K.	EU + CH	Overall Mean
'95 + '96			**0.9** (53)				**0.9** (53)
'97		**0.5** (49)*	**0.6** (87)*		**0.6** (20)	**0.8** (484)	**0.8** (504)
'98	**0.5** (11)		**0.5** (188)	**0.9** (28)			**0.6** (227)

* Finnish/German data in EU + CH screening

Table 2: Mean Daily OTA Intakes from All Coffee

in ng/day, based upon ICO per capita disappearance statistics (value estimated by original authors)

Publication Year	Denmark	Finland	Germany	Spain	U.K.	EU + CH
'95 + '96			15.4			
'97		12.3	10.0		3.4(4.0)	10.4(11)
'98	11.8/(14)		8.3/(5.6)	9.4/(16.9)		

10.0 ng/day = 0.17 ng/kg b.w./day, which is ~3% of SCF limit and ~1% of JECFA PTWI

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MODELLING AND HACCP TOOLS FOR COFFEE QUALITY IMPROVEMENT

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Introduction

The application of Hazard Analysis of Critical Control Points (HACCP) to solving agricultural problems is a new idea. Although very different from the traditional approach to solving agricultural problems, that of Good Agricultural Practice (GAP), the distinction between HACCP and GAP can be blurred because both call for the rigorous observance of guidelines. At the conclusion of The Pilot Study for the Enhancement of Coffee Quality Through the Prevention of Mould Growth, a philosophy and methodology for the approach had been developed and the first HACCP elements for coffee production had been proposed for refinement and validation. It is these aspects that we shall report in this paper.

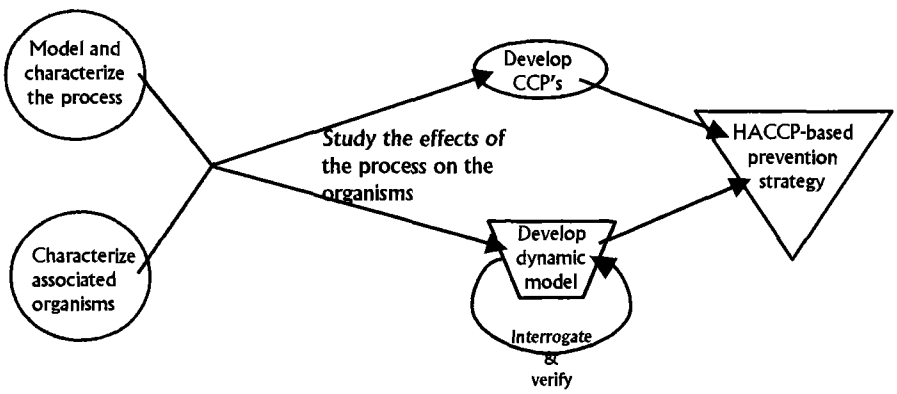
Development of a HACCP Plan

HACCP is dependent on a full understanding of the production system and the best test of this capacity is the generation of a predictive model. This requires the acquisition of a large amount of knowledge about processing methodology and parameters, biological features and the interactions between components of the whole system. Figure 1 illustrates the way in which this task is approached as has been developed in the course of the pilot study. Characterisation of the process and the biota are conducted simultaneously and comprise the initial focus of the study's effort. To 'characterise' in this context is to assemble quantitative field-based data to answer a few questions: What species, in what density and distribution comprise the 'normal' fungi associated with the host plant and what other organisms are important (bacteria, insects, other plants etc.)? What do these organisms do within the production ecosystem and particularly which organisms among these produce or influence identifiable problems? To what conditions are the associated biota exposed during production and what is the effect of these conditions on the biota? Are there objective criteria that can be devised to evaluate the success of any given production step so that different production protocols can be compared on the basis of function?

A coffee production system is conceptually identical to a purely natural ecological system except that human technology and activity play a greater but fully accountable role. At this stage of the study, intact elements of real production systems should be the subject of study and therefore experiments should mainly be of the nature of sampling experiments. Variables are too numerous and of too great a scale to control in this context thus any apparently significant determinants should be recorded and used to help interpret the results of the analysis and

design further sampling experiments. The in-built variation between sites and procedures constitutes the laboratory for this enquiry. Sampling experiments may appear to be less rigorous than conventional experiments but the reality is that equal rigour resides in the requisite careful design of a sampling and analysis routine designed to address specific objectives and to test enunciated hypotheses.

Figure 1. Flow diagram illustrative of how the elements required to found a HACCP analysis are related and best pursued.



The identifiable elements, whether they reside in the biotic or the processing sides, need to be accounted for in a model that functions as a testable hypothesis. This model seeks to relate the processing elements and biological elements such that changes in the microbial populations observed by the sampling experiments as described above, are explained. Once the physical conditions of processing parameters are established and the important organisms and consortia of organisms known, reductionist studies designed to address specific issues of interaction and dynamics can be conducted to inform the developing model. So, for example, if the hypothesis arises that bean infection by *Aspergillus ochraceus* might originate through contamination at the drying yard, that is, air sampling or patio surface swabs have shown the presence of *A. ochraceus* spores, controlled experiments where *A. ochraceus* spores are introduced to cherries or parchments at the commencement of drying can be conducted to evaluate this hypothesis. In this way, by iteration, the model is challenged and refined.

Through the modelling process the conditions that are deleterious and favourable to identified problem organisms, whether this relates to production of a mycotoxin such as ochratoxin A (OTA), disease or off-tastes, will become known along with information as to when these conditions are likely to occur in production. Putative critical control points (CCPs) can be established by applying this information to a working knowledge of the production protocol. A CCP is a point in the process when the problem organism requires to be within certain physical and biological conditions for it to grow and make an impact on the outcome of the process. The CCP remains 'putative' until it can be demonstrated to be effective in controlling the development of the problem. It is important to realise that there should be few CCPs, since if there are many, none may be said to be 'critical' and probably reflects an inadequate understanding of the origin and development of the problem. Having produced the dynamic model and ascertained which points are critical to the development of the problem, a HACCP assurance strategy must be devised. By this is meant raw material acceptability criteria; the definition of the acceptability limits of the controlling parameter; the method for measuring and monitoring this parameter; record-keeping protocols and remediation strategy in case of recorded process failures. One such CCP for coffee production will be developed below in some detail.

HACCP and Good Agricultural Practice (GAP)

Confusion sometimes arises between these two approaches to quality assurance yet they are fundamentally antithetical in approach. This is probably due to both systems setting down rules of practice that require adherence but this is no more than saying that both systems require adherence. HAACP focuses resources very narrowly on a very few production elements and replaces end-product analysis while GAP tries to up-grade the way in which each step is done to produce a cumulative improvement that is checked by end-product analysis. The main advantage of GAP is that little beyond a flow diagram of the process and a textbook knowledge of the suspected problem organisms is necessary to devise a GAP system. The draw-backs include dilution of resources and effort over the whole production chain since no step is any more or less important than any other, lack of monitoring to improve by experience, continued reliance on end-product analysis and lack of a theory of causality to explain failures contributing to the problem of 'application fatigue' where GAP elements are discarded due to a perception that they do not contribute to improvement.

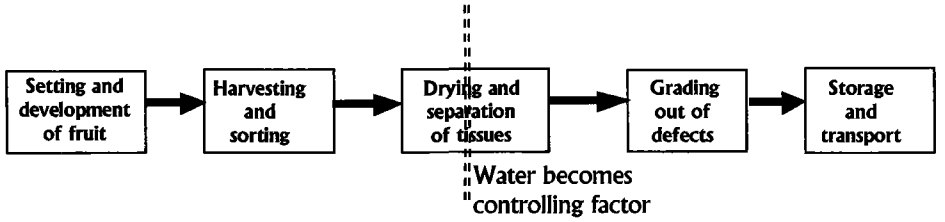
In contrast, HACCP is difficult to establish and much progress has to be made in understanding, but once in place the focus it provides maximises the efficient deployment of the improvement effort and helps acceptance on the part of those charged with its implementation since the advice is specific. End-product analysis is superfluous and further developments can be expected with experience because data will accumulate and can be interpreted using the model, which is, as described above, a theory relating specific conditions to outcomes.

Both approaches require a 'leap of faith', however. GAP relies on the idea that a big problem arises through an accumulation of a number of small problems. HACCP, for its part, relies on the concept that certain key points control the development of a problem and that these can be understood and controlled with adequate precision.

The Model for Coffee Production

Figure 2 illustrates the production model as proposed previously (Frank, 1997) but includes the criteria and rationale which act to unify all production protocols within this single model. From any particular production process, certain consistent objectives can be extracted and the efficacy of the procedure objectively evaluated. So, for example, the use of shade trees is intended to reduce flowering (and other) stress thus improving tree vigour, to reduce inputs such as fertilizer and pesticides and provide secondary crops. Growing coffee in full sun is to maximize yield but inputs have to be increased. The functional objective of both is the production of sound fruit and quality beans, an objective that can be evaluated. Separately or additionally, an

Figure 2. Production model for coffee. The five elements have been established through the interrogation of many observed production protocols with the questions 'Why is it done?' and 'When has it failed?' Some of the more salient unifying propositions are listed below the appropriate model elements.

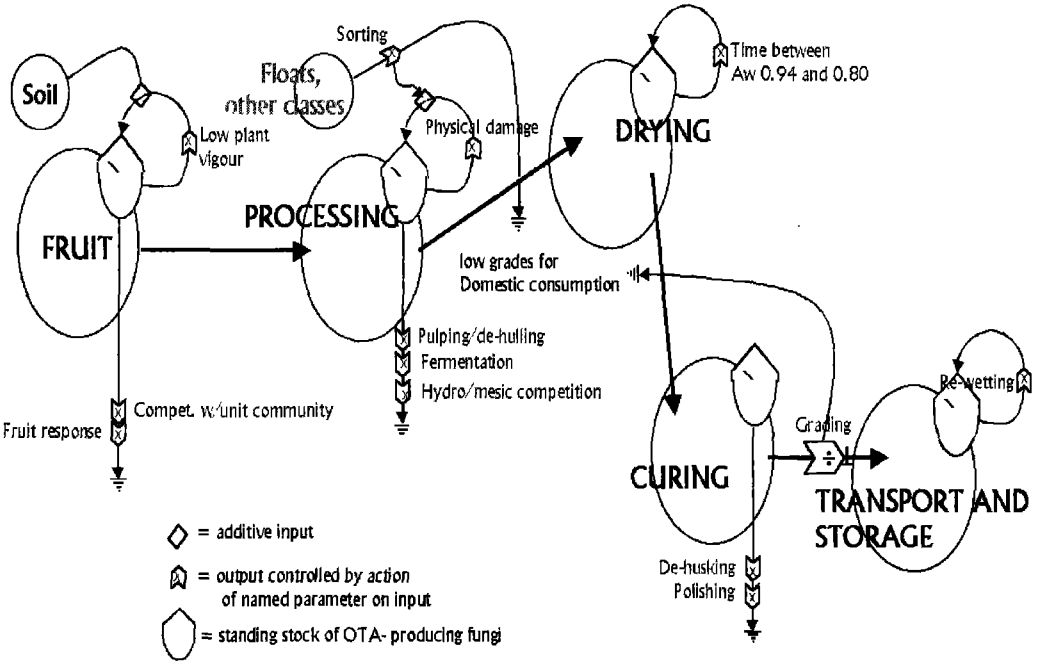


Why is it done? When has it failed?

Basis for the Evaluation of production Steps

Sound fruit?	**Uniformity within classes?**	**Is drying rapid? Parchments free from mucilage Beans free from hulls?**	**Clean coffee? loss of density?**	**Avoiding re-wetting: re-distribution**

Figure 3. Dynamic model of coffee production. As discussed in the text, the system model is a causal hypothesis that relates the identifiable, significant elements that have emerged in the study to each other and to the ecological conditions imposed by man's agricultural and manufacturing activities. It is oriented to ochratoxin A-producers as the problem organisms but could be reconstrued for other problems.



economic assessment of the cost and implied return of elements within each cropping system can provide a method for internal evaluation as well as an objective comparison of the two.

The dynamic model as construed in figure 3 is oriented to the ochratoxin A producing fungi, which, to a good approximation means *A. ochraceus*. In brief, the model provides the following hypotheses. *A. ochraceus* resides in the orchard soil from where it can infect the fruit by some, as yet unproven, mechanism but one aided by low plant vigour and mitigated by the associated microbial flora (unit community) and the host defenses.

During processing, cross contamination from floats and possibly other classes provide a second inoculum source of *A. ochraceus* to the parchment/cherry if sorting is inadequate. This infection route is encouraged by physical damage to the parchment/cherry. Competition with relative hyrophiles, particularly during fermentation in wet processing and during the first three or four days of cherry drying, and the elimination of microbial biomass in the pulping or dehulling steps of wet and natural processing, reduce the level of *A. ochraceus*. Drying is the period identified as the most favorable for the development of *A. ochraceus* with no mitigating factors known aside from the limitation of time which the drying commodity spends between water activity levels of 0.94 and 0.80.

The activities of de-husking and polishing removes any fungal biomass attached to these tissues (as well as any OTA deposited in them) during curing but we have found no good correlations between coffee grades and *A. ochraceus* occurrence. Lastly, during transport and storage, the adequately dried commodity is stable unless it is subject to rewetting or the water in the beans is allowed to redistribute itself such that pockets of high moisure content coffee arise.

As has been emphasised, the model incorporates hypotheses supported by data. Much of the data admits to more than one explanation, thus there is a requirement to challenge the model in its predictive mode to verify, qualify or refute its elements. For example, the model predicts that in the presence of an inoculum, physical damage to the fruit will lead to infection by *A. ochraceus*. This is almost certainly true in some conditions but are these conditions experienced in normal coffee processing and, if not, how close to the normal situation is there a measurable increase in infection? This can be established with a series of experiments.

Drying as a Critical Control Point in Coffee Production

The CCP we propose below sits in the middle of a long production chain and represents only the clearest mechanism we have relating to the occurrence of OTA in coffee. In reading the detail provided, bear in mind that assurrence must be established for the immediate product of the orchard, as discussed below, but also and in particular, during the transport and storage steps of the chain. In this connection the goal is obvious - avoid rewetting and redistribution of the stored moisture - but the practicality of assuring this is complex and is not discussed here.

The principle data we have that suggests drying controls *A. ochraceus* development and OTA accumulation are these. High *A. ochraceus* infection rates have been sustained for two seasons at a farm, the coffee from which is usually OTA-free but rain on one occasion resulted in an extended drying time course and high OTA contamination levels. Ripe cherries held in plastic bags for days showed no tendency for infection by *A. ochraceus* nor by fungi with similar physiological properties. The logic of HACCP runs that if the raw material feeding a process is satisfacory as defined in the HACCP evaluation and the process is well conducted as defined in the evalution then the product will be satisfactory because the parameters, as defined, have been shown to prevent the problem in specifically designed 'test to destruction' type of exercises. Other aspects include tools to measure the controlled parameter (when, how and with what), how to keep records connecting batches with drying time-course data for purposes of assurence and a strategy to deal with batchs that are dried out of specification as must happen from time to time.

Raw materials: Since the CCP at drying prevents development and is not a remedial step, there must be a means to asssure that the raw material does not already contain advanced *A. ochraceus* infection and OTA accumulation. Ideally this would take the form of another CCP but some other sort of check could suffice. Unfortunately, the orchard situation is not yet clear enough to be prescriptive. Although we know that infection can occur there we do not know to what extent *A. ochraceus* can develop and we were unable to find a correlation between *A. ochraceus* infection of beans and any simpler microbiological analysis (see Frank and Frisvad, this volume). Some assurance steps take place as a matter of course. In wet processing only ripe cherries that sink comprise the main production; in natural processing, if strip picking is employed, boia (tree dried fruit) is usually separated by floatation from ripe and green cherries or, if finger picking is the norm, only ripe cherries are dried. The floats coffee or mbuni is a low quality coffee but our field observations suggest this is often due to the poor drying applied to this grade. Further work is needed to develop well founded assurence criteria or, preferably, a CCP relating to the immediate product of the orchard.

Processs parameters: Subject to verification we can propose a specific drying specification. All coffee, cherry or parchment, must spend no more than 4 days between Aw=0.95 (≈27% moisture content) and 0.80 (≈16% moisture content). This proposal asserts that any wet coffee, whether containing a previous *A. ochraceus* infection or subject to *de novo* contamination will not support enough *A. ochraceus* growth to produce significant amounts of OTA if it resides in this moisture availability window only as specified. This provides a precise basis to evaluate the contention and verify, modify or refute it.

Measuring moisture content: The use of an Aw meter is probably a practical solution for large estates but for small holders, especially in remote areas, it is not. The traditional method of weight of a standard volume can be calibrated to provide a satisfactory method. The Aw meter is subject to error due to equilibration lag (which changes according to the moisture content), temperature differences and drift off of calibration. The gravimetric method avoids most of these problems but the standard volume will contain different amounts of coffee, hence different amounts of dry matter, due to shrinkage during drying. Using the Aw meter, which must be calibrated monthly against saturated salt solutions at a realistic measuring temperature, a litre of parchments/cherries from the centre of each lot on the yard must be measured in a closed container before the

day's drying commenses after allowing three minutes to equilibrate. Measurements must not be taken during or at the end of a drying day because equilibration times are variable and very long. The gravimetric method should be applied at the end of the drying day but the farmer will need to have received instructions on using a calibration curve to convert weight to moisture content although it may be possible to devise a scale for a purpose-built device that reads this value directly. A record of each measurement should be kept in a simple ledger and identified as to the day of harvest.

Intervention/remediation measures: Clearly, a day's harvest that has conformed to the criteria set out above is assured as containing no significant OTA. If the drying specification is violated there may or may not be a problem according to whether the toxin-producing fungus, *A. ochraceus*, is, in fact, present. Until a failed lot can be assured by end product analysis, however, it must be retained aside from the main production with certainty. In principle, a microbiological analysis for possible toxigenic fungi could provide the end-product analysis for failed lots but we require more experience before we are able to establish sampling and analysis guidelines to make this feasible. If a failed lot is found to contain the toxin as the result of end product analysis, a strategy of dealing with it that does not include blending must also be in place.

Conclusion

Coffee does not provide a particularly favourable substrate for *A. ochraceus* growth or for its production of OTA. Nevertheless, the occurrence of OTA is regular enough to be of concern to public health bodies. The apparent rarity of this fungus early in the production chain has made the study of its behaviour during production difficult but progress has been made. Our understanding of the ecology of the coffee production system has grown and progress in adapting the HACCP notion to an agricultural problem makes prevention of OTA and other fungus-based problems in coffee appear to be within reach.

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Fungi associated with the coffee berry borer *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae)

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Abstract. Insects have been shown to serve as vectors for a wide array of fungi. In some cases, insect-vectored fungi produce potent toxins in the host plant, which might create problems for the food industry. Recent findings indicate that the coffee berry borer (CBB), *Hypothenemus hampei* (Ferrari) (Coleoptera: Scolytidae) could be vectoring toxigenic fungi in coffee plantations.

Introduction

Based on then fossil record, it is known that insects have been associated with fungi for hundreds of millions of years (Borror *et al.* 1976; Brock 1974). These associations may range from casual to associations based on evolutionary benefits for the fungus, the insect or both (symbiosis).

An insect-fungus association may benefit the fungus in various ways. The insect may (1) move the fungus to a desirable location; (2) facilitate entry of the fungus into a host plant through damaging a host-derived barrier to the fungus (such as a seed hull); (3) serve as a host for the fungus, either in a pathogenic, commensal (benefitting one but not harming the other), or a mutualistic/symbiotic relationship. The association may also benefit the insect. For example, the fungus may serve as food for the insect. Fungi can be more nutritious than plant material due to relatively higher levels of proteins or sterols (Southwood 1973). The fungus may also modify a host material so it is more suitable for the insect. Appropriate modifications may include degrading the material to a suitably nutritious form (such as occurs for larvae of wood boring wood wasps; Kukor and Martin 1983) or detoxification of host-produced defensive compounds (Dowd 1992a). The insect, through carrying a toxin-producing fungus, may exclude other insect competitors from the food source provided the vector insect is resistant to the toxin (such as the sap beetle-toxigenic fungi relationship in maize, Dowd 1992b).

Insect vectoring ability can be specifically defined by criteria provided by Leach (1940): (1) a close, although not necessarily constant, association of the insect with diseased plants must be demonstrated; (2) it must be demonstrated that the insect also

regularly visits healthy plants under conditions suitable for the transmission of the pathogen; (3) the presence of the pathogen in or on the insect in nature or following visitation to a diseased plant must be demonstrated; (4) the disease must be produced experimentally by insect visitation under controlled conditions with adequate checks. However, even if all criteria are not fulfilled, the insect may still facilitate entry of the fungus, as discussed above (Dowd 1998). Although the vectoring criteria may only be critically and completely demonstrated for some insects for a particular plant-fungus relationship, many insect species may actually be involved in vectoring (Dowd 1998). Vectoring of fungi may be facilitated by sticky spore masses produced by a fungus (along with a feeding or odor attractant) (*e.g.,* Leach 1940), the presence of hairs on an insect that collect dry spores (much like bee hairs collect pollen) (*e.g.*, Juzwik and French 1983), or specific structures designed to house/collect the fungus, such as the mycangia of bark beetles (Scolytidae) (Beaver 1989; Berryman 1989).

Past reports of vector associations between insects and fungi have indicated significant economic losses in several instances. One of the earliest indications that insects vectored economically important fungal pathogens of concern was for ergot (Atanasoff 1920; Leach 1940 and references therein). Ergot is the sclerotia (a hard, long-term survival structure) of the fungus *Claviceps* (*paspalum, purpureum* or other species) that occurs in grain heads such as rye. In the times of Caesar and in the Middle Ages, outbreaks of ergot in grain often resulted in death of significant portions of regional populations of people or livestock due to the neurotoxic compounds present in the ergot sclerotia, which do not readily separate from the grain during threshing (Atanasoff 1920). Symptomatology of affected people was attributed to witchcraft or possession by evil forces due to the strange behavior caused by the active compounds, which include hallucinogens (Atanasoff 1920).

Other instances of economic consequences of insect vectored fungi have been of more recent concern. Although mycotoxin presence in crops such as maize is complicated, insects generally appear to be important contributors, and greatly reducing insect damage can greatly reduce mycotoxin levels (*e.g.* Anderson *et al.* 1975; Lillehoj *et al.* 1976; Smith and Riley 1992; Munkvold *et al.* 1997, 1999; Dowd *et al.* 1999). Levels of direct and indirect economic losses due to mycotoxins in maize in the U.S. have been estimated in the billion dollar range (Vardon 1998; USDA-ARS 1999). Many tree pathogens are vectored by insects such as sap beetles (*e.g.*, oak wilt disease; Juzwik and French 1983) or bark beetles (*e.g.*, Dutch elm disease; Leach 1940; Webber and Gibbs 1989). Even though economic losses are often hard to compute for these diseases due to variation in occurrence in "farmed" trees vs. ornamentals in populated areas, monetary losses due to Dutch elm disease have been estimated at billions of dollars (Karnosky 1979; Strobel and Lanier 1981). However, both the American chestnut and several native elms, both valuable as ornamentals and for commercial use, have been virtually eliminated in the U.S. due to pathogens carried by insects (Leach 1940).

The coffee berry borer

Hypothenemus hampei is a serious pest of coffee throughout the world (Le Pelley 1968). Endemic to Central Africa, it has now spread to most coffee growing regions, and has been reported in Uganda, Congo, Benin, Togo, Ivory Coast, Kenya, Nigeria, Angola, Ethiopia, Brazil, Colombia, Guatemala, Ecuador, Nicaragua, Honduras, Mexico, Malaysia, Indonesia, Sri Lanka, Jamaica, New Caledonia, India, and other countries.

The female adult bores a hole into the coffee berry where she deposits 20-50 eggs (Baker *et al.* 1992). Larvae feed on the endosperm, lowering the quality of the berry and possibly causing abscission of the fruit. There is a 10 to 1 female to male sex ratio, and females mate inside the berry; therefore, once they emerge, they are already inseminated and ready to deposit eggs into another coffee berry (Waterhouse and Norris 1989).

As part of a foreign exploration program aimed at finding biological control agents against CBB, coffee berries showing the characteristic hole bored by the female were collected in the field in Benin, Uganda, Cameroon, Honduras and Nicaragua, and brought into the laboratory. CBB's that emerged from the coffee berries were placed with their dorsum touching the surface of water agar in sealed Petri dishes, in order to promote growth of any fungal spores carried by the insect. The following fungi were isolated from *H. hampei*: *Aspergillus ochraceus, Aspergillus flavus, Aspergillus niger, Fusarium* sp., *Penicillium chrysogenum*, *Penicillium brevicompactum* and *Verticillium* sp., in addition to the fungal insect pathogens *Beauveria bassiana*, *Paecilomyces farinosus*, and *Paecilomyces lilacinus*. Many other fungi have also been isolated from CBB by other researchers throughout the world (Table 1).

A. ochraceus, as well as other members of the genus *Aspergillus*, are known to be important toxigenic fungi (Powell *et al.* 1994). For the coffee industry, the presence of ochratoxin A (produced by *A. ochraceus*) in coffee is a serious concern. Ochratoxin A has been reported in green coffee beans, roasted coffee, instant coffee, and coffee brews (Levi *et al.* 1974; Tsubochi *et al.* 1984; Micco *et al.* 1989; Studer-Rohr *et al.* 1994; Patel *et al.* 1997). CBB-infection rates with *A. ochraceus* in different countries is presented in Table 2. The isolation of *A. ochraceus* suggests that CBB might serve as a vector for this toxigenic fungus.

We hypothesize that the adult progeny emerging from coffee berries which were originally attacked by an *A. ochraceus*-contaminated female, could carry spores once they leave the berry. Thus, field studies should be conducted to determine if adult CBBs exiting coffee beans are carrying *A. ochraceus*, and if so, whether the fungus becomes established in a coffee berry once the female adult bores a hole to deposit her eggs. Plans aimed at managing ochratoxin levels in coffee should take into consideration the presence of *H. hampei* in the field, and its possible role as a mechanical vector for *A. ochraceus*.

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Table 1. Fungi isolated from coffee berry borers.

PATHOGEN	COUNTRY	REFERENCE
Aspergillus flavus	Benin India	Vega and Mercadier, unpubl. Kumar *et al.* 1994
Aspergillus niger	Benin	Vega and Mercadier, unpubl.
Aspergillus ochraceus	Benin, Uganda	Vega and Mercadier 1998
Fusarium spp.	Benin, Uganda	Vega and Mercadier, unpubl.
Fusarium avenaceum	India	Balakrishnan *et al.* 1995
Fusarium oxysporum	Colombia	Bustillo *et al.* 1998
Fusarium pallidoroseum	India	Kumar *et al.* 1994
Fusarium solani	Benin, Mexico	Rojas *et al.* 1999

Beauveria bassiana	Brazil	Averna-Saccá1930 Villacorta 1984 Jiménez-Gómez 1992
	Cameroon	Pascalet 1939
	Colombia	Bustillo *et al.* 1991, 1998 Varela and Morales 1996
	Ecuador	Klein-Koch *et al.* 1988 Klein-Koch 1990 Jiménez-Gómez 1992
	India	Balakrishnan *et al.* 1994
	Indonesia	Junianto and Sri-Sukamto 1995
	Ivory Coast, Togo	Vega and Mercadier, unpubl.
	Mexico	Méndez-López 1990 Barrera 1995
	Kenya, Togo, Mexico, Ecuador, Guatemala, Indonesia, New Caledonia, Brazil, Sri Lanka, Jamaica	Bridge *et al.* 1990
	Brazil, Kenya, Nicaragua, Togo Mexico	Humber 1992
	Thailand	Varela and Morales 1996
Hirsutella eleutheratorum	Colombia	Bustillo *et al.* 1998
Metarhizium anisopliae	Colombia	Bustillo *et al.* 1998
Nomuraea rileyi	Brazil	Le Pelley 1968
Paecilomyces amoeneroseus	India	Kumar *et al.* 1994
Paecilomyces farinosus	Ivory Coast, Togo	Vega and Mercadier, unpubl.
Paecilomyces fumosoroseus	India	Balakrishnan *et al.* 1995

Paecilomyces javanicus	Java, Indonesia	Friederichs and Bally 1923 Samson 1974
Paecilomyces lilacinus	Benin	Vega and Mercadier, unpubl.
Penicillium brevicompactum	Benin, Uganda	Vega and Mercadier, unpubl.
Penicillium chrysogenum	Uganda	Vega and Mercadier, unpubl.
Verticillium sp.	Benin, Uganda	Vega and Mercadier, unpubl.
Verticillium lecanii	India	Balakrishnan *et al.* 1995

Table 2. Percent *A. ochraceus* infection rates in coffee berry borers from different countries.

COUNTRY	# insects	% infected
Benin	564[1]	17.4
	258	47.3
Uganda	636[1]	5.3
Cameroon	355	0.8
Honduras	163[2]	0.6
Nicaragua	14[2]	0

[1]See Vega and Mercadier 1998; numbers given for Benin are for two separate collections.

[2]Preliminary data; research in progress.

FUNGI PRODUCING OCHRATOXIN A IN COFFEE

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1. INTRODUCTION

The presence of ochratoxin A (OA) in coffee is undesirable because it may be used as a trade barrier, affecting the economies of producing countries, many of which rely on coffee for much of their foreign exchange. OA has been found in samples from several coffee producing countries, including Brazil, which means that fungi capable of producing OA infected coffee beans at some stage of coffee production.

Brazil is the biggest coffee producer in the world. Research on fungi producing fungi OA in coffee in Brazil has been conducted since 1996, to gauge the importance of this problem and to understand the parameters permitting fungal growth, and hence OA contamination, in coffee.

The present project has following objectives:

- To identify which fungi are responsible for OA production in Brazilian coffee.
- To investigate which stages of coffee production are susceptible to infection by fungi capable of OA production.
- To understand which factors, such as water activity, control OA production in coffee.

2. MATERIAL & METHODS

2.1 Regions producing coffee: Coffee samples from three major Brazilian coffee producing states as follows:
(a) Farm 1: São Paulo State, southern area (Pirajú);
(b) Farm 2: São Paulo State, western area (Parapuã);
(c) Farm 3: São Paulo State, northern area (Franca);
(d) Farm 4: Minas Gerais State, Cerrado Mineiro area (Patrocinio);
(e) Farm 5: Paraná State, northern area (Londrina).

2.2. Samples: Samples for mycological and OA analysis were collected at different stage of ripening and processing from soil under coffee trees, ripe cherries and raisins from trees, cherries and raisins from the ground, from drying yard and from storage.

2.3. Mycological analysis: Coffee beans were surface disinfected with 0.4% chlorine solution for 1 min. A total of 50 beans were plated directly (10 particles per plate) onto the culture media Dichloran 18% Glycerol Agar (DG18, Hocking & Pitt, 1980), according to the methodology recommended by International Commission on Food Mycology (Samson *et al.*, 1992). The plates were incubated at 25°C for 5 days.

2.4. OA production by potential producers: Isolates of *Aspergillus ochraceus, A. carbonarius* and *A. niger* were grown on Yeast Extract Sucrose Agar (YESA) for 7 days at 25°C, and tested for OA production by the agar plug technique on thin layer chromatography plates (Filtenborg *et al.*, 1980). The plates were developed in toluene: ethyl acetate: formic acid (25:20:5) and observed under long wave (365 nm) UV light. Positive results were indicated by a green-blue fluorescent area with an R_f corresponding to the standard. When the plates were exposed to vapour of amonium hydroxide, the fluorescence from positive samples turned purple blue as described by Betina (1985).

2.5. Analysis of OA in coffee: Coffee samples were analysed for OA according to Nakajima *et al.*, (1990). The samples were extracted with methanol: 3% sodium bicarbonate, cleaned up through an imunoaffinity column (Ochratest™, Vicam, USA). OA was detected and quantified by High Performance Liquid Chromatography (HPLC). The HPLC system consisted of a Waters 600 system (Waters Corporation, MA, USA), set at 330 nm excitation and 470 nm emission. The HPLC was fitted with a ODS Hypersil (5 µm, 25mm x 4,6 mm) pre column and Spherisorb ODS (5 µm, 250 mm x 4,6 mm) column (Phase Separations, Inc. USA). The mobile phase consisted of acetonitrile/4mM sodium acetate with 0.5% acetic acid solution (42:58). The flow rate was 1ml/min. OA was detected by absorption at 333 nm and retention time of about 9 min.

2.6. Water activity: The water activity was measured in triplicate using Aqualab apparatus (Decagon Devices Inc, Washington-USA).

2.7 OA production in coffee: A. ochraceus were grown on Czapeck Yeast Extract Agar (CYA, Pitt, 1979) at 25°C for 5 –7 days. Spores were suspensed in 0.1% of peptone water and an inocula of 10^8/ml were inoculated in coffee beans with different a_w (0.80, 0.84 and 0.90) in several desiccators . The desiccators were incubated at 25°C. OA production was determined at various periods, according to the methodology of Nakajima *et al.*, (1990).

3. RESULTS & DISCUSSION

Occurrence of OA producing fungi in coffee:
Results of five representative farms from 12 farms will be presented below.
The fungi isolated from coffee from Farm 1 (from the south of Sao Paulo) is presented in Table 1. *A. ochraceus* was found only in samples from storage. All *A. ochraceus* isolates tested were OA producers. *A. niger* was found in raisins from ground, drying area and storage, however none of these isolates were OA producers. Several *Penicillium* spp. and *Fusarium* spp. appeared in this region. Most of samples were 100% infected with *Cladosporium* spp., *Mucor* spp., *Penicillium* spp., *Fusarium* spp yeasts and others

moulds. The water activity of most samples was high, even in the drying area. It was raining on the day the samples were collected.

Table 2 presents the fungi producing OA in Farm 2 (from the west of São Paulo State). As the coffee harvest was only beginning, samples were collected only from the drying yard and storage areas. From this farm, *Aspegillus carbonarius* isolates capable of producing OA in coffee were detected for the first time. Most *A. ochraceus* isolates from this farm were found to be OA producers, along with a few *A. niger* isolates. None of the *Aspergillus japonicus* isolates examined produced OA. This farm demonstrated very poor agricultural practice, with good quality samples being mixed with visibly mouldy ones.

Results from Farm 3 (From the north of São Paulo State) is shown in Table 3. There was only one *A. ochraceus* isolates capable of producing OA and was found olny in samples form the drying area. This species was present in soil. *Aspergillus niger* was present in samples from several areas, but none produced OA. *A. carbonarius* was not isolated.

Table 4 shows the data of Farm 4 (Minas Gerais State). Olny *A. ochraceus* were OA producers and they were more frequent from storage. This species was also present in the soil.

Coffee samples from North of Paraná (Farm 5) is shown in Table 5. OA producing *A. ochraceus* appeared in cherries and raisins from trees, in coffee from drying yard and from storage. The number of this species increased greatly in storage. *A. niger* was not isolated from this farm.

The levels of OA found in some coffee samples are shown in Table 6. The presence of OA was high in samples from storage of Farm 2. It corresponds to presence of *A. ochraceus, A. carbonarius* and few *A. niger* OA producer.

Effect of water activity on OA production:
OA production by *A. ochraceus* in coffee with very high water activity (approaching 1.0) in an environment with 100% relative humidity was analysed over time (Table 7). Production of OA increased up to 245 µg/Kg after 45 days and decreased to 194 µg/Kg after 50 days. When a_w was reduced, OA production was reduced considerably as shown in Table 8. Coffee does not appear to be a good substrate for OA production as cereals and dried fruits.

This work is still preliminary. More studies are been conducted with further coffee samples from a wider range of regions. However some conclusions can be drawn from this work to date:

- Two to or at most three species are involved in the production of OA in coffee: *A. ochraceus, A. carbonarius* and rarely *A.niger.*

- The incidence of fungi capable of producing OA in coffee increases during drying and storage.

- From admittedly limited data, no evidence was found of fungi capable of producing OA infecting coffee beans while still on the tree.

- It seems likely that only badly handle samples actually contain OA. Good agricultural practice is probably sufficient to prevent OA in coffee. At the same time the quality of coffee would be improved.

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Summary

Coffee samples (*C. arabica)* were collected from the three major Brazilian coffee producing states as follow: São Paulo, Minas Gerais and Parana. Coffee samples were collected at different stage of ripening and processing from soil under trees, ripe cherries and raisins from trees; cherries and raisins from ground; from the drying yard and from storage. Coffee beans were surface disinfected with 0.4% of chlorine and plated on Dichloran 18% Glycerol Agar (DG18), at 25°C for 5-7 days.

Coffee beans from different regions were infected, with *Penicillium* spp., *Fusarium* spp., *Aspergillus niger* and *Aspergillus ochraceus.* The frequency of *Aspergillus ochraceus* was lower in beans from trees and soil, however from the drying yard and storage area, the presence of this species increased. *Aspergillus carbonarius* was found only in one farm from West of São Paulo States.

Most isolates of *A. ochraceus* and *A. carbonarius* were capable of producing OA, but few *A. niger* isolates produced OA. The presence of OA in coffee was at the level of 0.3 to 19.75 µg/Kg.

A. ochraceus was capable of producing OA in coffee with high water activity (approaching 1.0), however when was a_w was reduced, OA production was reduced considerably.

Table 1. Occurrence of fungi and OA producing fungi (positives in brackets) in coffee, from Farm 1 (southern São Paulo State).

Samples	*A ochraceus*		*A . niger*		*Cladosporium spp.*		*Penicillium spp.*		*Fusarium spp.*		Yeasts, others		Infected beans		Water activity
	Nº	%	Nº	%	Nº	%	Nº	%	Nº	%	Nº	%	Nº	%	
Cherries (tree)	-	-	-	-	19	38	-	-	9	18	17 34		40	80	0.978
Cherries (ground)	-	-	-	-	15	30	11	22	15	30	13 26		43	86	0.974
Raisin (ground)	-	-	3 (0)	6	2	4	39	78	22	44	3 6		50	100	0.978
Drying area 1 (Mouldy Floaters)	-	-	16 (0)	32	-	-	1	2	3	6	24 48		50	100	0.925
Drying area 2	-	-	1 (0)	2	-	-	39 48		34 68		-	-	50	100	0.979
Storage	5 (5)	10	1 (0)	2	-	-	18 32		1	2	31 62		50	100	0.618

Table 2. Occurrence of OA producing fungi (positives in brackets) in coffee from Farm 2 (western São Paulo State).

Samples	*A. ochraceus*		*A. niger*		*A. carbonarius*		*A. japonicus*		Water activity
	Nº	%	Nº	%	Nº	%	Nº	%	
Drying yard 1	-	-	13 (0)	26 (0)	12 (4) (8)	24	1 (0) (0)	2	0.728
Drying yard 2	3 (2) (4)	6	6 (0)	12 (0)	13 (6) (12)	26	-	-	0.579
Drying yard 3	-	-	23 (0)	46 (0)	1 (1)	2 (2)	-	-	0.514
Drying yard 4	-	-	4 (0)	4 (0)	-	-	-	-	0.502
Drying yard 5	-	-	1 (0)	1 (0)	4 (2)	8 (4)	-	-	0.491
Storage 1	-	-	13 (0)	26 (0)	5 (4) (8)	10	1 (0) (0)	2	0.5888
Storage 2	-	-	12 (2)	24 (4)	6 (3) (6)	12	-	-	0.579
Storage 3	1 (1) (2)	2	12 (0)	24 (0)	3 (3)	6 (6)	1 (0) (0)	2	0.595
Storage 4	-	-	2 (0)	4 (0)	1 (1)	2 (2)	-	-	0.603
Storage 5	4 (2) (4)	8	18 (0)	36 (0)	6 (4) (8)	12	1 (0) (0)	2	0.562

Table 3. Occurrence of OA producing fungi (positives in brackets) in coffee from Farm 3 (northern São Paulo State).

Samples	*A ochraceus*		*A . niger*		Water activity
	N°	%	N°	%	
Soil	Presence (+)		Presence (-)		-
Cherries (tree)	-	-	-	-	0.971
Cherries (ground)	-	-	6 (0) (0)	12	0.969
Raisin (ground)	-	-	1 (0) (0)	4	0.942
Drying area 1	1 (1) (2)	2	-	-	0.937
Drying area 2 (Bottom of washer)	-	-	32 (0)	64 (0)	0.956
Storage	-	-	1 (0)	2 (0)	0.508

Table 4. Occurrence of OA producing fungi (positives in brackets) in coffee from Farm 4 (southern Minas Gerais State).

Samples	*A ochraceus*		*A . niger*		Water activity
	N°	%	N°	%	
Soil	Presence (+)		presence (-)		-
Cherries (tree)	-	-	-	-	0.973
Cherries (ground)	-	-	-	-	0.948
Raisin (ground)	-	-	-	-	0.780
Drying area 1	-	-	-	-	0.962
Drying area 2 (floaters)	-	-	1 (0) (0)	2	0.943
Storage	2 (2)	4 (4)	7 (0) (0)	14	0.551
Storage	8 (6) (12)	16	-	-	0.572

Table 5. Occurrence of OA producing fungi (positives in brackets) in coffee from Farm 5 (northern Parana State).

Samples	*A. ochraceus*		*A. niger*	
	N°	%	N°	%
Cherries (tree)	2 (2)	4 (4)	-	-
Raisins (tree)	1 (1)	2 (2)	-	-
Raisins (ground)	-	-	-	-
Drying yard	4 (4)	8 (8)	-	-
Storage	15 (15) (30)	30	-	-

Table 6. Level of ochratoxin A (µg/Kg) found in coffee samples [a, b]

Region	Drying yard	Storage
Parapuã	1.62	19.75
Piraju	-[a]	1.15
Franca	ND[b]	-
Patrocinio	-	-
Londrina	0.3	-

[a] Not Determined.
[b] Not Detected (Limit of detection 0.2 µg/Kg).

Table 7. Production of ochratoxin A (µg/Kg) by *A. ochraceus* on green coffee stored in 100% relative humidity

Time (days)	Production of OA (µg/Kg)
10	59
20	64
30	117
40	213
45	245
50	194

Table 8. Production of ochratoxin A (µg/Kg) by *Aspergillus ochraceus* on green coffee with different water activities, after 10 and 20 days incubation at 25°C[a].

Water activity	OA production (µg/Kg)	
	10 (days)	20 (days)
0.80	0.4	ND[a]
0.84	0.1	0.6
0.90	0.7	5.1

[a] Not Detected (Limit of detection 0.2 µg/Kg).

RECENT INTERNATIONAL DEVELOPMENTS IN THE FIELD OF MYCOTOXINS

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Since 1997, at least three major events of international importance took place, that dealt with mycotoxins in general and Ochratoxins in particular.

A. The Thirteenth Session of the Codex Committee on Food Additives and Contaminants, The Hague, The Netherlands, 9-13 March 1998

This committee discussed a position paper on Ochratoxin A (prepared by Sweden). The paper discusses the mycology, chemistry and occurrence, toxicological evaluations and intake data. It also provides information on the maximum limits enforced by a number of countries and finishes with a number of conclusions and recommendations which are of direct relevance to this Workshop. The parts of the paper related to maximum limits and to the conclusions and recommendations are reproduced below:

Maximum limits for Ochratoxin A

Nine countries have specific regulations for Ochratoxin A. Legislative limits range from 5 to 50 µg/kg, although lower limits may apply to infant foods. Limits apply either to some foods, particular cereals, or all food. No barriers to trade have been reported, although existing national regulations or guidelines may lead to such barriers.

Depending on the method used in the assessment of TDI, these estimations would yield maximum limits (MLs) in cereals ranging from approximately 1 to 10 µg/kg. If the risk assessment is based on the carcinogenicity of Ochratoxin A, it would lead to a ML not exceeding 5 µg/kg in cereals.

Physical treatment of grain, such as scouring while cleaning the grain prior to milling, have resulted in a >50% reduction of Ochratoxin A contamination in the resultant wheat flour. Milling seems to have none or minor effect on the level of Ochratoxin A and it is only partly destroyed during bread making.

Several other food items have been identified as sources of Ochratoxin A, for example pulses and coffee. However, the existing data for these commodities are too limited or inconsistent to form the basis for a proposal for a maximum level. Nevertheless, commodities other than cereals may contribute a considerable part of the Ochratoxin A intake and further efforts should be made to obtain data to better assess dietary exposure from these products.

Conclusions and Recommendations

The present Position Paper on Ochratoxin A in Food leads to the following broad recommendations for consideration at the 30th Session of the CCFAC:

1. Based on all the toxicological evidence available to date, levels of Ochratoxin A need to be as low as technologically feasible, taking into account economic and social factors. Since "prevention is better than cure", the ultimate way to protect the consumer from the toxic effect of Ochratoxin A is to encourage and ensure a good agricultural practice by:
   (i) revealing the critical points where the fungi start growing and producing Ochratoxin A during the agricultural production.
   (ii) including quality control programmes in agricultural production.
   (iii) improving the training of individuals involved at all stages of production
   (iv) supporting research on methods and techniques to prevent fungal contamination in the field and during storage.

2. It is recommended that a code of practice be established by Codex for the reduction of Ochratoxin A in cereals;

3. It is recommended that a Codex maximum limit for Ochratoxin A be established at a level of 5 µg/kg for cereals and cereal products intended for direct human consumption.

4. It is recommended that Codex establish sampling plans and methods of analysis for Ochratoxin A in cereals. There are no evaluated sampling plans existing for Ochratoxin A in cereal grain today. Standardization of methods for determination of Ochratoxin A in cereals and cereal products are on-going (European Committee for Standardization: prEN ISO 15141-1, prEN ISO 15141-2) and have been evaluated in interlaboratory studies.

5. In order to evaluate the benefits and possible problems which may arise from the adoption of these proposals, governments and international organizations are requested to consider the following points concerning the above proposal, in particular:
   - from producing countries: the consequences for international trade they anticipate if a Codex ML for Ochratoxin A in cereals was established at the level of 5 µg/kg for cereals and cereal products intended for direct human consumption.
   - estimates of the possible public health consequences that might arise from the establishment of a ML for Ochratoxin A at levels of 5 µg/kg for cereals and cereal products intended for direct human consumption.
   - sampling plans and methods of analysis for Ochratoxin A.

B. The Joint FAO/WHO/UNEP International Conference on Mycotoxins, Tunis, Tunisia, 3-6 March 1999

This Conference discussed among other matters Ochratoxin, as one of the emerging mycotoxins (along with Zearalenone, Fumonisins, Tricothecenes), and reviewed a number of papers related to mycotoxin prevention and control. These include a case study by Mick Franck on "HACCP and its Mycotoxin Control Potential: an evaluation of Ochratoxin A in Coffee production". These papers are available on FAO Webpage.

The Conference conclusions and recommendations are reproduced below:

Recommendations of the conference

Regarding **the health and environmental considerations** of mycotoxins, the Conference concluded that mycotoxins can pose significant risk for human and animal health. National regulations establishing limits for mycotoxins have been intended to reduce human exposure, but these limits have varied widely and resulted in international trade problems.

In attempting to address the problem of non-tariff barriers to trade, the signatory countries of the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) of the World Trade Organisation obligated themselves to base their health and safety requirements for food on sound scientific risk assessment. In addition to other principles, such as consistency and transparency, countries are also required to take into account the risk assessment methodologies developed by international organisations. The regulation of mycotoxins should be based on sound scientific risk assessment and take into account the work on the occurrence and toxicology of relevant mycotoxins. In general, there is inadequate information on the role of the mycotoxins in human health to complete the risk assessment paradigm. Therefore, only a few mycotoxins have been evaluated by the JECFA, and progress in adoption of maximum limits by the Codex Alimentarius Commission has been slow.

In this context, the following recommendation shave been made to improve understanding of the health effects of mycotoxins and to acquire sufficient appropriate information to conduct risk assessments as required by the SPS Agreement.

It was concluded that the effects of mycotoxins on human health were the principal concern, although control measures need to take account of the social and economic consequences.

Regulation and surveillance should be targeted at the major sources of exposure in order to be cost effective. It may be inappropriate to employ limited resources for commodities which make a non-significant contribution to intake.

Considering the importance of mycotoxins as a threat to human health and as a potential impediment to the availability of, and trade in, food and feedstuffs, the evaluation of mycotoxins by the JECFA should be allocated a high priority. In view of the urgency of this matter, FAO and WHO should consider convening a meeting of the JECFA devoted specifically to risk assessment of mycotoxins as soon as the requisite data bases can be compiled. Member States are recommended to supply FAO and WHO all available data on levels of exposure and related effects determined experimentally, clinically, or epidemiologically. Where gaps are identified, measures should be taken to generate the

required information. Priority should be given to those mycotoxins where there are human data on the effects and levels of exposure.

Risk assessment of mycotoxins should be conducted in a transparent manner and according to sound scientific principles with any gaps in knowledge and consequent assumptions made clear.

As previously indicated by the JECFA, where good quality human data are available these should take precedence over animal data in assessing the risk to human health. As a corollary, there is a need to improve procedures for obtaining human data related to exposure and health consequences. In cases of acute outbreaks of mycotoxicoses, methods need to be developed to monitor these in real time. In view of the transient nature of such events, there is a need for speedy response to such disasters. Procedures should be established to ensure adequate exchange of information between the responsible national authorities and the responsible international agencies.

In the evaluation of data used for regulations, surveys and the establishment of toxicological properties, the reliability of sampling procedures should be considered.

Research should be conducted to evaluate the modification of the toxic potential of mycotoxins due to the presence of natural constituents in complex food and feed matrices, including co-contamination with other mycotoxins.

In order to provide a more secure basis for assessing risk, WHO and its Member States are encouraged to acquire human mortality and morbidity data in populations known to be highly exposed.

Children represent a special "at risk" group because of the likelihood of higher exposure on a body weight basis. In most cases, it is not known whether children might be more susceptible to the same dose as adults and there is a need, where possible, for more data to determine potency in children.

Population groups are often exposed to several mycotoxins concurrently with the possibility of interactions. For agriculturally important mycotoxins, and where there are plausible mechanistic reasons to suspect such an interaction (e.g. between a carcinogenic mycotoxin and another which is a promoter or immunosuppressant), such potential interactions should be considered.

Some agriculturally important mycotoxins have properties (e.g. immunotoxicity, increased gastrointestinal permeability) that may increase the susceptibility of a person to secondary sequelae such as infections with pathogenic bacteria. This may give rise to symptoms not recognised as a classical mycotoxicosis but should be investigated.

There is a need for harmonisation of Methods for assessing dietary exposure, and these should be appropriate for the purpose. The need for sound intake estimates should be emphasised since this is a means by which potency of mycotoxins in relation to chronic effects can be estimated. They are also indispensable in assessing the distribution of risks as a function of dose and identifying groups at risk.

In relation to human health, occupational exposure may represent an additional route of exposure, e.g. in agriculture, milling etc. and the associated risks should be explored .

In assessing human exposure, more emphasis should be placed on total diet and duplicate studies rather than individual foods. Where validated biomarkers are available, these should be used for assessing human exposure.

Mycotoxin prevention and control

Harmonization of regulatory limits for mycotoxins worldwide, should be promoted to reduce problems in international trade. An explanation of the risk assessment supporting specific regulations should be provided.

Enacted regulations and those under development should be the result of cooperation between interested parties from the industry, the ranks of the consumers, the scientific sector and official government circles.

To minimize intake of mycotoxins, measure should be taken to ensure that lots which do not meeting regulatory limits are not mixed with other lots but used for other purposes.

Regulatory standards can provide a benchmark against which the effectiveness of food safety programmes can be measured to provide a guarantee of successful implementation of HACCP systems.

In order to facilitate exposure assessment in the establishment of regulations, there is a need to obtain reliable survey data from laboratories utilizing a quality assurance programme. National governments and international and regional bodies, as appropriate, should develop and fund such studies.

Integrated mycotoxin control programmes should incorporate HACCP principles in the control of risks associated with mycotoxin contamination of foods and feeds.

HACCP principles can be used to highlight the roles that fungal econology, crop physiology, and agricultural practices play in mycotoxin contamination prevention and control.

There is a need for sensitization of all stakeholders in the production chain, particularly farmers, of the importance of measures to reduce or avoid mycotoxin contamination.

Due to limited information on mycotoxin contamination in developing countries such as those in Sub-Saharan Africa, collaborative and partnership research between scientists and laboratories in developing and developed countries should be encouraged to assess the extend of mycotoxin contamination in these countries.

Research should be conducted on new varieties on those agricultural commodities susceptible to mycotoxin contamination with a view to enhance their resistance to fungal infection, insect damage and toxin formation.

Before recommending the introduction of crops or genotypes into new environments, consideration should be given to the potential for increased fungal infection in such regions.

In drying procedures, the time period when the water activity is most favorable to mould growth should be minimized .

Training programmes for the development of practical control and management strategies, essential to ensure consumer's safety should be conducted, in particular in developing countries.

Training programmes should be developed and conducted on HACCP principles, applications and programmes.

Training programmes for those involved in agricultural and food processing activities addressing good agricultural, processing and handling practices should be conducted in order to set up strong mycotoxin management programmes.

More research should be conducted in the development of decontamination processes for fumonisins, ochratoxins, trichothecenes, zearalenone and other mycotoxins.

Aflatoxins

Research should be conducted on the development of cultivars and procedures for preventing fungal contamination and formation of aflatoxins in pistachios in the field.

Research should be conducted to determine the importance and critical effects that water used in processing may have on the aflatoxin levels in pistachios.

Training programmes should be conducted on the prevention of aflatoxin contamination in pistachios.

Research should be conducted to study the etiology of contamination of minor commodities such as Brazil nuts by aflatoxins and to develop industrial processes for reducing aflatoxins levels in these commodities.

Fumonisins

Research should be supported on the genetic variability of corn germ plasm to determine the effects on fumonisin production.

Research should be conducted to develop biomarkers and indicators of contamination by fumonisins.

Safe limits for human dietary exposure to fumonisins should be established for those population groups that consume large amounts of corn products.

Accurate and extensive surveys of corn-based human food and animal feed, including processed products, should be conducted using methods verified for their efficiency of extraction of fumonisin analogs.

Ochratoxins

Research should be conducted to determine the significance and presence of ochratoxin in human milk.

Research should be conducted to identify and establish factors that affect pre-harvest contamination by ochratoxins.

Sampling and analysis of mycotoxins

Additional sampling plans should be developed to cover a wide range of commodities and for a wider range of mycotoxins. These sampling plans should be realistic, based on sound science and on a careful balance between consumer and producer risks.

The reasons for undertaking and applying specific sampling plans should be made explicit as should the intended aim of the sampling plan.

A manual which sets out in some detail the recommended manner of taking samples should be developed and also shows how samples should be prepared for homogenisation and for subsequent sub sampling.

Research should be undertaken to examine the relationship between the analysis of multiple small samples from a consignment and the analysis of a single sample taken from a homogenised large composite sample.

Analytical methods should be developed which are simple, robust and of a low cost for use by developing countries. FAO, WHO, UNEP, IAEA, IUPAC and others concerned agencies should consider assisting in the transfer of appropriate technology. For example, they could make antibodies available that might be used to prepare ELISA kits or affinity columns locally rather than necessitating purchase of expensive commercial materials.

Research is needed on the development and validation of rapid testing methods.

More analytical methods for mycotoxins should be validated for a wider range of food and feed matrices than has been achieved to date. Recognising the cost and length of time involved in full collaborative trial validation, the AOAC International 'Peer Verified Methods' process should be encouraged and more widely employed. Methods so generated should be widely disseminated.

The development of reference materials should be encouraged for a wider range of mycotoxins and a wider range of foods and feeds than is presently the case. It should be recognised that certified materials with a high degree of confidence in the true values may not always be required and that lower cost, more stable materials may be more appropriate and relevant to needs.

There still remains a need to provide analytical standards, particularly for the less common mycotoxins, and these should be made available centrally by FAO, IAEA and WHO to appropriate official and other control and monitoring laboratories.

FAO, IAEA, WHO and UNEP should develop a mechanism for the transfer of small quantities of mycotoxin reference standards among countries to overcome recently introduced export restrictions.

There is a need to establish and confirm physicochemical constants for a number of analytical standards that can be used for confirming quantification of analytical calibrant solutions.

FAO, IAEA, WHO and UNEP should encourage the development of analytical methods which do not employ environmentally damaging chemicals or those that might present health risks to laboratory personnel.

In order to encourage wider participation in proficiency testing, FAO, IAEA, WHO and UNEP should consider regional training in establishment of local proficiency testing schemes which would include preparation of test materials, homogeneity testing and the production of test reports based on the International Harmonised Protocol for proficiency testing.

On other matters

FAO, IAEA, WHO and UNEP should consider setting up a portable, self sufficient 'turn key' mycotoxin laboratory that could be transported anywhere in the world and could be rapidly operational. Such a laboratory could be used as a model for what would be required for a self-contained laboratory in a developing country.

FAO, IAEA, WHO, UNEP and other concerned organizations should encourage the authors of reports and publications containing analytical data on mycotoxins to include information on sampling and Quality Assurance undertaken so that judgements can be made on the quality of the reported data.

C. Twenty third Session of the Codex Alimentarius Commission, Rome, 28 June - 3 July 1999

The last session of the Codex Alimentarius Commission discussed among other matters the maximum levels and sampling plans for aflatoxins in peanuts intended for further processing and the maximum level of aflatoxin M1 in milk. The outcome is summarised below.

Draft Maximum Level and Sampling Plans for Total Aflatoxins in Peanuts Intended for Further Processing

The Observer of the European Community and other delegations suggested an alternative sampling plan to more thoroughly address sample selection, sample preparation and analytical methods for the detection of aflatoxins. These delegations suggested that the proposed sampling plan should either be held in abeyance or adopted on an interim basis so that the Codex Committee on Food Additives and Contaminants could consider alternative sampling proposals in collaboration with the Codex Committee on Methods of Analysis and Sampling.
Other delegations supported the adoption of the draft level and sampling plan as proposed because the Committee had discussed the issue over many years, and the importance of linking

the maximum level with the applied sampling plan was consistently recognized. It was noted that the maximum level and sampling plans were developed on the basis of expert advice arising from an FAO Consultation and an extensive risk assessment was recently conducted by the 49th Session of the Joint FAO/WHO Expert Committee on Food Additives. It was also stated that further processing significantly reduced the level of contamination and that in any case, a further reduction in the draft maximum level or significant modification in the sampling plan could create severe negative impacts on trade with no significant increase in health protection for consumers.

The Commission adopted the maximum level of 15 μg/kg for total aflatoxins in peanuts intended for further processing. The Commission also adopted the draft sampling plan on an interim basis, with the understanding that the issue would be further considered by the Committee and the Codex Committee on Methods of Analysis and Sampling on the basis of proposals to be developed by an electronic working group prior to their next Sessions.

Draft Maximum Level for Aflatoxin M_1 in Milk

Many delegations expressed opposition to the adoption of the draft level of 0.05μg/kg for various reasons. These delegations noted that the availability, application and costs associated with methods of analysis for the determination of aflatoxins at the lower level were significant. They noted that adoption of the lower level would result in severe disruptions to trade in feeding stuffs and that liquid milk for direct consumption was not widely traded internationally. The Commission noted that the risk assessment conducted by the 49th JECFA had shown that the potential carcinogenicity of Aflatoxin M_1 was approximately ten times less than that of Aflatoxin B_1.

Many other delegations supported the level of 0.05 μg/kg as proposed. These delegations noted that the lower level was needed to protect high intake milk-drinkers and vulnerable populations such as infants and young children, and that the level was set as low as reasonably achievable. They pointed out that this low level could be easily detected analytically.

As the Commission could not reach a consensus, it agreed to return the Draft Maximum Level for Aflatoxin M_1 in Milk to Step 6 for additional comments and further consideration by the Codex Committee on Food Additives and Contaminants. This decision was taken with the understanding that information should be provided on the public health and the potential economic implications of a higher level or a lower level as proposed, and the levels of aflatoxin contamination found in milk.

ICO/FAO/CFC PROJECT : ENHANCEMENT OF COFFEE QUALITY THROUGH PREVENTION OF MOULD FORMATION

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Dr. Boutrif presented the main objectives, outputs and activities of the CFC/FAO/ICO International Project on "Enhancement of Coffee Quality through prevention of Mould Formation" which is currently under negociation for funding by CFC, the European Coffee Industry and possibly UNDP. CIRAD would make a contribution in kind by putting 2 of its experts at the disposal of the project and by providing the physical space for accommodating the Project Management.

The project aims at assisting all coffee producer countries in dealing with the problem of quality loss caused by mould infestation and to prevent the possible contamination of green coffee by OTA. Dr. Boutrif indicated that the 4-year project strategy is based on the following: (i) addressing the problem in a comprehensivbe way; (ii) use of participatory approach; (iii) capitalising on existing institutional framework; (iv) transfer of technologies to producers, traders and transporters; and (v) involving producer countries in all aspects of project implementation.

He informed that the project will establish 6 Collaborating Centers, one in each coffee production region of the world. The selection of these Centres take into account the type of coffee produced (arabica/robusta) as well as the the processing methods used (wet/dry). These Centres will be strengthened to assist the project in providing the following services to the nearby coffee producer countries: training and extension, collaborative research, specialist advice, and networking.

In addition to these Collaborating Centres the project will involve the participation of specialised research institutions from coffee consumer countries, through contractual arrangements e.g.: CIRAD, France; CBS, Baarn, The Netherlands; University of Surrey, U.K.; and Denmark Technical University.

The project activities are grouped under 3 phases: a first phase (diagnosis phase) in which optimal parameters for coffee processing and storage would be investigated in selected participating countries; it will build on the work already carried out under the "Pilot Study on the Prevention of Mould Formation in Coffee" commissioned by the European Coffee Association. The second phase (dissemination phase) would be devoted to the dissemination of IPSM tools and HACCP process monitoring systems among producer countries and
the development of training aids. During the third phase (implementation phase) the HACCP-IPSM methodology would be widely disseminated through training, first of the trainers and then, through these, of the farmers/producers themselves.

A Project Advisory Board, consisting of Mycotoxin Experts and Representatives of CFC, FAO, ICO and CIRAD would monitor the project implementation and provide overall guidance. The project execution would be with FAO (HQ and decentralised offices), and the daily management entrusted to the Project Manager who will be recruited for the entire duration of the project.

Dr. Boutrif gave an account of the various inputs to be provided (personnel, equipment, training, contractual services, etc...) and informed that out of the entire budget of US$ 5.593,500 the CFC would contribute by US$ 2.6 Million and the European Coffee Association by US$ 350,000. The remaining balance is to be sought from other potential donors (UNDP, EU, US Coffee Association).

The discussion which followed the presentation gave a clear indication of the importance of the project for producer as well as consumer countries and for the coffee industry and trade in general.

First Uganda FAO mission - How near is a HACCP system in dry Robusta coffee production?

R. Viani

According to the Joint FAO/WHO Food Standards Programme of the Codex alimentarius Commission[1] the HACCP consists of the following seven principles:

1. *Conduct a hazard analysis*
2. *Determine the CCP's*
3. *Establish critical limit(s)*
4. *Establish system to monitor the CCP*
5. *Establish corrective action*
6. *Establish procedures for verification*
7. *Establish documentation on procedures and records*

On the occasion of the FAO study described by H. Ngabirano[2] a preliminary hazard analysis of the production of green Robusta coffee in Uganda has been made:

Flow diagram for dry-processed Robusta

Operation	Information to be recorded	Possible hazard
Fresh cherries on tree		over-ripe cherries non-uniformity of ripening
Picking		mixing with fallen cherries
▪ strip ▪ selective	Time to yard, uniformity of ripening, temperature	storage of bags in sun
Drying		layer too thick
▪ concrete yard ▪ tarpaulin ▪ wire net ▪ soil	Time in yard, raking per day, thickness of layer, weather conditions, temperature	inefficient raking mixing of lots of different dryness lack of protection from showers/dew incomplete/non-uniform drying
Storage/transport	Time, weather conditions, temperature	contamination with soil contamination with vegetable material cleanliness of bags
Dry cherries		
Re-drying at curing station	Location of curing station	non-uniformity of moisture level
Hulling	Number of defects associated with increased OTA risk (hulls/husks/cherries/moldy beans)	insufficient cleaning/sorting re-bagging in bags used to transport dry cherries disposal of husks in vicinity
Green beans		
Storage/transport	Time, weather conditions, temperature	cleanliness of bags storage on (wet) soil storage against a wall

At the farmer level, corrective actions, which would already lead to an improvement of the mold situation, are:

- Avoiding the storage of ripe cherries in polythene bags on the subsequent development of mold, with risk of OTA contamination, as, according to a work conducted by Bucheli et al.[3] in Thailand, keeping ripe cherries in sealed bags leads to OTA formation at the end of drying.
- Protecting the cherries in the drying yard from showers and dew with plastic tarpaulins, now available to only one fifth of the farmers.
- Assistance in procuring tarpaulins and an incentive for quality (mainly, uniform dryness of beans) should also be given.

At the curing station the hygiene of operations could already be enhanced by:

- Locating the plant in well-drained areas, and disposing of husk material, which according to Bucheli et al. contain more than 90% of the OTA contamination, away from the plant.
- Optimizing the cleaning of green coffee and the sorting of defects associated with OTA, such as hulls, husks, cherries, moldy beans.
- Avoiding bagging of green beans in polythene bags already used to transport cherries.

A simple one page pictorial representation of the do's and don'ts already identified shall be established with the help of FAO training experts and distributed to the farmers.

Further work is, however, needed to establish critical limits, procedures for verification and documentation on procedures and records, and to monitor critical control points. The forthcoming enhancement of mycological expertise already planned for this project, and the synergy given by the integration of the project into the multi-center CFC/ICO/FAO program shall lead to a more complete application of the HACCP system and to a quality improvement.

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[1] Anon., General requirements (Food hygiene), *Supplement to Vol. 1B, 2nd edition, FAO/WHO*, Rome 1997, p. 20.

[2] H. Ngabirano, Initial experiences on the enhancement of coffee quality through prevention of mould growth project in Uganda, *Proc. 18th ASIC 1999*, In print.

[3] P. Bucheli et al., Development of ochratoxin A during Robusta (*Coffea canephora*) coffee cherry drying, *J. Agric. Food Chem.* In print

ENHANCEMENT OF QUALITY IN COFFEE BY PREVENTION OF MOULD FORMATION PROJECT

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In February 1996 the International Life Sciences Institute (ILSI) convened a workshop on Ochratoxin A, which had been first reported in green coffee in 1974 and in the beverage in 1988. This expert meeting strongly
recommended that prevention of mould growth was the best solution to the OTA issue. As a result of this the European coffee industry through the Institute of Scientific Information on Coffee (ISIC) financed a pilot study to develop a strategy for studying causative factors in the field.

This work resulted in the development of what is known of as the Enhancement of quality in coffee by prevention of mould formation project, which will be described in greater detail by my fellow speakers. The project was presented in February 1997 to the Executive Board of the International Coffee Organization (ICO) at its meeting in Bali, Indonesia. The project, which was formally backed by Papua New Guinea and Thailand, was endorsed in principle by the Board, which requested the ICO's Executive Director to submit it for financing by the Common Fund for Commodities (CFC), a body established by the United Nations to provide concessionary finance for projects of benefit to primary commodities.

The ICO is an international intergovernmental organization, also established by the United Nations in 1962, designed to foster international cooperation on coffee. It was established because of the great economic importance of coffee, particularly for a large number of developing countries, some of which depend on coffee for up to 80 per cent of their foreign exchange earnings. Total world exports of coffee are valued at some US$10 billion a year and retail value exceeds US$50 billion. The ICO is the international commodity body recognized by the CFC as qualified to submit coffee projects. It has 44 exporting and 18 importing country governments as its Members, with the exporting Members representing over 98 per cent of world exports of coffee. After some revisions on technical grounds the CFC's Consultative Committee approved the project, to a total value of US6.9 million, in July 1998, which was followed by approval by the CFC Executive Board in October 1998.

The project is being managed by the Food and Agriculture Organization of the United Nations (FAO), which is uniquely placed to ensure that the relevant authorities in producing countries are engaged in the OTA prevention process. The scope of the project, although activities are initially concentrated in representative coffee producing countries, is completely worldwide with provision for universal dissemination of the prevention guidelines.

This project development process is important because it implies the endorsement of the world coffee community to the project. ICO delegates are government representatives, often from a high level including cabinet ministers, and this support for the project confirms the commitment of both coffee exporting and importing countries to take action to ensure that OTA formation in coffee is largely eliminated.

Studies on Ochratoxin A in Indian Coffees and its Management Strategies

Naidu, R.*, Ramesh V Bhat**, Vasanthi.S**

Coffee in India, occupies an important position among the export commodities, particularly in the plantation sector. Coffee is cultivated in an area of 306 thousand ha, mainly in the southern states of Karnataka (56 %), Kerala (27%) and Tamil Nadu (10%) and to a lesser extent, in non-traditional areas like Andhra Pradesh, Orissa and North Eastern States (7 %). Coffee is grown in a varied agroclimatic regions in altitude ranging from 500 to 1500 MSL , temperature ranging from 15° C to 30^0 C and rainfall ranging from 1000 to 2500mm. India produces both Arabicas and Robustas. In all, India produced 228 thousand tonnes of green coffee during 1997-98 consisting of 43% arabica and 57% robusta coffees. The Indian Coffee Industry is largely export driven. One-third of the production is consumed within the country and two-third of the production is exported.

India is perhaps one of the few coffee producing countries that received information on the problem of Ochratoxin in coffee as early as 1995. As an outcome of the round table discussion on mycotoxin in coffee held at ICO London in the year 1997, a project has been initiated dealing with indexing fungal flora associated with mycotoxin in coffee in collaboration with School of Biological Sciences, University of Surrey, UK and Technical University of Denmark. The studies conducted in this project have thrown up some interesting results on the extent of mould growth, types of fungi associated at different stages of coffee cultivation and processing.

Subsequent to the decisions taken at the Nairobi Conference, studies were intensified at Central Coffee Research Institute (CCRI) in collaboration

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with the National Institute of Nutrition, Hyderabad to assess the extent of OTA contamination in Indian coffees and also to evolve management strategy to prevent OTA contamination in green coffee. The various studies conducted includes, mycological examination of coffee samples, estimation of naturally occurring OTA and study on the effect of roasting on the ochratoxin content in coffee and identification of critical control points in Coffee production, processing, storage and transport.

Coffee samples of different types viz., cherry, parchment and green coffee of Arabica and Robusta, representing various agro-climatic zones of entire coffee growing areas, were subjected to mycological examination by using moist chamber techniques, agar plate method (PDA and CDA), dilution plating and slide culturing for isolating and identification of the fungi. The mycological examination of the coffee sample recorded the presence of both storage and field fungi. Species of Aspergillus were dominant particularly *A.niger, A. fumigatus, A. flavus, A .ochraceous* and *A. restrictus*. *Penicillium* species were encountered in Cherry coffee samples. Field fungi like *Fusarium, Alternaria, Curvularia* were also observed. The incidence of *A. ochraceous* was found to range from 20 to 76%.

Thirty five different isolates of *A. ochraceous* representing different agroclimatic zones were used to assess the toxigenic potential. A known toxigenic isolate of *A. ochraceous* (NRRL3174) was also included for comparison. All the isolates were grown both on wheat and coffee substrates by providing ideal conditions such as moisture, temperature etc. for the production of toxins. Ochratoxin was analysed both in the culture material and in the green coffee beans using HPLC as the mode of detection and quantification. Extraction was carried out following the method of Studer-Rohr et. al. (1995). The HPLC analysis was performed on a 15 cm Bondclone RP C18 Spherisob Column using acetonitrile (55%), water (45%), acetic acid (2ml) as the mobile phase at a flow rate of 1ml/min and at an excitation wavelength of 330nm and emission wave length of 450 mm. The limit of detection on HPLC was 0.3 ng. Out of 35 isolates tested, only 23 were found to be toxigenic with a variation of toxin production ranging from 0.002 to 622 mg/Kg when wheat is used as substrate. Majority of the isolates (14) produced ochratoxin at levels ranging from 0.002 to 0.25 mg/Kg, while 4 isolates produced moderately between 1.02 to 6.31 mg/Kg and 6 produced levels above 10mg/Kg. One of the strains was found to produce as high as 622 mg/Kg. The A.ochraceous isolate NRRL 3174, a

known toxigenic strain was found to be producing 625mg/Kg of OTA. The production of OTA was found to be less when coffee beans were used as substrate. Studies are being further carried out to standardise favourable conditions for the production of OTA in coffee under controlled conditions.

Natural occurrence of OTA in green coffee beans at various stages of coffee production starting from harvesting, processing, drying, storage and trade, both random and purposive samples were studied. Out of 86 samples, 73 samples were from estates of various agro-climatic conditions and 13 were commercial samples. Most of the estate samples, where the coffee was processed as per the guidelines of the Coffee Board, were found to be negative except few cases where the OTA level are to the tune of 2.72ppb. Among the different types of coffee, in unwashed Arabica and Robusta, more percentage of samples were found to contain OTA. In general, most of the trade sample selected randomly were found to be free from OTA except a few samples where the maximum level is 4 ppb, which is below permissible limit. Highest OTA content i.e., 32ppb was recorded in a purposive sample collected from coffee stored under high humid and moist condition for long time, which was not fit for human consumption.

As per the decisions taken at the Round Table Conference held at ICO, London, a project dealing with indexing fungal flora associated with mycotoxin in coffee was proposed for implementation, including India as one of the participating nations, by the University of Surrey, UK and Technical University of Denmark. The Research department of Indian Coffee Board is actively involved in implementing the above project sponsored by the ISIC, Italy. The studies under this project were carried out for two years from 1996-97. This collaborative studies have generated some interesting results on the extent of mould growth, types of fungi associated at different stages of coffee cultivation and processing. Based on the results of these studies, Critical Control Points (CCPs) were identified. The Central Coffee Research Institute (CCRI), Coffee Board, initiated in depth studies during '98 cropping season, based on the results of the studies carried out by Dr. Mick Frank, to understand the role of various microorganisms, both targeted (toxigenic) and non-targeted (non-toxigenic), associated right from coffee habitat to drying process. The microbial population was studied in samples of different types viz.,soil, rhizosphere, rhizoplane, phyllosphere, caulosphere, different stages of fruit development (ripe and over- ripe),

remnants samples collected from pulping unit and samples of both parchment and cherry during the process of drying. The microbial population in the samples were isolated using the serial dilution technique and the population was expressed in terms of colony forming unit.

The samples collected from the Coffee habitat indicated the presence of microorganisms to the tune of 5.3 to 13.6 CFU x 10^4 . The sample collected from rhizosphere showed the highest population of fungi and the least recorded population was in the sample from rhizoplane. Analysis of samples collected at different stages of berry development, remnants samples from pulping unit and samples of both parchment and cherry during the process of drying, recorded the presence of various fungi viz., Yeast, *Fusarium*, *Cladosporium*, *Aspergillus*, *Penicillium*. In general, the presence of yeast dominated in all the samples analysed. The presence of toxin producing organisms like *Aspergillus* and *Penicillium* was found to be lower in all the samples.

The technical information available on OTA in coffee was transferred to the Coffee Industry (Producer, processors, traders and exporters) through the extension machinery of the Board. Latest extension tools such as farmer's participatory programmes through on-farm training, creation of more trainers trainees and liberal use of print and electronic media were used in transfer of technology.

ENHANCEMENT OF COFFEE QUALITY THROUGH THE PREVENTION OF MOULD GROWTH: START UP EXPERIENCES IN UGANDA

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ABSTRACT

A two year project on Quality Improvement through Prevention of Mould Growth financed through the Technical Co-operation Programme between Government of Uganda and Food Agricultural Organisation started in May 1999. Initial experiences in the start up areas which are in the high density natural robusta districts show that this project forms a good starting basis and cross fertilisation for the global project.

Through evaluation of the coffee production, processing and marketing systems in the project areas, potential problems and critical control points relevant to health safety and quality deterioration due to mould contamination have been identified especially at drying and storage levels.

The paper illustrates using data so far gathered, how production costs, availability of labour and inputs and producer margins affect adoption and application of GAP/GMP. Preliminary or first experiences show that differential pricing greatly influences farmer and processor adoption of GAP/GMP.

Operational parameters which can be important in mould growth reduction employed at each critical control point at farmer and processor levels in the Gourmet Project have shown considerable quality improvement through organoleptic evaluation. Use of tarpaulins and or trays during the coffee drying stage seem to offer the best applicable solution at this stage.

INTRODUCTION

In Uganda coffee is the most economic important commodity employing directly about 3 million people and accounting for 65% of the national foreign exchange earnings.

Coffee is produced entirely on small scale holding in 35 out of the 45 administrative districts in the country. Robusta is mainly concentrated in the radius of 300 km from shores of Lake Victoria and Arabica in the mountain ranges on the borders with Kenya and Democratic Republic of Congo.

Total acreage under coffee is about 272,000 ha out of which arabica accounts for only 10%. From this acreage, only around 4 m 60 kg bags are produced per annum which is indicative of relatively low yields.

Production and season patterns follow the climatic/rainfall conditions which are distinctly different according to the hemisphere. The northern hemisphere has the main season between November - February with a fly crop in May - August and the southern hemisphere is vise versa. This makes it possible to practically harvest coffee throughout the year.

The Government of Uganda attaches great importance to coffee and is very concerned about any threat to the health safety of the consumer. Given this concern and sensitivity of the possible health risk attached to mould induced quality deterioration the Government of Uganda fully supports any effort that will reduce mould contamination of coffee.

The Government of Uganda approached FAO to take the initiative in this area pending the proposed global project on prevention of mould growth on coffee. A project financed through the Technical Co-operation Programme with FAO to be implemented over a 2 year period was designed. The primary goal of the project is to improve quality of coffee through development of sustainable quality assurance programmes which will have a positive impact on the incomes of producers and eliminate the potential health risk to consumers.

In this regard a team of consultants composed of international, regional and national coffee experts have been recruited to work hand in hand with UCDA and the private coffee sector in Uganda. Work started in May 1999.

METHODOLOGY

Study area

The selection of the project study area was based on the production capacity and coffee seasons. The districts of Mukono, Mpigi, Masaka, Iganga, Mubende and Bushenyi which are major Robusta coffee producers in the country accounting for more than 60% of national robusta production and which were in season were selected.

Sample frame

Data was collected from all the three levels in the production and marketing chain i.e. farm, processor and export levels using a pre tested questionnaire and field observations.

Farm level

Random sampling was done to cover villages, parishes and counties in each district using groups of farmers and extension staff. A total of 50 groups of farmers (each of 10 farmers) were interviewed and their operations observed.

Processor level

A random sample of 40 buying stores and 40 hulling factories was used.

Export level

A random sample of 10 exporters out of the 25 operating exporters was used.

RESULTS/DISCUSSIONS

The farm level agronomical practices applied in the project areas seem to be in line with the normal GAP. Weed control is mainly through cultural practices where hand hoeing was practised by 90% of the sample farmers. Use of herbicides was limited to the medium scale farmers representing 10%. Limited accessibility and affordability were given as the main reason for not using herbicides and chemical fertilisers. Only 5 - 10% indicated they use chemical fertilisers (NPK, CAN and UREA). Use of chemical insecticides was also reported to be used on a nominal scale. Pruning, desuckering, mulching and manuring were largely practised by the farmers.

Farmer yields, costs and margins

The average age of the coffee trees ranges between 35 - 40 years. This together with the low input/output levels that characterised the production in the project areas and Uganda as a whole translate to a low yield of about 700 kg of clean coffee per hectare.

The average wage price in the coffee areas is about Shs. 1,200 equivalent to less than one United States dollar. The average total man days required for all farmer activities are estimated to 135 man-days.

Table 1a gives a rough estimate of costs and margins. Although the coffee farmer in the study seems to earn up to 127% per hectare above cost, we need to recall that the average size of the farm is about one acre. Therefore the farmer's income is miserably lower than his essential household costs/expenditure as shown in Tables 1 b and c.

Farm level processing and handling

Processing and handling at farm level is characterised by a mixture of practices some of which are inconsistent with GMP as shown in Table 2 below. The main problem areas were strip harvesting, mixing cherries of all sorts including those fallen down before harvesting and in some cases selling the crop before harvest. More than 75% of the farmers practice selective harvesting using baskets and hesian cloth/bags in the districts under study.

However this is negated by the factor that more than 80% of the farmers dry their coffee on bare ground and also practice mixed storage.

All coffee at farm level is sun dried and can take 7 - 14 days depending on the weather (t°, RH, and wind). Table 3 gives a comparative cost analysis of the concrete drying yard and other materials. Use of tarpaulins and portable trays seem to offer the best option given the cost and ease of utilisation.

Most of the farmers who practice strip harvesting method and dry coffee on bare ground named luck of incentives and high costs of drying trays and tarpaulins as the main limitations.

Processor/exporter costs and margins

The processor/exporter costs and margins are shown in Tables 4 and 5 below. The processor and exporter margins are U.Shs. 50 and U.Shs. 89 per kg respectively. Although the processor and exporter margins are much lower than the farmers, given the economies of scale through the quantities handled, the processor and exporter are much better off than the farmer.

Processing and storage level

Field observations carried out at hulleries pointed out possible areas that can cause hazards.

- Inadequate storage facilities where dry coffee waiting to be processed can be stored along the wet lots.
- Inadequate drying facilities resulting in thick layers of coffee.
- Some factories are located in swampy and humid areas.
- Some factories have inadequate quality control systems and equipment for carrying various quality control tests like moisture content and defect estimation.

- Efficient separation of coffee beans and husks was found important in order to avoid bean contamination from husks and spillage on dirty floors.
- Use of same gunny bags to carry cherry, husks and beans.
- Mixed storage of graded export coffee with rejects and husks.
- Insufficient grading according to bean size and weight can result in failure to remove the infected beans and reduction of mouldy beans.

Quality Evaluation

Evaluation of coffee exports done at Uganda Coffee Development Authority over a period of three years shows that all cup quality faults encountered resulted from poor handling and manufacturing practices most of which are important in mould contamination. Table 6 shows that the biggest percentage of cup defects is over fermentation which can be related to mould growth and contamination.

Gourmet Project cross fertilisation

Uganda is among the five countries implementing the Gourmet Project funded jointly by Common Fund for Commodities/International Coffee Organisation and Uganda.

The project generally follows the GAP/GMP in the production and marketing. Quality evaluation results show tremendous improvement when compared with the conventional coffees produced from the same region as is illustrated in Chart 2. The Gourmet coffee shows higher intensity of the positive and desired attributes while the conventional coffees show has higher intensity of the negative and undesirable attributes.

The Gourmet Project can therefore be complementary to the Mould Prevention Project. It may be worthwhile to carry out an OTA evaluation on the Gourmet Project coffee.

CONCLUSIONS AND RECOMMENDATIONS

There are some critical areas in the coffee production chain that can constitute possible hazards of mould contamination especially through rewetting and soil contact during the drying stage.

Farmer incentives like premia payment seem to be very important for implementation of GAP/GMP.

Availability of inputs and cost of materials required to carry out easily controlled drying operations like portable trays and tarpaulins are crucial factors in mould growth prevention. This seems to be a bigger problem since cost of inputs/materials is quite high compared to farmer's income from coffee.

Even where the farmers/processors can afford there should be effective training programmes to demonstrate GAP/GMP.

Training manuals clearly showing the corrective action or **DO's** and **DON'Ts** should quickly be developed and circulated.

Sampling plan and procedures should be established and documented.

Chart 1: - Robusta Coffee Harvesting Periods by Regions in Uganda

Region/District	**Coffee Seasonal Variation by Months During the Year**			
	Nov.- February	**March - April**	**May - August**	**Sept. - October**
1: Northern (a) Mukono (b) Mpigi (c) Mubende (d) Iganga	Main Crop *(Harvesting)*	Flowering and Fruiting *(for Main Crop)*	Fly Crop *(Harvesting)*	Flowering and Fruiting *(for Fly Crop)*
2: Southern (a) Masaka (b) Bushenyi	Fly Crop *(Harvesting)*	Flowering and Fruiting *(for Fly Crop)*	Main Crop *(Harvesting)*	Flowering and Fruiting *(for Main Crop)*

Source: Uganda Coffee Production Profile, 1999.

Table 1(a): Farm level Costs and Margins (ushs); per Ha. of Robusta Coffee in Uganda

Cost item	Physical measure (man/days, or units)	Unit cost	Total cost
Maintenance activities	135	1,200	162,000
Inputs	0	0	0
Tools appreciation			100,000
Sub total			262,000
Opportunity cost at (20)%			52,000
Total cost			314,000
Estimated yield kgs of kiboko	1300		
Cost per kg (314,000/1300)	242		
Farm gate price	550		
Margin	308		

Source: Survey Estimates for June/July, 1999

Table 1 (b): - Monthly Expenditure (ushs); for a typical (I) ha of old Robusta Coffee Farmer from Uganda with average HH of (5) people.

Expenditure Items per HH	Units	Unit Cost	Annual Cost
1. 5 bars of Soap	5 per month	1,000	50,000
2. 5 kgs of Sugar	5 per month	1,000	50,000
3. School fees for	2 persons per term	75,000	-
(2) Children At Sec.	(x3 terms)	per term	300,000
4. HH equipment	10	5,000 x 2	100,000
5. HH improvements	-	-	100,000
6. Clothing	5	x 2 shs 10,000	100,000
8. Medial	5	5,000 per month	300,000
9. Food Supplements	-	20,000 per month	240,000
Total HH expenditure			1,240,000
* Yield old Robusta coffee per ha per year			1,300
* Average price shs/kg			600
* Total income			800,000
* Farmer costs at shs 242 per kg.			- 340,000
* Farmers Margin per ha.			500,000
Expenditure Deficit			-(740,000)

Source: Survey Estimates for June/July, 1999

Table 1 (c): - Monthly Expenditure (ushs); for a typical (1) ha of old Clonal Robusta Coffee Farmer from Uganda with average HH of (5) people.

Expenditure Items per HH	**Units**	**Unit Cost**	**Annual Cost**
1. 5 bars of Soap	5 per month	1,000	50,000
2. 5 kgs of Sugar	5 per month	1,000	50,000
3. School fees for	2 per term	75,000	-
(2) Children At Sec.	(x3 terms)	(Per term x 3)	300,000
4. HH equipment	10	(5,000 x 2)	100,000
5. HH improvements	-	-	100,000
6. Clothing	5	(x 2 shs 10,000)	100,000
8. Medial	5	5,000 per month	300,000
9. Food Supplements	-	20,000 per month	240,000
Total HH expenditure			1,240,000
* Yield old Clonal coffee per ha per year			2,800
* Average price shs/kg			600
* Total income			1,680,000
* Farmer costs at shs 200 per kg.			- 420,000
* Farmers Margin per ha.			1,260,000
Expenditure Surplus			shs 20,000

Table 2: Estimates on the levels of Post-harvest Coffee Handling

Post-harvest Parameters	**Mukono** *(percent level)*	**Mpigi** *(percent level)*	**Masaka** *(percent level)*	**Bushenyi** *(percent level)*
Harvesting				
Strip method	22	14	21	26
Selective	78	86	79	74
Drying				
Bare soil	82	92	80	75
Mats/others	18	8	20	20
Storage				
Own house	88	92	82	90
Separate store	12	8	18	10
Marketing				
From tree	15	24	22	14
Wet coffee	50	55	58	44
Dry coffee	35	21	20	42

Source: Survey Data, June/July, 1999

Table 3: Comparative Cost Analysis (ushs); for Coffee drying Space at farm level in Uganda

Drying Equipment inputs Costs Comparison		
Barbecue	**Wire Meshes**	**Tarpaulins**
Area: (20 m x 10) m Inputs (i) 15 bags of cement = 225,000 (ii) 1 lorry of Sand = 50,000 (iii) 1 lorry of Stones = 80,000 (iv) labour = 50,000 Total Cost = 405,000	Area: (20 x 10) m Estimated cost = 300,000	Area: (20 x 10 m) Estimated cost = 75,000

Source: Survey Estimates for June/July, 1999

Table 4: Costs and Margins (ushs); per Tonne of hulled Robusta in Uganda

Cost parameters	Estimated Average Cost (ushs)
1. Farmer Price (kiboko)	550,000
2. Out-turn	55%
3. FAO Equivalent price	1,000,000
4. Processing/hulling cost	200,00
5. Processor Cost	1,200,000
6. FAQ price	1,250,000
7. Processor Margin (6-5)	50,000

Source: Survey Estimates for June/July, 1999.

Table 5: Costs and Margins (ushs); per Tonne of Export Processed and Marketed in Uganda

Cost Parameters	**Estimated average cost**
A: Export processing Costs	
1. Pre-Processing costs	50,000
2. Sec. Processing costs	80,000
3. Export Grading costs	30,000
4. Sub-total	160,000
5. Cost of the money (10-20%)	24,000
6. Total Cost	184,000
7. FAQ price	1,250,000
8. Total Exporter Cost	1,434,000
9. Average Export Price in US $ per tonne	1,050
10.Average Exchange rate; ushs/dollar	1,450
11.Total Receipts (9 x 10); ushs per tonne	1,523,000
12.Exporter margin (11-8); ushs per tonne	89,000

Source: Survey Estimates for June/July, 1999

Table 6: Trends in Coffee Quality Defects: 1995/96 - 1997/98

Defect	**1995/96**	**1996/97**	**1997/98**
1. Over Fermentatinon	24.4	26.2	39.8
2. Earthy	26.5	28.5	21.9
3. Potato	10.3	7.8	13.1
4. Taints	29.5	31.5	22.5
5. Others*	9.3	6.0	2.7

* Others includes Woody, Winey, Bitterness, Harsh, Fruity, Grassy and Musty.

Source: UCDA

Levels of Ochratoxin A in Blood from Norwegian and Swedish Blood Donors: Estimated Intake and Correlation between Blood Levels and Food Consumption Habits

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ABSTRACT
To study the influence of dietary habits on exposure to ochratoxin A, blood levels of the toxin were determined in 400 Scandinavian blood donors, using a HPLC method. In conjunction with the bloodletting, the subjects were asked to fill in a food questionnaire.
No strong correlation was found between plasma levels of the toxin and the intake of various foods. However, low but statistically significant correlations were seen for some food items, including red wine, certain kinds of bread, chickpeas, roast pork and liver paste either in the Norwegian (Oslo) or the Swedish (Visby) study population. It was also found that individuals with the highest plasma levels of the toxin consumed relatively more of certain foods, including red wine, coffee, beer and certain types of bread and other cereal products, as compared to individuals with low plasma levels.

INTRODUCTION
The mycotoxin ochratoxin A can be found in a number of foods, most importantly in cereals, coffee, wine, beer, vinefruit, pulses and pork. Even if cereal products are considered to contribute to the major part of the intake, occurrence of ochratoxin A in other foods, such as coffee and wine, have recently drawn much attention.
Since ochratoxin A is carcinogenic, as well as nephrotoxic, immunotoxic and teratogenic, it is crucial to keep human exposure to a minimum. Thus, the sources of exposure and their relative contribution to the exposure are important to identify. Previous studies have shown that analysis of ochra-toxin A in plasma is a useful method to assess exposure to the toxin, although further validation of this biomarker is needed.
In the present study, blood levels of ochratoxin A were measured and compared with dietary habits of Swedish and Norwegian blood donors.
The food items that significantly contribute to the ochratoxin exposure were identified by:
- Evaluating the correlation between plasma levels of ochratoxin A and the individuals' intake of various foods or food groups.
- Comparing the food habits between individuals with high and low plasma levels of ochratoxin A, respectively.

MATERIALS AND METHODS
Collection of blood and data on food consumption
The study included 100 women and 106 men in Oslo (Norway), and 66 women and 134 men in Visby (Sweden). The mean age of the subjects was 40 years in Oslo and 44 in Visby, and the mean body weight was 75 kg in Oslo and 79 in Visby. Blood samples were taken in connection with the ordinary bloodletting at the hospital Blood Banks of Oslo and Visby, respectively.
In conjunction with the bloodletting, the subjects were asked to fill in a frequency questionnaire concerning their consumption of foodstuffs in which ochratoxin A is known, or suspected, to occur. No food analyses were included in the study. To calculate the intake of ochratoxin A from the dietary sources included in the questionnaire, data from previously published studies were used.

Analysis of ochratoxin A

HPLC analyses were performed at an alkaline pH using the ion-pair technique introduced by Breitholtz et al. (1991). The mobile phase consisted of 10 mM tetrabutyl ammonium bromide in a methanol: potassium phosphate buffer mixture. The retention time for ochratoxin A was 10 min. The analyses were performed at the excitation wavelength 380 nm, the emission wavelength 450 nm and the temperature 30°C. The HPLC system consisted of two pumps, Shimadzu LC-6A, an autoinjector, Shimadzu SIL-9A, a column oven, Shimadzu CTO-6A, a fluorescence detector, Shimadzu RF 551, and a chromatography data system and controller, Axxiom Model 727. The column was a Spherisorb S3ODS2 (C-18) column with 3 μm particles. The column size was 4.6x150 mm.

RESULTS

Levels of ochratoxin A in plasma and corresponding dietary intakes

Plasma levels of the studied subjects are shown in Table 1. The levels of ochratoxin A in subjects from Oslo were slightly lower than those found in Visby (P=0.046). There were no differences in levels of the toxin between men and women in any of the centres.

The dietary daily intake of ochratoxin A was calculated on the basis of plasma toxin levels according to Breitholtz et al. (1991). The calculated dietary intakes for the subjects in this study are shown in Table 1.

Table 1. Levels of ochratoxin A in plasma and corresponding calculated dietary intakes.

Study group	Ochratoxin A in plasma (ng/ml)		Calculated intake (ng/kg bw/day)[a]	
	Mean ± SD	95th-percentile	Mean ± SD	95th-percentile
Oslo:				
All participants	0.18 ± 0.14	0.38	0.24 ± 0.14	0.51
Women	0.20 ± 0.14	0.42	0.27 ± 0.14	0.56
Men	0.17 ± 0.14	0.37	0.23 ± 0.14	0.50
Visby:				
All participants	0.21 ± 0.17	0.48	0.28 ± 0.22	0.64
Women	0.21 ± 0.17	0.57	0.28 ± 0.22	0.77
Men	0.21 ± 0.16	0.38	0.28 ± 0.22	0.52

[a]The dietary daily intake of ochratoxin A was calculated on the basis of plasma toxin levels according to Breitholtz et al. (1991), using the equation $k_0 = Cl_p \cdot C_p / A$, where k_0 is the dietary intake (ng/kg bw/day), Cl_p is the plasma clearance (ml/kg bw /day), C_p is the plasma concentration of ochratoxin A (ng/ml), and A is the bioavailability of the toxin. Cl_p and A have previously been estimated at 0.67 ml/kg bw/day and 0.5 (i. e. 50%), respectively (Breitholtz et al., 1991), and these figures were also used in the present calculations.

Correlation between intake of individual foods and plasma levels of Ochratoxin A

The correlation between individual intakes of each food and the plasma level of ochratoxin was tested (table 2). Only those who consumed a certain foodstuff were included in the correla-tion test on that particular food. As can be seen in table 2, the correlation was generally weak, although highly statistically significant for some food items (figure 1).

Figure 1. Correlation between plasma levels of ochra-toxin A and intake of beer in Swedish (Visby) women.

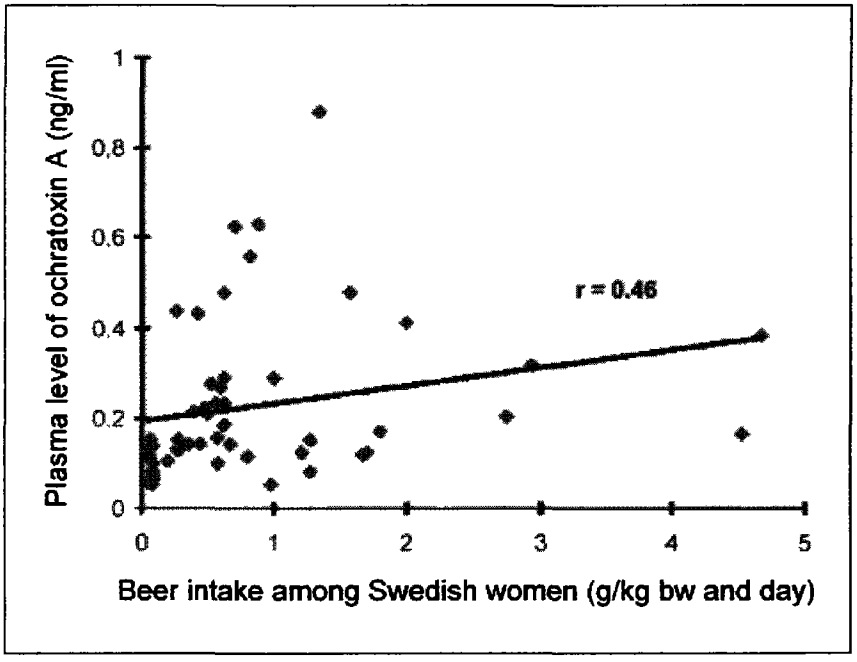

Table 2. Correlation coefficients (r) for food consumption and plasma levels of ochratoxin A among consumers. *Only foods for which statistically significant correlation coefficients were obtained are shown.*

Foods	All consumers		Female consumers		Male consumers	
	r	*p**	*r*	*p*	*r*	*p*
Oslo:						
Medium brown bread	**0.26**	**0.005**	**0.42**	**0.001**	**0.26**	**0.04**
Ham	0.06	0.50	**0.26**	**0.040**	-0.05	0.68
Roast pork	**0.22**	**0.01**	0.10	0.46	0.18	0.12
Liver paste#	**0.19**	**0.03**	0.14	0.26	**0.26**	**0.04**
Yellow pea stew	0.20	0.08	-0.01	0.94	**0.32**	**0.04**
Jam#	0.17	0.06	-0.003	0.98	**0.28**	**0.02**
Visby:						
Brown crisp bread	-0.02	0.27	**0.28**	**0.04**	0.003	0.97
Rusks	**0.18**	**0.03**	0.28	0.06	0.17	0.09
Chick-peas	0.20	0.14	0.001	0.99	**0.39**	**0.02**
Maize snacks	0.11	0.17	**0.35**	**0.02**	0.07	0.48
Beer	0.13	0.08	**0.46**	**0.001**	0.03	0.77
Red wine	**0.19**	**0.02**	0.22	0.12	**0.21**	**0.03**
Oslo + Visby:						
No statistically significant correlation was found						

*Figures given i bold indicate that the correlation is statistically significant ($p < 0.05$)
#Not included in the Visby study.

Comparison of food habits of the populations that display the 90 th percentile and 10 th percentile of plasma ochratoxin A

What characterise the food habits of individuals who have high plasma level of ochratoxin compared with those who have low level? One hypothesis was, that if the plasma level of ochratoxin A is solely derived from food intake, then the differences in plasma levels stem from foods that the population with high plasma levels (defined as the 90 th percentile) eat more of than do the population with low plasma level (defined as the 10 th percentile).

Food items consumed in higher quanti-ties by individuals with high plasma levels are shown in table 3. No statisti-cally significant differences were found in food consumption between the 10:th and the 90:th percentile among the subjects in Oslo. Among the subjects in Visby, individuals with high plasma levels of ochratoxin A were shown to have a higher intake of black pudding, chick-peas, figs, coffee, beer, and red wine. The same food items, except for beer and wine, were also shown to be consumed in higher quantities among individuals with high levels of ochratoxin A in plasma when the two study-populations were combined.

Table 3. Intake of foods in subjects with high plasma levels of ochratoxin a (90 th percentile) in relation to the intake in subjects with low plasma levels of the toxin (10 th percentile). Food items for which the difference in intake was signifi-cantly higher by the 90:th percentile ($p<0.05$) are included.

Food item	Intake by 90:th /10:th percentile		
	All participants	Women	Men
Oslo:			
No statistically significant differences were found.			
Visby:			
Black pudding	1.8	**5.1***	0.3
Chick-peas	**14***	-#	**15***
Figs	**2.9***	2.4	3.1
Coffee	1.1	**3.3***	0.9
Beer	**2.6***	**5.3***	1.3
Red wine	**3.0****	**17****	2.9
Oslo+Visby:			
Porridge	**6.5***	7.1	**11****
Sweet rolls, buns	**1.3***	0.8	**2.1***
Black pudding	**2.1***	**3.8****	0.9
Chick-peas	**2.5***	1.9	**3.4***
Figs	**1.7***	1.5	2.3
Coffee	1.1	**1.6***	1.1

* $p< 0.05$; ** $p<0.01$

Intake = 0 in by the 10^{th} percentile

DISCUSSION

The levels of ochratoxin A in plasma found in the present study (0.2 ng/ml) correspond very well with the levels found in previous studies of the Swedish population (Breitholtz et al., 1991, Olsen et al., 1993), and indicate an exposure below the temporary tolerable daily intake (tTDI) of 5 ng/kg body weight proposed by a Nordic Working Group (1991). Despite the extensive studies of ochratoxin A in human plasma, the correlation between plasma levels and dietary intake of the toxin has so far not been fully evaluated. In our study, the correlation between intake of food items which often are contaminated with ochratoxin A, and levels in plasma, was weak. This finding is in agreement with the study of duplicate diets reported by MAFF (1999), in which a weak, but statistically significant correlation was seen between plasma levels and dietary intake of ochratoxin A. The low degree of correlation between food intake and plasma levels probably reflects the wide-spread occurrence of the toxin at rather low levels in a number of food items. At higher levels of contamination, the relationship between plasma levels and intake of certain food items would be easier to disclose.

The analysis of dietary intakes in the individuals with the highest (90 th percentile) and lowest (10 th percentile) plasma levels of ochratoxin A indicated an higher consumption of certain food items in individuals with high plasma levels in Visby, as well as in the whole study population. Especially the consumption of red wine was high in subjects in Visby with high plasma levels, which is in agreement with the positive correlation found be-tween plasma levels and consumption of red wine. The plasma levels of ochratoxin A differed by a factor of approximately 10 between individuals in the 90 th and the 10 th percentile. This difference was not reflected in the calculated dietary intake, which was similar in both groups (data not shown). The reason for this discrepancy needs to be further investigated. Since the calculations of dietary intake are based on literature data, they will only give very approximate figures. Still, it appears likely that individual differences in uptake and/or metabolism of the toxin contribute significantly to the individual variation in plasma levels.

CONCLUSIONS

- The intake of ochratoxin A in this study was below the tTDI of 5 ng/kg body weight.
- At the present level of ochratoxin A exposure, there is no marked correlation between plasma levels and the intake of any specific type of food in the general population.
- Several types of food were to some degree related to high plasma levels of ochratoxin A, including cereal products, wine, beer, coffee and pork.

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POTENTIAL, PROGRESS AND FUTURE THRUST AREAS OF COFFEE BIOTECHNOLOGY RESEARCH IN INDIA

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Summary

Intensive research efforts of Central Coffee Research Institute (CCRI) during the past seven decades have resulted in sustained increase in production and productivity of Indian coffee. But still there are many old as well as new challenges to be overcome to make the Indian coffee cultivation sustainable in the next millenium. Biotechnology can make significant contributions in overcoming these challenges. In India, coffee biotechnology research activities, almost exclusively concentrated at CCRI, are focussed on micropropagation, anther culture, embryo rescue, endosperm culture, protoplast culture, genetic transformation, marker aided selection and *in-vitro* preservation. CCRI has achieved plant regeneration in more than 20 Indian selections of both *C. arabica* (arabica) and *C. canephora* (robusta) through somatic embryogenesis. Hardening procedure has been evolved for micropropagated plants. Large scale trial plots of micropropagated plants are being established in different agroclimatic zones using 3

selections viz., Cauvery, Sln.9 and CxR. For the first time in any plant species, plant regeneration is achieved from the integument tissues in the CxR cultivar. Protocols have been optimized for direct and indirect plant regeneration from embryo culture. Plants derived from embryo culture of interspecific crosses between cultivated *C. canephora* (robusta) and indigenous wild species, *C. bengalensis*, *C. travancorensis* and *C. wightiana* have been established in the field. Plant regeneration is achieved for the first time from the anther culture in the diploid CxR cultivar and from the endosperm cultures of *C. arabica* (S. 2803). Zygotic embryos of *C. arabica* have been preserved for 2 years under slow growth conditions and successful cryopreservation of zygotic embryos is achieved in three species. Protocols have been developed for isolation of protoplasts from the embryogenic calli, and for isolation of DNA and producing RAPD and AFLP markers. The RAPD markers are being used for evaluating leaf rust differentials and genetic fingerprinting. Transient expression of gus gene is achieved in leaf tissues of CxR cultivar after co-cultivation with *Agrobacterium tumefaciens*. The future thrust areas include, evaluation and management of biodiversity with molecular markers, identification of molecular markers linked to leaf rust resistance and drought tolerance, engineering leaf rust resistance with antifungal genes from heterologous sources, isolation of leaf rust resistance genes and caffeine degradation genes, and developing Bt based biopesticides to combat major pests, apart from using the protoplast technology for introgressing useful genes from the wild relatives.

Introduction

The genus *Coffea* coming under the family Rubiaceae is described to contain about 70 species. However, only two species are commercially important. C. *arabica* popularly known as arabica coffee accounts for about 70% of the coffee consumed in the world because of its superior beverage quality. *C. canephora* supplies the remaining

30%. Cultivated varieties of this species are generally called robusta coffee. A few other species of coffee like *C. dewevrei, C. liberica and C. racemosa* are consumed in the local producing areas.

Coffee is the most important agricultural export commodity in the world and many countries are involved in its production, trade and consumption. It generates 15 billion US dollars per year in the green coffee trade. The average world production is estimated to be 93 million bags (60 kgs each) (about 56 lakh tonnes) from more than 50 coffee producing countries in the tropical regions of Latin America, Africa and Asia. For most of these countries, coffee is the major source of hard currency earnings. India is among the top ten coffee producing and exporting countries along with Brazil, Columbia, Indonesia, Vietnam, Mexico, Ivory Coast, Ethiopia, Uganda and Guatemala. In India both arabica and robusta coffee are cultivated almost in equal areas. Coffee production in India has reached 2.3 lakh tonnes in 1998 from around 19 thousand tonnes in 1950, while the average productivity has increased to over 850 kg/ha from 204 kg/ha during the same period.

Major challenges to coffee cultivation in India

Managing the existing biodiversity, tackling major biotic and abiotic stresses, quality improvement and protection of environment are the major challenges to be addressed to make coffee cultivation more sustainable.

The most important disease affecting Indian coffee is the leaf rust caused by the fungus *Hemileia vastatrix*. The disease first recorded from India in 1869, has developed into the most challenging disease of arabica coffee in India. The disease is noticed throughout the year, but at different intensities with peak incidence from August to November. Severe attack results in defoliation, die-back, debility and crop loss upto

70%, if timely control measures are not taken up. The pathogen of leaf rust exists in physiological forms or races and 23 races have been reported from India alone (Anonymous, 1998) out of around 40 races recorded from all over the world. Black rot, also called as 'Koleroga' caused by the fungus *Koleroga noxia,* is a seasonal disease usually prevalent in South-West monsoon period. Blackening and subsequent rotting of the affected leaves, berries and young shoots are the common symptoms. Eventhough, coffee berry disease (CBD), which is a major disease in Africa caused by the fungus (*Colletotrichum coffeanum),* is not reported from India, research efforts need to be geared up to identify resistance source against this pathogen in the Indian germplasm, to be prepared to face any eventuality. A common nursery disease is damping off caused by *Rhizoctonia solani*, which can occasionally be observed in one year old field plantings. Apart from the above, four fungal root diseases also affect Indian coffee.

The major pests attacking Indian coffee are, the coffee berry borer *(Hypothenemus hampei)*, white stem borer *(Xylotrechus quadripes)*, mealy bugs *(Planococcus citri* and *P. lilacinus)*, shot hole borer (*Xylosandrus compactus)* and root nematode (*Pratylenchus coffeae*) (Anonymous, 1998). The minor pests include, brown scale (*Saissetia coffeae*), cockchafers or white grubs (*Holotrichia* spp), hairy caterpillars (*Eupterote* spp.), termites, thrips etc. Berry borer is the serious pest of coffee all over the world. Recently introduced to India, possibly from Sri Lanka in 1990, it has become a major pest. If uncontrolled, the pest can reach very high infestation levels. For berry borer control, research has so far focussed mainly on chemical insecticides, and biological control with parasitoids like *Prorops nasuta* and *Cephalonomia stephanoderis* and the fungal pathogen *Beauveria bassiana.* There is no known source of resistance to this pest in the coffee germplasm. While the white stem borer is a very serious pest of arabica coffee in India, the shot hole borer is a major pest of robusta coffee. Mealy bugs attack both the robusta and arabica, but prefer the former.

Coffee improvement programmes relying so far on conventional hybridization techniques were focussed on agronomic traits like increased yield and resistance to diseases. However, strong market demand for quality coffee has now directed the attention to breed new varieties with enhanced aroma and taste. Eventhough highly susceptible to environmental conditions and post harvest processing, coffee quality depends primarily on the genotype.

Water stress and oxidative stress are the most important abiotic stresses affecting the coffee plants and responsible for severe yield losses. With the expansion of coffee into non-traditional areas, high temperature stress also needs attention. Apart from the biotic and abiotic stresses, increasing stringency of environmental protection is gaining prominence.

Conventional breeding in coffee improvement

C. arabica is economically the most important species of coffee, although other species of *Coffea* have such potentially desirable genetic traits such as, for example, the absence of caffeine production, disease resistance, pest resistance, abiotic stress tolerance, wide adaptability, early maturity etc., these species have other undesirable factors such as, low yield of beans or beans which produce a poor quality coffee. However, it would be desirable to combine useful genetic traits of wild species with those of cultivated species. Employing conventional plant breeding methods of individual plant selection followed by progeny evaluation, hybridization and pedigree selection, coffee plants with high yield and vigorous plant growth have been developed. In addition to these, back cross methods have been used to transfer specific traits such as, short internodes, and disease and pest resistance to *C. arabica* from other cultivars or related species. During the last 60 years, Central Coffee Research Institute (CCRI), India, has developed 12 improved cultivars of arabica and 3 improved cultivars of robusta, through conventional breeding (Anonymous, 1996). A few more promising

selections are in the advanced stages of evaluation. However, inspite of tremendous contribution to coffee genetic improvement, conventional breeding suffers from the limitations like, the genetic barrier of chromosome number (diploids vs. tetraploids), autoincompatible alleles (diploid species), lack of genetic understanding and long breeding cycles. As a result of these factors, the transfer of genetic traits from wild outbred species of the genus to the cultivated species is quite difficult. A further complication is *Coffea*'s lengthy period of fruit development and the 2-4 year bean to bean generation time, which make such traditional approaches costly and time consuming. Because of these difficulties, development of a new coffee variety has been estimated to take atleast 24 years of continuous breeding.

Potential of biotechnology approaches for coffee improvement

Biotechnology can supplement the efforts of coffee breeders with additional tools which can overcome the limitations of traditional plant breeding to a great extent. There is tremendous scope for application of crop biotechnology tools for genetic improvement of Indian coffee. Coffee improvement through biotechnology can focus on three different areas of applications: agronomy, processing industry and consumers. Agronomic benefits should focus on reducing direct and indirect farming costs. To reduce coffee farming costs, the new technologies can address fertilizer efficiency, disease and pest resistance and crop management aspects that will reduce labour utilization (herbicide tolerance, mechanized harvesting etc.).

Various biotechnology approaches like micropropagation, embryo rescue, anther culture, cell line selection, somaclonal variation, protoplast culture, *in-vitro* preservation, marker aided selection and genetic transformation have tremendous potential for genetic improvement of coffee. Eversince the first report on tissue culture regeneration of coffee

by Staritsky (1970), there has been a steady progress in coffee biotechnology research (Sreenath & Naidu, 1997; Sondahl & Lauritis, 1992; Sondahl & Loh, 1988).

Micropropagation can be an efficient method of propagating individual plus coffee trees from a segregating population. It reduces the time for varietal development and helps to preserve the heterozygosity and plasticity in coffee plantations. Large scale micropropagation could be used to establish new plantations of hybrids that carry disease resistance genes. For transferring resistance genes, interspecific crosses could be achieved by embryo rescue methods. Alternatively, protoplast fusion between cultivated and wild coffee species could be used to transfer genes from wild diploid species to high yielding arabica or robusta varieties. As an alternative to propagation of segregating individuals, anther culture could provide homozygous lines for seed propagation in a short period (4 years vs 24 years). *In-vitro* variability could be induced in embryogenic suspension cultures. Chemical or physical mutation methods could also be attempted for this purpose. In addition, somaclonal variants could be isolated from embryogenic cultures of commercial coffee varieties. *In-vitro* preservation techniques, especially cryopreservation, could prove very useful for germplasm conservation. DNA markers could provide powerful tools for managing the biodiversity and improving the efficiency of conventional breeding. Genetic engineering can make most important contribution for genetic improvement of coffee. Possible applications of genetic transformation are engineering insect resistance, use of male sterility genes for production of F1 hybrids, engineering disease resistance and genetic manipulation for caffeine free beans (Sreenath, 1998c, d).

Apart from the above mentioned approaches for genetic improvement, biofertilizers, biocontrol of pests and pathogens, biodegradation and recycling of wastes from coffee processing units and bioremediation of coffee soils from the agrochemical residues, can be of great help in protecting coffee eco system and the consumer from the harmful residual effects of toxic agricultural chemicals.

Progress of coffee biotechnology research in India

Tissue culture is one of the major components of plant biotechnology and useful for rapid clonal propagation as well as genetic improvement. Realizing the importance of tissue culture for coffee improvement, Tissue Culture Division was set up at CCRI, as a Seventh Five Year Plan (1985-89) Project and the Division started functioning at the main station at Balehonnur in Chikmagalur district from 1989. The short term and long term objectives of the Division were micropropagation and genetic improvement respectively. During 1993 the main Tissue Culture Laboratory was shifted to Mysore and more facilities were added for increasing the activities and keeping in mind the long term need for diversifying into more advanced areas of biotechnology. Coffee Biotechnology Centre was set up at Mysore as an Eighth Five Year Plan project during 95-96 and Tissue Culture Laboratory was integrated in this Centre. Necessary infrastructure was created at this Centre to conduct research in advanced areas of biotechnology like molecular biology, genetic transformation, protoplast culture and cryopreservation. Research was conducted at the Tissue Culture Division and subsequently at the Biotechnology Centre on various aspects of coffee biotechnology. For achieving the short term and long term objectives, research efforts were focussed on stem, leaf, integument, apical bud and node cultures for micropropagation, anther culture, embryo culture, endosperm culture, protoplast culture, genetic transformation and molecular markers for genetic improvement and *in-vitro* preservation techniques, including cryopreservation, for conserving the biodiversity. Significant achievements made in these areas of research are summarized here.

Micropropagation

In coffee, micropropagation is possible by micro-cutting production and somatic embryogenesis. Various experiments conducted to develop micropropagation protocols for Indian cultivars of coffee has resulted in plant regeneration through somatic embryogenesis from stem and leaf tissues in more than 20 genotypes/clones of arabica, robusta and hybrid selections (Raghuramulu *et al.*, 1987; Babu *et al.*,1993; Jayashree *et al.*, 1995; Muniswamy & Sreenath, 1995b; Sreenath, 1998b). Genotypic differences were found in respect to callus induction, somatic embryogenesis and plant regeneration in arabica coffee (Naidu et al. 1999). Encapsulation techniques were standardized (Muniswamy & Sreenath, 1995a). Hardening protocols are developed for small scale (Muniswamy *et al.*, 1994) and medium scale plant production and trial plots of tissue cultured plants are being established in different agroclimatic zones (Sreenath, 1998b). Three improved selections viz., Cauvery, Sln.9 and CxR are being used for large scale field evaluation of micropropagated plants against seedling progeny. Plant regeneration is achieved from the apical bud and nodal explants (Ganesh & Sreenath, 1997), but further refinement of the technology is required. Effect of TIBA and BAP is tested on integument cultures of *C. canephora* (Babu *et al.*, 1997). For the first time in any plant species, plant regeneration is achieved from integument tissues of CxR cultivar (Sreenath *et al.*, 1995).

Genetic improvement

With the objective of using the tissue culture technology for genetic improvement, research was done in embryo culture, anther culture and endosperm culture. The research has resulted in plant regeneration through embryo culture by direct germination as well as somatic embryogenesis (Muniswamy *et al.*,1993; Sreenath *et al.*, 1989; Muniswamy & Sreenath, 1997). Plants produced from the embryo culture have been established in the field. In addition to this, plants regenerated from 3 interspecific

(Sreenath *et al.*, 1992) and 5 intervarietal crosses through embryo rescue methods are established in the field and are being evaluated. Plantlets have been regenerated from the anther culture of CxR cultivar (Muniswamy and Sreenath, communicated). This is the first achievement in a diploid species of coffee. Callus cultures were established from endosperm tissues (Raghuramulu, 1989) and plantlets are regenerated through embryogenesis from the endosperm calli of S. 2803 (Muniswamy & Sreenath, communicated).

Protocols have been optimized for isolation of protoplasts from embryogenic calli (Mamatha & Sreenath, 1998). DNA markers are becoming powerful tools in the hands of coffee breeders (Sreenath 1988a, 1999). Protocols have been developed for isolation of DNA and producing RAPD (Ram & Sreenath, communicated) and AFLP markers (Sreenath *et al.*, communicated). The RAPD markers are being used for evaluating leaf rust differentials and genetic fingerprinting (Ram & Sreenath, 1998). Genetic transformation protocols are being developed with the objective of genetic engineering of coffee. Transient expression of gus gene is achieved in leaf tissues of CxR cultivar after co-cultivation with *Agrobacterium tumefaciens* (Naveen & Sreenath, unpublished).

In vitro preservation

In vitro preservation of zygotic embryos upto 2 years under slow growth condition has been achieved in *C. arabica* (Naidu & Sreenath, communicated). Successful cryopreservation of zygotic embryos is achieved in three species (Krishna & Sreenath, unpublished).

Future thrust areas

There is great potential for application of biotechnology for genetic improvement of Indian coffee cultivars and growing coffee in an eco-friendly way. Results obtained so

far are encouraging. Now the efforts are being focussed on converting the vast potential of biotechnology into reality. However, it has been realised that concerted efforts, rather than individual efforts, are essential for realizing the enormous potential of coffee biotechnology. CCRI has proposed to make cordinated efforts involving national and international collaborators, for harnessing the potential of coffee biotechnology. A two day Workshop on Coffee Biotechnology was conducted at Mysore, during March 98 jointly by Coffee Board and Dept. of Biotechnology, Govt. of India (DBT) and with the help of more than 30 experts from different Institutes, priority areas in coffee biotechnology research were identified. Action is taken to implement the recommendations of the Workshop in a phased manner. Based on the recommendations of the Workshop, a Network Programme on Coffee Biotechnoloy has been drawn up by CCRI for implementation with the support of DBT. The salient feature of this programme is the participation of various National Laboratories and Universities with CCRI.

Developing molecular markers linked to leaf rust resistance, characterization of coffee germplasm with DNA markers, engineering leaf rust resistance in coffee with antifungal genes from heterologous sources, isolation of genes of leaf rust resistance from resistant cultivars, developing Bt based biopesticides to combat major pests, engineering insect resistance using Bt and proteinase inhibitor genes, isolating caffeine degradation genes for manipulation of caffeine content in the beans, are identified as thrust areas of coffee biotechnology research in India.

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A TECHNICALLY AND ECONOMICALLY ATTRACTIVE WAY TO PROPAGATE ELITE *COFFEA CANEPHORA* (ROBUSTA) CLONES : IN VITRO SOMATIC EMBRYOGENESIS

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INTRODUCTION

The *Coffea canephora* (Robusta) species is strictly self–incompatible and should be multiplied by vegetative propagation in order to maintain the genetic potential of a selected clone. Although cuttings is a well validated method, propagation by seed is still the most widely encountered multiplication process.
As they allow a rapid mass vegetative propagation, somatic embryos represent a new powerful method for clonal propagation of elite Robusta varieties. Large quantities of coffee somatic embryos can be produced in liquid medium (Zamarripa *et al.*, 1991; Ducos *et al.*, 1993; Noriega and Söndhal, 1993). We have reported a maximum production of 400,000 embryos within 2 months starting from one g of callus in the case of a genotype of Robusta (Zamarripa *et al.*, 1991).
Before to start any large scale commercial applications of this technology (described in the figure 1), it is necessary to determine:

- the regeneration capacities of embryogenic cell lines issued from selected genotypes,
- if and how the process can be transferred in coffee producing country conditions,
- a first estimate of the cost production of a plant issued from somatic embryos,
- the true-to-type status of the regenerated plants.

This paper reports data obtained during various large scale experiments recently run in order to clarify the four above mentioned issues.

MATERIALS AND METHODS

Plant material, callogenesis and establishment of embryogenic cell lines:

Primary explants were collected from 5 Robusta clones selected for their agronomic value (clones 01, 07, 62, 44, 84). Young leaves collected from greenhouse-grown trees were disinfected for 30 min in a 4% solution of sodium hypochlorite. The explants (500 per clone) were placed on the callogenesis semi-solid medium described by Yasuda *et al.* (1985) containing 6-benzyladenine (BA) as the only growth hormone (table I). This medium contains 1/4 strength macro salts and half strength micro salts of MS medium (Murashige and Skoog, 1962). After 8 months, yellowish friable primary calli were transferred (0.1 g/10ml) to liquid medium of the same composition (multiplication medium). Every two weeks, the volume of suspension was increased by the

addition of new medium; starting from 0.1 g, we obtained about 1.0 g of callus in 100 ml of medium within 2 months. After that time, embryogenic cell lines were subcultured every 2 weeks in this multiplication medium with an inoculation density of 10.0 g /l. Routine testing for latent bacteria was carried out on screening medium according to Viss *et al.* (1991).

Measure of the regeneration capacity of the embryogenic cell lines:
Callus tissues were directed towards embryo formation in liquid medium by decreasing the inoculation density from 10 to 1.0 g/l and by transferring them into a liquid production medium in which the salt concentration is equivalent to the MS medium. Old medium was replaced by fresh medium every two weeks. After 2 to 3 months, the torpedo embryos were spread out onto a semi-solid pre-germination medium (Zamarripa *et al.*, 1991): 0.5 g of embryos / 40 ml of medium/ plating dishes. When most of the torpedoes turned green (1 to 2 months), all the embryos contained in plating dishes were individually subcultured onto a germination medium identical to the previous one but without BA (25 embryos/ 40 ml of medium/Petri dishes or jars with a transparent cover). The embryos were classified according to their morphology: normal embryos ("Y" form due to small cotyledonary-like appendages) and abnormal embryos ("club" and "trumpet" form). After 4 months, the embryos having developed at least one pair of leaves were counted. Recorded data are the total embryos, "Y" form and plantlet numbers produced per g of callus inoculated at the beginning of the production phase.

RESULTS

Evolution of regeneration capacity of cell lines subcultured in liquid medium

The embryogenic potential of 38 cell lines (7 or 8 lines per clone) was measured every 3 months (table II). Over a one-year period, the average number of plantlets produced per g of callus decreased from 56,000 to 13,000. Considering the most productive cell line of each clone, the maximal mean regeneration capacity reached 160,000 plantlets per inoculated g after 6 months of subculture.
After one year, 26% of the cell lines could not be maintained because of a total evolution of the callus towards embryo development, probably due to the absence of auxine in the multiplication medium: indeed, auxine is generally used to maintain the plant tissue in a non-differentiated status during the multiplication phase. 26% more were lost due to other reasons, including a stop of growth, a complete loss of embryogenic potential and contamination due to some fastidious bacteria.
The germination rate of the total embryo number decreased from 31 to 16% over one year. Based on the "Y" form embryos, this rate also decreased (from 56 to 29%). As only a few percent of the abnormal embryos developed into plantlets, the selection of the embryos based on morphologic criteria allows to double the germination rate.
In conclusion, it looks necessary for commercial propagation to produce the embryos from young embryogenic cell lines in order to limit the waste of labor time due to the loss of cell lines during their regular subculture. In addition, old cell lines are less productive. However, a 3 month-multiplication duration is for example sufficient to achieve a large scale propagation considering the following data:

- 500 explants produce at least 1.0 g of friable primary callus
- 60 g of fresh tissue are obtained from 1.0 g after 3 months of multiplication in liquid medium
- 1.0 gram of a 3 month-old cell line produces 56,000 plantlets.

So, one run starting from 500 explants should allow to produce about 3 million plantlets. It must also be emphasized that these capacities were achieved using the same medium and protocol for the 5 clones. Possibly a higher potential could be observed optimizing them for each clone. Moreover, as reported by Yasuda *et al.* (1985), it is possible to subculture callus on semi-solid medium: they maintain a high embryogenic potential for more than 2 years, contrary to what has been observed with liquid medium. Consequently, an interesting alternative is to maintain the cell lines on semi-solid medium and to multiply them for only 2 or 3 months in liquid medium before starting a production batch. Cryopreservation could also allows to overcome the difficulty of embryogenic potential loss (Florin *et al.*, 1995) and would give a better flexibility for planning the embryo productions.

Simplification of the germination protocol

A mass propagation procedure was experimented with the collaboration of a public Philippino laboratory of the Department of Plant Industry located in the Mindanao island. A large quantity of torpedo or cotyledonary embryos were sent to this laboratory in which about 70,000 plants were developed. The germination rate under local *in vitro* conditions was 4 to 5 times lower than in our laboratory due to the unavailability of some chemicals and materials in the Philippines (gelrite powder, adequate jars with transparent covers...).
Consequently, an adapted new protocol has been developed in which the embryos are directly transferred from pre-germination plating dishes to *ex vitro* conditions (figure 2): they are acclimatized earlier at the cotyledonary stage and for most of them without roots. Data of an *ex vitro* germination experiment are shown in table III: depending on the development stage of the embryos, the germination rate on a substrate bed made of coconut fibers varies from 20 to 68% (47% on average). Considering all similar experiments we have run so far in the Nestlé Research Center, out of a total of 11,000 *ex vitro* embryos transferred, the global germination rate is 37%. A similar success rate is observed with peat miniplugs (Jiffy pellets).
This simplified protocol allows a drastic reduction of the labor at the laminar flow because the only individual embryo handling takes place for transfer to *ex vitro* conditions. It also allows a reduction the required area in the culture room.

Cost evaluation of a somatic embryo-derived plant

We can reasonably consider that with the simplified protocol, in a commercial production context, it should be possible to regenerate 15,000 plants from 1.0 g of callus, instead of 56,000 which is the current maximum obtained with the initial protocol (table IV).
We evaluated the production cost of 1.2 million plants over 2 years based on the following hypothetical figures:

- from 80 g of callus, 4 million embryos produced and *ex vitro* transferred using the simplified protocol
- 30% germination rate (1.2 million plants at the end)
- the implementation of the whole process in the Philippines (including the *in vitro* steps)

Compared to cuttings, the ready-to-transfer to the field embryo-derived plants have a slight over cost of 1.15 US$ cents (table V), i.e. 7%, which corresponds to a planting material over cost of 18.4 US$ per hectare (1,600 plants/ha). This estimate takes into account all the component costs (evaluated in the Philippines), except the renting cost of the laboratory facilities (requiring a culture room of at least 15 m2 of workable area). We can therefore conclude that up to now the cost of the plants obtained by the two different technologies is quite similar in spite of a low final germination rate with the new simplified protocol.

Field trial evaluation of the somatic embryo-derived trees

Clonal mass propagation by somatic embryogenesis must be assessed in terms of true-to-type characteristics before being run at a commercial level. To evaluate the true-to-type quality of the Robusta trees, field trials have been set up in 5 countries corresponding to a total of 90,000 trees issued from somatic embryos of 9 clones (table VI). Developed through the mass propagation experiment in the Philippines, 70,000 plants will be grown in local coffee production farms and will be compared to plants issued from cuttings or seeds.
Presently, out of a total of 8,000 visually inspected trees, all the plants have normal behavior: they developed flowers and fruits during the second year after plantation. In the oldest trial, set up in a Nestlé experimental farm in the Philippines, the yield of the third year after plantation already reaches 1 ton of green beans per hectare. More detailed observations and measures will be made during the three coming years for each field trial, with special attention paid to yield and cup quality.

CONCLUSION

Based on Philippines cost figures, a slight planting over cost of about 18.4 US$ per hectare would be required for the use of somatic embryos instead of cuttings. It is not really significant and must be quite acceptable, especially if the multiplied clones are over performing the seed-derived population trees and therefore creating a higher revenue per ha to the grower. Furthermore, several improvements of this *in vitro* technology are expected in various steps of the process (increase of the percentage of embryos ready to be *ex vitro* transferred, of the embryo-to-plantlet conversion rate...). On the contrary, as it has already been optimized, a cost reduction of the cutting method cannot be expected. Moreover, associated with the

cryopreservation technology, somatic embryogenesis offers more flexibility and rapidity than cutting to adapt plant production to the demand (in terms of quantity and quality of the clones). Indeed, a clonal garden cannot offer such a flexibility as it has to be managed whatever the demand: it immobilizes land and it has to be renewed when new selected clones are ready to be introduced for commercial use.

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SUMMARY

The plantlet regeneration capacity of 38 embryogenic cell lines (issued from 5 Robusta clones selected for their agronomic value) has been measured. On average, starting with 1 g of callus per liter of medium, 56,000 plantlets can be regenerated. A collaborative project between NRC Tours and a Philippino laboratory led to a simplification of the process, achieving embryo germination in *ex vitro* conditions rather than in *in vitro* ones. Based on the results of this project, an assessment shows that the cost per plant is competitive compared to the cost of cuttings, particularly if one takes account of the advantages that somatic embryogenesis offers. Ongoing field trials have been set up in 5 countries (90,000 trees). According to the first observations, these plants do not express any morphological abnormality, they are flowering and producing normal beans.

RESUME

Le potentiel de régénération en plantules de 38 souches embryogènes (issues de 5 clones de Robusta choisis pour leur valeurs agronomiques) a été mesuré. En moyenne, 1 g de cal par litre de milieu permet d'obtenir 56,000 plantules en final. Un projet commun entre Nestlé et un laboratoire Philippin a conduit à une simplification du procédé: les embryons sont mis à germer *ex vitro* et non plus *in vitro*. Une estimation de coût, basée sur les résultats de ce projet, montre que la propagation en masse par embryogenèse somatique est compétitive par rapport au bouturage surtout si on prend en compte les avantages qu'offre cette technique. Des essais au champ ont été mis en place dans 5 pays (90.000 arbres). Selon les premières observations, ces arbres ne présentent pas d'anormalités et leur floraison/ fructification sont normales.

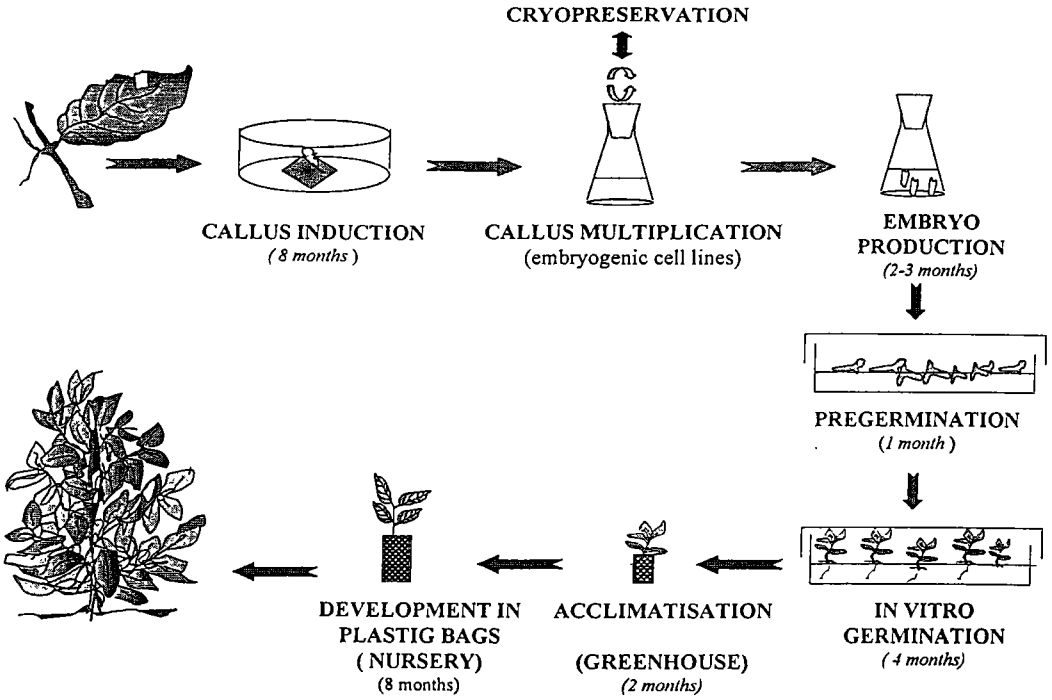

Figure 1. Mass propagation of Robusta by somatic embryogenesis

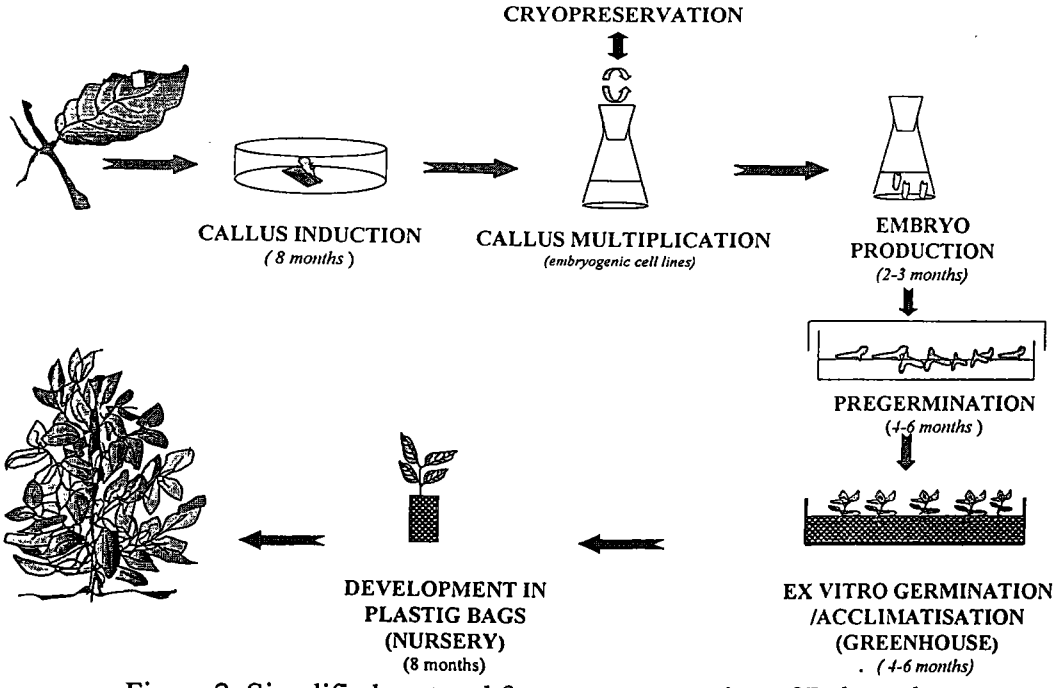

Figure 2. Simplified protocol for mass propagation of Robusta by somatic embryogenesis: "ex vitro" germination

Table I. Culture media for Robusta somatic embrogenesis

		Callogenesis	Multiplication	Production	Pregermination	Germination
salts		Yasuda	Yasuda	MS	MS X 0.5	MS X 0.5
inositol	*mg/l*	100	100	100	100	100
nicotinic acid	*mg/l*	1	1	1	1	1
pyridoxine HCl	*mg/l*	1	1	1	1	1
thiamine HCl	*mg/l*	10	10	10	1	1
pantothenic acid	*mg/l*	–	–	–	1	1
biotine	*mg/l*	–	–	–	0,01	0,01
BAP	*mg/l*	1,125	1,125	1,125	0,225	–
sucrose	*g/l*	30	30	30	10	10
agar	*g/l*	8	–	–	–	–
gelrite	*g/l*	–	–	–	4	4
PH		5,7	5,7	5,7	5,7	5,7

Table II. Evolution of the regeneration capacity of embryogenic cell lines cultivated in liquid medium (Mean ± Standard Error on the Mean)

Age	Lost cell lines	Total embryos*(1)*	"Y" form embryos*(1)*	Plantlets*(1)*	Plantlets*(2)* (Maximum)
months	nbr	nbrX1000/ g	nbrX1000/ g	nbrX1000/ g	nbrX1000/ g
3	5	182 ± 23	99 ± 13	56 ± 9	153 ± 20
6	11	227 ± 54	105 ± 24	47 ± 13	160 ± 57
9	15	142 ± 62	40 ± 9	23 ± 6	77 ± 18
12	20	65 ± 23	44 ± 17	13 ± 6	67 ± 24

(1) n = 38 (counting the lost cell lines as "0")
(2) n = 5 (average of the "maximum" cell line of each clone)

Table III. Simplified protocol : *ex vitro* transfer of somatic embryos at the cotyledonary stage (Mean + Standard Error on the Mean)

protocol : embryos were transferred into VitroVent containers containing 100 ml of coconut fibers soaked with germination medium devoid of sugar (40 ebr/container)

Stage	Description	Germination (%) *(1)*
I	elongated hypocotyl, well developed cotyledons and roots	68 ± 19
II	elongated hypocotyl, well developed cotyledons but no roots	50 ± 26
III	no open cotyledons no roots	20 ± 19

(1) counted after 4 months of *ex vitro* germination (n= 32)

Table IV. Comparison of the regeneration capacity using simplified protocol *vs* initial protocol

Initial Protocol *(in vitro germination)*		Simplified Protocol *(ex vitro germination)*	
Callus	1 g	1 g	Callus
Laboratory			Laboratory
selected embryos nbr	100,000	No selection	
germination rate	56%		
Greenhouse			Greenhouse
acclimatised plantlets nbr	56,000	50,000	selected embryos nbr
		30%	germination rate
Nursery			Nursery
plant nbr	56,000	15,000	plant nbr

Table V. Evaluation of the cost per plant at the plantation (in US $ cents)
(1 $ US = 38.1 Php Peso)

Somatic Embryos (estimated cost) *(1)*		Cuttings (real cost in 99)	
Laboratory *(2)*			
labor	0,34		
chemical/perishables	0,79		
equipment*(3)*	0,99		
Greenhouse			Greenhouse *(4)*
labor	1,89	3,94	
material	0,76		
construction	0,32		
Nursery			Nursery
labor/material /construction	11,81	11,81	labor/material /construction
Total	**16,90**	**15,75**	

(1) all costs evaluated in the Philippines (salaries, chemicals, perishables....) to produce 1.2 millions over 2 years
(2) not including the renting of a laboratory
(3) equipment cost with depreciation on a 4 year basis
(4) including the clonal garden establishment and maintenance

Table VI. Field trials for the quality evaluation of the Robusta trees issued from somatic embryos

Country	Embryogenic cell lines multiplication*(1)*	age (months)	Number of trees	Year of Plantation	Comparison
Philippines	L	5	4,000	96	*vs* microcutting
Thailand	S	12	4,000	97	
Mexico	S	6 to12	4,000	98/99	*vs* S.E. issued from lines cultivated with auxine
Nigeria	L	5 to 12	4,000	99	*vs* S.E issued from frozen cell lines
Brazil	L	5 to 12	4,000	99	*vs* S.E issued from frozen cell lines
Philippines	L or S	2 to 16	70,000	99	*vs* cuttings/ clonal seeds

(1) Embryogenic cell lines cultivated in liquid medium (L) or on semi solid medium (S)

COFFEE TISSUE CULTURE AS A NEW MODEL FOR THE STUDY OF SOMACLONAL VARIATION

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ABSTRACT

Coffee (*Coffea spp.*) is one of the most important agricultural commodities in the world. *C. arabica* and *C. canephora* are the principal commercial varieties of the genus. Somatic embryogenesis was first described in *C. canephora* by Staritsky (1970) and in *C. arabica* by Söndal & Sharp in 1977. Both systems are based on the use of growth regulators (auxins and citokynins) to induce a de-differentiation stage and the formation of calli. We had modified the protocol described by Yasuda et al., (1985) to create a model that allow us: 1) to use "*in vitro*" cultured plants as starting material and thus generate lines from selected plants, 2) the induction of direct somatic embryogenesis avoding the callus stage and 3) a rapid responding system (globular embryos are obtained at day 22) in contrast with other systems where they are obtained in 2 or 3 months. Histological studies using this system have shown that embryos are originated directly from the explant cell rather than from callus. When we analyzed the regenerated plants, we could not find any somaclonal variation at the molecular level.

INTRODUCTION

Somatic embryogenesis is the process by which somatic cells diferentiate into embryos, which are capable to germinate and produce a new plant. Somatic embryogenesis was discovered 40 years ago by two independent groups. Since them the applied potential of the discovery was visualized. Actualy there are hundred of hectars plantled with diferent crops that where obtained by somatic embryogenesis.

In vitro somatic embryogenesis of *C. canephora* was first reported by Staritsky (1970), who described the induction of callus tissue from orthotropic internudes; later, Herman and Hass (1975) obtained somatic embryogenesis in *C. arabica* from callus cultures derived from leaf explants. Söndahl and Sharp (1977, 1979), developed a two phases experimental protocol for somatic embryogenesis from leaves of *C. arabica* of the var. Bourbon. Dublin (1981) reported somatic embryogenesis from leaf explants of Arabusta using a medium with cytokinins but without auxins. Yasuda *et al.*, (1985) induced embryogenic calli from *C. arabica* leaf explants using 5 □M benzylamino purine (BAP), white and friable calli were obtained after 16 weeks and somatic embryos were obtained four weeks following calli initiation.

Vigorous research using both *C. canephora* and *C. arabica*, has allowed the use of different explants such as orthotropic stem fragments, plagiotropic branches, leaves, ovules, etc., (Dublin 1980a, 1980b, 1981). Leaves has proved to be the best explants for this purpose. The systems propused by Dublin (1980a, 1980b, 1981) and Söndhal and Sharp (1977, 1979) are among the most sucessful; in both methods indirect somatic embryogenesis is promoted.

The somatic embryogenesis in coffee is very different from somatic embryogenesis in other species. The somatic embryogenesis process in carrot takes only a few days, while in coffee takes several weeks in the

better of the cases. In the other hand, in some cases, as the one described by Söndhal group, the process takes place in two steps; after several weeks a small amount of somatic embryos are produced in a process that Söndahl called Low Frecuency Somatic Embryogenesis (LFSE), after this and on the top of the brown tissue a new production of somatic embryos takes place. This was called High Frecuency Somatic Embryogenesis (HFSE).

Morphogenesis in tissue culture depends mainly of two major parameters: the kind and amount of growth regulators and the nature of the nitrogen source used. In general, the research on coffee somatic embryogenesis has been focuced in the use of different growth regulators and nothing has been done with the nitrogen source.

We are interested into answer several questions such as: why the somatic embryogenesis process in coffee taakes several months? Which is the role of the nitrogen source on coffee somatic embryogenesis? Does the coffee tissue culture secrete proteins into the culture medium? If so, are they involve in the somatic embryogenesis process?

The protocol described here is a simple one step system for the induction of direct somatic embryogenesis of *C. arabica*, which can be used to answer some of the questions addressed before.

RESULTS

For the induction of somatic embryogenesis, leaf fragments (0.25 cm^2) from plants cultivated *in vitro*, under asceptic conditions, were cultured in the medium previously described by Yasuda *et al.*, (1985), using Gelrite as gelling agent.

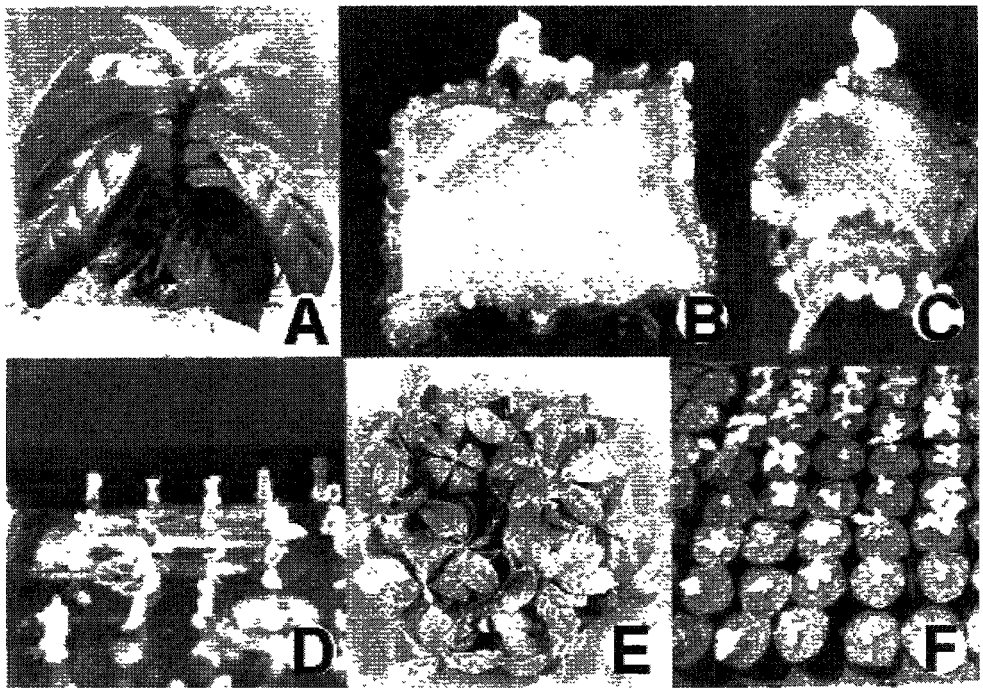

Figure 1. Direct somatic embryogenesis in *Coffea arabica*. A) *In vitro* cultured plant used as explant source. B and C) Embryos at different stages of development. D) Germinatino of the somatic embryos. E and F) Plants in the green house.

Two weeks after the explants were puted under the incubation conditions, a callus was formed in some of the injured edges of the leaves. On other sites of the leaf edges, globular somatic embryo-like structures began to form. These structures developed in true somatic embryos after day 21. These structures were separated and puted in Magenta boxes containing the germination medium. The somatic embryos germinated and produced plants after a few weeks. Actually we have more than 700 plants, produced by this method, under greenhouse conditions, and several thousands moved in Magenta boxes.

More than 100 plants have been analyzed by the AFLP technique, looking for somaclonal variation. However, all plants have showed exactly the same patterm, suggerting that there are not genetic differences between them (data not shown). Similar results, no somaclonal variation, has been obtained by the french group in Central America and by Dr. Söndhal in Brasil in plants obtained by different somatic embryogenesis methods.

Histological studies showed the formation of proembryos directly from cells rather than from the callus. Although Dublin (1981) reported direct somatic embryogenesis from Arabusta (*C. arabica* x *C. canephora*), no histological evidence supported his results. As can be seen in figure 2 (A – D), proembryos originated from the mesophyll cells and in some few cases, from epidermal cells. Indirect embryo formation was also observed from microcalli.

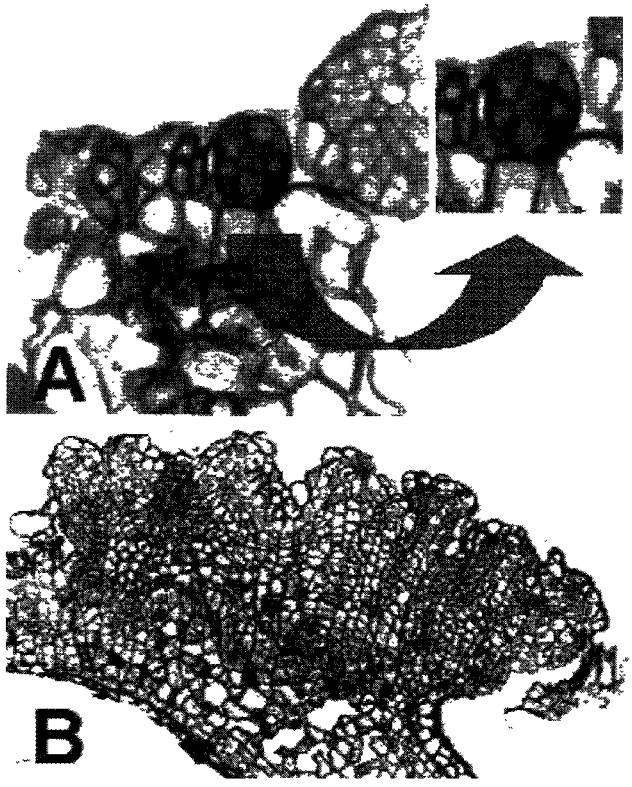

Figure 2. Histological study of direct somatic embryogenesis in *Coffea arabica*. A) Development center at the edge of the leave. B) Somatic embryos at initial development state.

It is know that the origin of the explant has a strong influence in the response of the explants to somatic embryogenesis. We tested if coffee is the case. The leaf number 3 showed the best results for the induction of somatic embryogenesis in the number of embryos per explant. Explants obtained near the apex showed a disminished response compared to those obtained from the basis of the leaf.

Since the early 60s it is know that the nitrogen source has an important effect on plant differentiation. The total nitrogen, the nitrate/ammonium ratio and the type of the nitrogen source, have a deep effect on the response of the explants to the somatic embryogenesis process. However, as already was mentionated, the effect of nitrogen source on somatic embryogenesis has not been studied in coffee.

As the total nitrogen in the medium increased, the response to somatic embryogenesis decreased. A 60 mM nitrogen concentration had a strong inhibitory effect on the response as well as the number of embryos-explant (Figure 3); the lowest concentrations, between 4 - 9 mM, give the maximun response. (Figure 4).

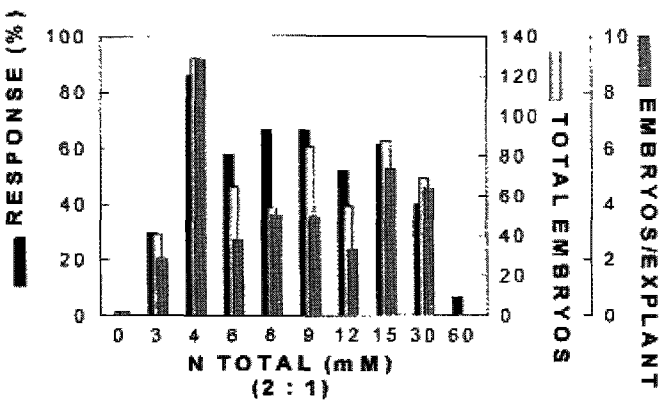

Figure 3. Effect of nitrogen source on somatic embryogenesis induction in *Coffea arabica*.

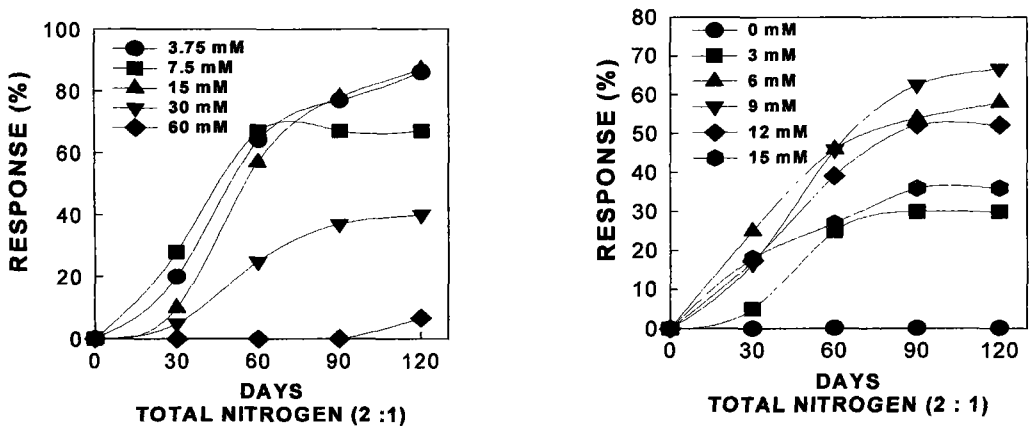

Figure 4. Effect of nitrogen source on somatic embryogenesis induction in *Coffea arabica* after 120 days of culture.

The nitrate/ammonium ratio also modified the response, the better ratio was 1 (nitrate):2 (amonium). The presence of ammonium is essential to obtain a response and nitrate has an inhibitory effect. In fact, both nitrogen sources are essential to induce the response.

CONCLUSIONS

Direct somatic embryogenesis is the formation of somatic embryos from the explant without the formation of an intermediate calli phase (Raghavan and Sharma, 1995). The production of callus is usually undesirable if embryogenesis is the goal of the research. Unfortunately, in most plants, direct somatic embryogenesis is difficult to obtain. This process in leaves does not happen frecuently. Conger *et al.*, (1995) and Trigiano et al., (1987) have reported direct somatic embryogenesis from leaves of *Dactylis glomerata* and Dubois *et al.*, (1991) in *Cichorium*.

To our knowledge this is the first report of direct somatic embryogenesis from explants of leaves in *Coffea arabica* supported by histological evidence. We had modified the protocol described by Yasuda *et al.*, (1985) to create a model that allows us:

a) To use *in vitro* cultured plants as starting material and this generate lines from

selected plants.

b) A fast and responsive system (globular embryos are obtained at day 22) in contrast with other systems where embryos take 2 or 3 months to develop.

The separation between the leaf tissue, the callus and the somatic embryos, let to obtained a pure sample to analyze what is happened at the biochemistry and molecular level.

Using this model and using differential display and RT-PCR, we isolated 5 cDNA, that are involved in the response of the tissue to somatic embryogenesis. Three of them have been sequenced. One of then has a high homology with quitinase, this sequence has been already registered in the Gene Bank; a second one with an oxygenase; the third is an unknown gene. The other two are actualy been sequenciated (R. Rojas-Herrera & V. M. Loyola-Vargas, data not published).

Actually we are studying the enzymes of nitrogen assimilation as well as the expression of their genes in orden to learn how the genetic program of the tissue is changed.

ACKNOWLEDGMENTS

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CRYOPRESERVATION OF SEEDS FOR LONG-TERM CONSERVATION OF COFFEE GERMPLASM AND ELITE VARIETIES : SUCCESSFUL APPLICATION AT CATIE

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Introduction

Though *C. arabica* seeds can withstand desiccation down to 0.06-0.08 g $H_2O.g^{-1}$ dw (Becwar *et al.* 1983 ; Ellis *et al.* 1990), they cannot be considered orthodox because they remain cold-sensitive and desiccation does not improve their longevity (Van der Vossen 1977; Ellis *et al.* 1990). *C. arabica* seeds are also characterized by their very short lifespan in the hydrated state (Couturon 1980).

Whatever their water content, *C. arabica* seeds do not withstand direct immersion in liquid nitrogen (Becwar *et al.* 1983). However, successful cryopreservation of zygotic embryos extracted from mature seeds has been achieved with *C. liberica* (Normah and Vengadasalam 1992), *C. arabica* (Abdelnour-Esquivel *et al.* 1992 ; Florin *et al.* 1993), *C. canephora* and the interspecific hybrid arabusta (Abdelnour-Esquivel *et al.* 1992). With all species tested, partial dehydration of excised embryos to 0.2 g H_2O/g dw was sufficient to obtain high survival after their direct immersion in LN.

Even if cryopreservation of excised zygotic embryos represents an interesting alternative strategy for long-term preservation of *C. arabica* genetic resources, this technique presents some drawbacks for routine use in coffee genebanks: i) in the case of coffee seeds, embryo extraction is very time consuming and labour intensive ; ii) low reproducibility was observed when desiccation zygotic embryos using classical desiccation methods (air-flow or silica gel); iii) all stages of the cryopreservation procedure have to be performed under aseptic conditions, which does not allow to avoid using *in vitro* culture techniques. Cryopreservation of whole seeds, instead of zygotic embryos, would allow to eliminate these drawbacks.

In this aim, the effects of several parameters of the cryopreservation protocol (desiccation, cooling, thawing and post-treatment) were investigated with *C. arabica* seeds to define conditions which would ensure survival of both the endosperm and the embryo (Dussert *et al.* 1997, 1998). It was shown that: i) the optimal water content for cryopreserving whole coffee seeds was 0.2 g H_2O/g dw; ii) a two-step freezing procedure including precooling at 1°C/min to -50°C was imperatively required to recover normal seedlings after cryopreservation; and iii) there was no effect of the thawing rate on survival of cryopreserved seeds. Under these conditions, the maximal percentage of normal seedlings produced after cryopreservation was about 30% (Dussert *et al.* 1997).In addition, it was observed that, whatever the cooling process, the survival rate of zygotic embryos extracted from cryopreserved seeds after thawing was always very high (80-90%). In this study, the reproducibility of seedling recovery was investigated by carrying out several cryopreservation experiments using the same optimal conditions. In addition, the effect of various post-thawing treatments was studied in order to improve the production of normal seedlings after cryopreservation of whole seeds.

CATIE coffee (*C. arabica*) collection is one of the most important in the world. This collection has been well characterized using molecular markers and agronomic traits. One of the objectives of CATIE is now to rationalize this collection in order to improve its long-

term conservation. Besides, excellent cryopreservation facilities are available in CATIE. Therefore, a collaborative project between CATIE and IRD, supported by IPGRI, was initiated in 1998 for the development of coffee seed cryopreservation techniques at CATIE. The aim of this project was to transfer to CATIE the methodologies for cryopreservation of coffee germplasm developed by IRD, to adapt them to local conditions and to test them on several accessions of the collection.

Materials and methods

Plant material, desiccation and cryopreservation

Fresh mature seeds of *C. arabica* var. Typica were employed. After the testa was removed, seeds were desiccated to 0.2 g H_2O/g dw by equilibration for 3 weeks under 78% RH obtained using a saturated NH_4Cl solution (Dussert *et al.* 1997). The different cryopreservation procedures (rapid cooling, two-step cooling, rapid rewarming) were carried out following the protocols described by Dussert *et al.* (1997, 1998).

Osmo-conditioning

After thawing, some seeds were osmo-conditioned for two weeks at 27°C in the dark using PEG solutions. Osmo-conditioning was carried out by placing batches of 10 seeds in Petri dishes sealed with Parafilm Ribbon, on a thin layer of cotton wool imbibed with 20 ml of aqueous PEG 6000 solution. PEG concentrations were calculated to achieve osmotic potentials of -1, -2 and -4 MPa at 27°C using the equation given by Michel and Kaufmann (1973).

Culture conditions

After thawing and/or osmo-conditioning, seeds were cultured *in vitro* for survival assessment. Disinfection and *in vitro* culture were performed as described by Dussert *et al.* (1997). Extraction of zygotic embryos and *in vitro* culture were carried out according to the method of Bertrand-Desbrunais and Charrier (1989).

Survival assessment

Both germination *sensu stricto* and development of normal seedlings were used to assess seed survival. Emergence of the hypocotyl and radicle was used as the criterion for estimating the germination rate. Seedlings which stood upright on the medium were considered normal. Excised embryos were considered viable when they stood upright on the culture medium and when their first pair of leaves was developed. The time to reach half of the final proportion (P_f) of normal seedlings, T_{50}, was estimated using the least square regression and the following model where P is the proportion of normal seedlings, T the time in days and A a treatment-dependant variable describing the synchronisation of seedlings development : $P = P_f / (1 + \exp(A (T - T_{50})))$.

Test at CATIE of cryopreservation procedures set up at IRD

In order to verify the reproducibility of results obtained at IRD (France), two cryopreservation procedures resulting from the experiments described above were performed at CATIE using *C. arabica* var. Typica seeds. In both procedures, seeds were desiccated to 0.2 g $H_2O.g^{-1}$ dw by equilibration for 2 weeks over a NH_4Cl saturated salt solution. In procedure 1, seeds were cooled rapidly (direct immersion into LN) and thawed rapidly in a 40°C water-bath for 3 min before zygotic embryos were extracted from cryopreserved seeds and germinated *in vitro*. In procedure 2, seeds were cooled slowly by precooling them at 1°C/min to -50°C prior to immersion into LN, thawed rapidly and germinated *in vitro*.

Table 1. Number of experiments (n) and percentage (mean, minimal and maximal values) of normal seedlings recovered from whole seeds or extracted zygotic embryos after desiccation of seeds to 0.2 g H_2O/g dw, followed by rapid cooling (direct immersion in LN; 200°C/min) or slow cooling (1°C/min to -50°C prior to immersion in LN).

	Whole seeds		Zygotic embryos	
	n	Seedlings (%) (min-max)	n	Seedlings (%) (min-max)
Rapid cooling	4	0	3	91 (83-98)
Slow cooling	7	17 (6-29)	1	70

Results

Effects of slow and rapid cooling

When cooled rapidly (200°C/min) by direct immersion in LN, none of the cryopreserved seeds produced normal seedlings. By contrast, viability of zygotic embryos extracted from rapidly cooled seeds was always very high. If seeds were precooled to -50°C at 1°C/min before immersion in LN (slow cooling), over 7 repeats, a mean value of 17% of cryopreserved seeds developed into normal seedlings. Under these conditions, some normal seedlings were always recovered after germination of whole seeds, but high variability in the final survival rate was observed (6 to 29%).

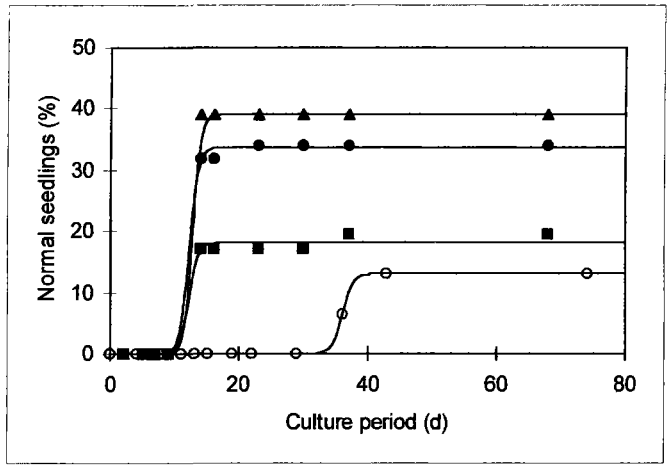

Figure 1. Evolution with time in culture under germination conditions of the percentage of normal seedlings recovered from cryopreserved seeds after a 2 weeks osmo-conditioning treatment on PEG solutions at -1 (▲), -2 (●) and -4 (■) MPa or without osmo-conditioning treatment (O).

Effect of osmo-conditioning of seeds after rewarming

Osmo-conditioning of seeds after rewarming improved the final proportion of normal seedlings recovered from cryopreserved seeds but the gain in survival decreased in line

with decreasing osmotic potential of the PEG solution used for osmo-conditioning treatment (Fig. 1). Production of normal seedlings under optimal osmo-conditioning conditions (-1 MPa) was three-fold higher than that of non osmo-conditioned cryopreserved seeds. Post-thawing seed osmo-conditioning drastically reduced T_{50}, the time to reach half of the final proportion of seedlings: when cryopreserved seeds were placed under germination conditions directly after thawing, T_{50} value was 36 d, while with osmo-conditioned seeds, it was about 12-13 d, independently of the osmotic potential of the osmo-conditioning solution.

Test at CATIE of cryopreservation procedures set up at IRD

Desiccation to 0.2 g $H_2O.g^{-1}$ dw by equilibration over a saturated NH_4Cl solution had no detrimental effect on seed viability since 100% of desiccated seeds developed into normal seedlings.

One hundred percents of embryos extracted from cryopreserved seeds according to the Procedure 1 (desiccation to 0.2 g $H_2O.g^{-1}$ dw, rapid cooling and rapid thawing) developed into normal seedlings. These seedlings showed a normal development after transfer under greenhouse conditions

All seeds cooled slowly and placed under germination conditions directly after rewarming (Procedure 2) showed hypocotyl extrusion and radicle growth. Further development into normal seedlings occurred in 24% of cryopreserved seeds. This final percentage of normal seedlings is very similar to those observed at IRD over 7 repeats (Table 1).

Discussion

When seeds of *C. arabica* at 0.2 g H_2O/g dw were cooled rapidly (200°C/min), none of them developed into normal seedlings. This result is consistent with those of Becwar *et al.* (1983) who showed that *C. arabica* seeds did not survive after immersion in LN, even if all freezable water had been removed from the seeds. By contrast, when seeds were slowly precooled to -50°C at 1°C/min before immersion in LN, an average value of 17% (over 7 repeats) of cryopreserved seeds developed into normal seedlings. It is thus clear that slow precooling of *C. arabica* seeds had a dramatic effect on their survival and their capacity to develop normally (Dussert *et al.* 1998). However, in view of the high variability observed for the survival rate over 7 experiments, improvement to the method appeared necessary before routine use and various post-thawing treatments were investigated.

It was shown for the first time that seed osmo-conditioning (osmo-conditioning) carried out after thawing had a dramatic beneficial effect on the proportion of normal seedlings recovered after cryopreservation and on seedling growth rate: under optimal osmo-conditioning conditions (-1 Mpa for 2 weeks), the percentage of cryopreserved coffee seeds which developed into normal seedlings was three-fold that of non osmo-conditioned cryopreserved seeds (39 % *vs* 13%) and the time to reach half of final percentage of normal seedlings (T_{50}) was about three-fold lower (13 *versus* 36 d). To our knowledge, the effect of seed osmo-conditioning after cryopreservation has been investigated previously in one study on celery seeds only (Gonzales-Benito *et al.* 1995). In this study, no effect of osmo-conditioning could be found since cryopreservation did not affect germination rates and T_{50} values. Seed osmo-conditioning was first employed to improve their rate and uniformity of germination (Heydecker *et al.* 1975). Since then, a beneficial effect of osmo-conditioning after seed ageing has been observed both on germination percentage and germination rate with numerous species (Bewley and Black 1994; Bray 1995).

Thus, even if the percentage of seeds which developed into normal seedlings remained relatively low in comparison with that obtained from excised zygotic embryos, a combination of slow cooling and osmo-conditioning treatment could represent a simple and

efficient complementary option to field conservation for genebanks which cannot afford *in vitro* culture facilities. Moreover, this method might be simplified by using a -80°C freezer for precooling seeds to -50°C and could thus become more easily employed routinely in a larger number of genebanks maintaining coffee genetic resources. However, additional research should be undertaken to optimize osmo-conditioning conditions and to carry out direct germination tests under greenhouse or nursery conditions.

In cases where very high survival rates are required for routine use, we propose a second alternative approach based on the extraction of zygotic embryos after rewarming of seeds, which would allow to avoid most of the drawbacks of cryopreservation protocols developed for zygotic embryos. Equilibrating coffee seeds under 78% RH allowed seeds to reach optimal water content for cryopreservation in a very easy and reproducible manner. This method also allows the processing of large amounts of seeds at the same time. Moreover, aseptic culture conditions are requested after thawing only.

Both procedures proposed in this study were successfully applied at CATIE. Optimization of post-thawing osmo-conditioning conditions and testing of the two procedures on 14 accessions of *C. arabica* are currently under investigation at CATIE. Some preliminary results indicate that up to 70% of normal seedlings could be recovered from cryopreserved seeds (without zygotic embryo extraction) under some osmo-conditioning conditions. Testing of optimized protocols on 14 accessions of *C. arabica* will provide preliminary information on the feasibility of establishing a *C. arabica* germplasm cryobank at CATIE.

Abbreviations

CATIE : Centro Agronomico Tropical de Investigacion y Enseñanza ; dw : dry weight ; LN : liquid nitrogen ; IRD : Institut de recherche pour le développement ; IPGRI : International Plant Genetic Resources Institute.

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MOLECULAR CHARACTERISATION OF THE CULTIVAR BOURBON LC.

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INTRODUCTION

Arabica coffee has being world-wide recognised as a species providing fine, aromatic, sweet/bitter beverage to consumers. Beverage quality of coffee is controlled by multigene traits. In reciprocal crosses between Arabica and Robusta, cup quality behaves as a dominant characteristic and in general, Arabica improves the cup quality of the resulting hybrid (Teixeira *et al.*, 1974; Fazuoli *et al.*, 1977). Differences in coffee quality beverage were also described for espresso coffee preparation by Petracco (1997). *Coffea arabica* var. Laurina (Smeathman) D.C. has being cited as source of a fine quality beverage and for this reason it has being used in different hybridisation programs (Krug et al., 1954). The most accepted origin of this variety is an spontaneous mutation out of Bourbon plantations occurred in Reunion Island (Froehner, 1898; Cramer, 1913; Choussy, 1935; Krug *et al.*, 1938). According with Raoul (1897), Laurina variety was cultivated by coffee producers in Reunion Island (ex-Bourbon Island) around year 1810 because of its rusticity to drought in substitution to variety Moka.

Studies on the genetic control of caffeine demonstrated that the variety Laurina has 0.6-0.7% caffeine and that it is controlled by the *Irlr* gene (Carvalho *et al.*, 1965). Subsequently, Sylvain (1969) revealed that the variability for caffeine in Arabica species from Ethiopia varies from 0.6% to 1.7%.

Despite its excellent beverage properties, the variety Laurina did not succeed in commercial coffee plantations due to its low yield potential. In a 19-year comparative study with 9 leading Arabica varieties it was demonstrated that Laurina produces 31% less than the variety Mundo Novo (Carvalho *et al.*, 1973).

A coffee improvement program based on cell and plant genetic methods has being carried out to select new lines of Arabica coffee with agronomic, industry and consumer benefits (Sondahl and Bragin, 1991). This program was successful in isolating high-yielding Laurina lines with twice the productivity of the original donor plants but similar 50% reduced caffeine and superior beverage (Sondhal et al., 1995). The top high-yielding lines were pooled together and released as a new cultivar called "Bourbon LC".

Morphological and agronomic characterisation of Bourbon LC were already described by Sondahl and Lauritis (1992) and Sondahl *et al.* (1995), but no molecular data is yet available. The purpose of this paper was two fold: (a) molecular characterisation of this new Arabica cultivar using

subtractive DNA analysis and Ribosomal Spacer Genes (ITS 1 and 2); and (b) based on RAPD analysis, make comparison of Bourbon LC with other commercial Arabica cultivars.

MATERIAL AND METHODS.

Samples.

About 20 young leaves, 2-5cm, were collected from each sample plants and kept on dry ice until they underwent freeze-drying. Some samples were kept in absolute alcohol. The samples were obtained from a number of cultivars as reported in Table I.

Table I

Cultivar	# of plants	Origin
Mundo Novo	20	Instituto Agronomico, Campinas (Br)
BourbonYellow	1	Instituto Agronomico, Campinas (Br)
Maragogipe	1	Instituto Agronomico, Campinas (Br)
San Bernardo	1	Instituto Agronomico, Campinas (Br)
Catuai Red	1	Instituto Agronomico, Campinas (Br)
Icatu Somaclone 4055	12	Serra do Salitre (Br)
Caturra	10	Instituto Agronomico, Campinas (Br)
Bourbon LC Somaclone 2165	20	S. Sebastiao Paraiso (Br)
Laurina	9	Instituto Agronomico, Campinas (Br)
Bourbon LC Somaclone 2154	150	S. Sebastiao Paraiso (Br)

DNA extraction.

The DNA extraction and purification was carried out combining the methods reported by Murray and Thompson (1980) and Orozco-Castillo et al (1994).

RAPD analysis.

The amplification conditions adopted for the RAPD analysis were the following:
20-50 ng DNA; 1X PCR Buffer (Perkin Elmer); 2 mM $MgCl_2$; 0,2 µM primer; 100µM dNTPs each: 1.25 U *Taq Polymerase* (Perkin Elmer). The thermocycler setting for thin wall tubes were the following: first denaturation 4' at 94°C; 43 cycles 30" at 94°C (denaturation); 30" at 37°C (annealing); 1' at 72°C (elongation); final elongation 3' at 72°C

The primer sequence together with the Operon Technologies nomenclature are reported in Table II.

Table II

Primer	Sequence		Primer	Sequence
BO7	GGTGACGCAG		P12	AAGGGCGAGT
B08	GTCCACACGG		P14	CCAGCCGAAC
B14	TCCGCTCTGG		P15	GGAAGCCAAC
B17	AGGGAACGAG		P16	CCAAGCTGCC
B19	ACCCCCGAAG		P17	TGACCCGCCT
B20	GGACCCTTAC		P19	GGGAAGGACA
I07	CAGCGACAAG		P20	GACCCTAGTC
I20	AAAGTGCGGG		V01	TGACGCATGG
J19	GGACACCACT		V06	ACGCCCAGGT
M04	GGCGGTTGTC		V08	GGACGGCGTT
N18	GGTGAGGTCA		V14	AGATCCCGCC
P02	TCGGCACGCA		V15	ACACCCCACA
P06	GTGGGCTGAC		V17	ACCGGCTTGT
P07	GTCCATGCCA		V19	GGGTGTGCAG
P09	GTGGTCCGCA		X09	GGTCTGGTTG
P10	TCCCGCCTAC		X16	CTCTGTTCGG
P11	AACGCGTCGG			

The gel electrophoresis of the RAPD amplification products were scanned and the molecular weight of each band was calculated. The data were then used in a Multivariate Data Analysis as reported by Podani (1993).

Subtractive PCR.

Genomic DNA of C.a var Bourbon LC, Caturra and Mundo Novo was digested with *Bam HI* and the digests were used for the subtractive PCR as described by by Lisitsyn et al (1993,1994,1995) with minor modifications. Both the Caturra and Mundo Novo DNA was used as driver, while the Bourbon LC DNA was the tester.

Two amplicons obtained in the subtractive PCR were cloned in Bluescrip according the specifications of the producer and then sequenced as reported below.

ITS1 and ITS2 amplification.

The Internally Transcribed Spacers (ITS) 1 and 2 of the ribosomal DNA was amplified using the universal primers reported by White et al (1990). The following conditions were adopted: first denaturation 35" at 94°C; 34 cycles 35" at 94°C (denaturation); 35" at 50°C (annealing), 2' at 72°C (elongation); final elongation 3' at 72°C.

The amplification products which underwent cloning or sequencing were purified on agarose gels by The High Pure™ PCR Product Purification kit (Boehringer Mannheim).

DNA cloning and sequencing.

Two amplicons obtained from the subtractive PCR, ITS1 and ITS2 of Bourbon LC were cloned using the PCR-Script™ Amp Cloning Kit (Stratagene) following the specifications of the producer.

The sequencing was performed with the DNA Sequencer ABI 373 using the Thermo Sequenase™ dye terminator cycle sequencing pre-mix kit (Amersham Pharmacia Biotech) as suggested by the producers of the kit. The sequencing was performed on both strands.

RESULTS.

The general strategy adopted for the characterisation of Bourbon LC relays on three approaches differing for the technique adopted as well as for the rationale. The first approach, based on RAPD analysis, was aimed to a general characterisation of different cultivars through a well established and generally accepted technology. The other two approaches were aimed to the identification of a peculiar characteristic of Bourbon LC, as for instance one base substitution, and made use of well established techniques for an innovative target.

RAPD analysis.

A number of primers was used but, for the final analysis, we considered only the 33 more informative primers. Each RAPD pattern showed a few strong bands and a number of faint bands. Usually the strong bands were reproducible while the faint bands were very variable in intensity depending upon many experimental conditions and therefore they were not taken in consideration. In total we obtained 292 reliable bands, 186 of which were polymorphic

Different plants of the same cultivar sometimes showed band variability. Bourbon LC Somaclone 2154 also showed individual variability for some primers. To solve the problem of individual variability, the final characterisation was carried out on one single reference plant as well as on a mix of 10 plants.

Different cultivars frequently showed strong band variation but each variable band was present in more than one cultivar. In particular we never found one specific band which was present only in Bourbon LC Somaclone 2154 or 2165. The lack of a specific marker band for Bourbon LC was overcome by the characterisation of the cultivar by the Multivariate Data Analysis. This approach allowed for the discrimination of Bourbon LC from the other Cultivars here considered as well as from the donor Laurina plants

The results of the Multivariate Analysis is reported in fig.1 where the branches of the dendrogram are proportional to the differences among the samples.

Subtractive PCR.

The method is based on the PCR-amplification of DNA sequences unique to a sample population (tester, Bourbon LC), after subtractive hybridisation with DNA sequences of a related population (driver, Mundo Novo and Caturra). The tester DNA sequences were annealed to a large molar excess of driver DNA following denaturation. Removal of hybrids as well as of self-reassembled driver molecules resulted in a strong increase of tester specific sequences (Fig.2).

The two upper bands of lane II in fig.2 were cloned and sequenced. The sequences of the two clones are reported below.

Clone A, 964 bp

```
   1  GATCCCCGCT GGTGCGGTGA CGTCTTCCTT CACATTCCCC TTCAATCGTT
  51  GGCGCAAGAG CAGCATCGTT AGCCTTGGCC GCCCACGGGT TTCCTGTGTT
 101  GCATACCTAT TAGAAGGAAT TCGGATGCCA CAACATTCAA CGTTCTCCCA
 151  ACGCCGTCCC GCCCGGTCGG GCTGCGGCGG CGTCGGGGAA CGCAAAGGCG
 201  AGGCCGTGTT CCGAGTCGCA GCCAAGCGAT GCGTCTCGGC CCACGAACTG
 251  TAGCCCGAGC TCTTGGACGC GGAACACCGG GAGGGCAGGA GATCGTCGAT
 301  CTCTATTTGC CTGAACTTGG CGTCAATCGC CCGCATCGAA CGACTGCCAT
 351  CGTCGCCTCG AGACGTCACG TCTCCTTCGA GCTCGTTGAC CTCGTGCGAC
 401  GTCGGCGTCG GTGAGGAATG CTACCTGGTT GATCCTGCCA GTAGTCATAT
 451  GCTTGTCTCA AAGATTAAGC CATGCATGTG TAAGTATGAA CTAATTCAGA
 501  CTGTGAAACT GCGAATGGCT CATTAAATCA GTTATAGTTT GTTTGATGTA
 551  CCTGCTACTC GGATAACCGT AGTAATTCTA GAGCTAATAC GGTGCAACAA
 601  ACCCCGACTT CTGGGAAGGG GTGCACTTAT TAGATAAAAG GTCGACGCGG
 651  GCTCTGCCCG TTGCTGCGAT GATTCATGAT AACTCGACGG ATCGCATGGC
 701  CTTCGTGCTG GCGACGCATC ATTCAAATTT CTGCCCTATC AACTTTCGAT
 751  GGTAGGATAG TGGCCTACCA TGGTGGTGAC GGGTGACGGA GAATTAGGGT
 801  TCGACTCCGG AGAGGGAGCC TGAGAAACGG CTACCACATC CAAGGAAGGC
 851  AGCAGGCGCG CAAATTACCC AATCCTGACA CGGGGAGGTA GTGACAATAA
 901  ATAACAATAC CGGGCTCTTC GAGTCTGGTA ATTGGAATGA GTACAATCTA
 951  AATCCCTTAA CGAG
```

Clone B, 804 bp

```
   1  GATCCTTTTC TATCAGATAG GAAGGGCTGT AGCACAAAAT GTATTTCTAA
  51  GTAATTGCCC CATAGATCCT ATATCTATCT ATATGAAGAA GAAATCATGT
 101  AACGAAGGGG ATTTTTATTT GTACAAATGG TACTTCGAAC TTGGAACGAG
 151  CATGAAGAAA TTAACGATAC TTCTTTATCT TTTGAGTTGT TCTGCCGGAT
 201  CGGTCGCCCA AGATCTTTGG TCTCTACCCG GACCCGATGA AAAAAATGGG
 251  ATCACTTCTT ATGGACTCGT TGAGAACGAT ATCGGATCTA GTTCATGGCC
 301  TATTAGAAGT TAGAAGGCCG CTCTGGTGGA ATCTTCACGG ACAGAAAAAG
 351  ATGCAGTCAG TTTGATAATG ATCGAGTGAC ATTGCTTCTT CGGCCCGAAC
 401  CGAGGAATCC CTTAGATATG ATGCAGAGCG GATCTTGTTC TATCCTTGAT
 451  CAGAGATTTC TCTATGAAAA ATATGAGTCG GAGTTTGAAG AGGGGGAGGG
 501  GGAAGGAGCC CTTGACCCGC AACAGATAGA GGAGGATTTA TTCAATTACA
 551  TAGTTTGGGG GGCTCCTAGA ATATGGCGCC CTTGGGCCTT TCTATTTGAT
 601  TGTATTGAAA GGCCCAATGA ATTGGGATTT CCCTATTGGT CCAGGCCATT
 651  TTGGGGCAAG CGGATCATTT ATGATGAAGA GGATGAGCTT CAAGAGAATG
 701  ATTCGGAGTT CTTGCAGAAT GGAACCGTGC AGTACCAGAC ACGAGATAGA
 751  TCTTCCAAAG AACAAGGCCC TTTTCGAATA AGTCAATTCA TTTGGGACCC
 801  TGCG
```

Clone A had 964 bp, from base 1 to base 415 it was homologous to part of the externally transcribed spacer of the ribosomal DNA region of many plants, while the rest of the sequence was homologous to the 5' region of the 16S ribosomal gene. Clone B had 804 bp and it was homologous to a plastid ORF of many plants.

ITS1 and ITS2.

The Internally Transcribed Spacers 1 and 2 are part of the ribosomal genes whose structure is reported in fig.3. Notably the ribosomal gene sequence is expected to be tandemly repeated many times along the same chromosome. The boundaries of ITS1 were established by comparison to other vegetal species. The complete consensus sequence is reported below.

Consensus sequence for *C.a.* ITS1

```
  1   TCGAATCCTC CAGAGCAGGC GACCGTAGAC TTGTGTAACC GTTGGGTGTC
```

```
  51   AGGGGAGAGG CGGCGAGATT GAACCCTTCC CTCTTTTCTT GTCACTCCCT
 101   AGGCACTCAT CACCCAAATG ACCAACTCAA CCTTAGCGTG GAAAGTGCCA
 151   TGGAATACGT AAAGGGATCG TTCCACCCCC GACTGCCCCA TATGCGGAGT
 201   GTAGGAAAGG AAGCTATTGT GCCTGTTGTA ACC
```

The sequence of ITS2 that we obtained was the same of that reported by Lashermes *et al.* (1998).

ITS 1 and 2 were sequenced in a number of different cultivars and specific base substitutions were observed for specific cultivars. For instance Bourbon LC had a "G" in position 216 of ITS1, while the other cultivars had an "A" (reported in bold in the consensus sequence above).

Nevertheless we noticed that the direct sequencing of both ITS's frequently showed ambiguities, which suggested some degree of sequence variations amongst repeats. To solve this doubt we cloned ITS2 of Bourbon LC and of Mundo Novo and then we sequence a number of independent clones. Seven clones out of 32 from Bourbon LC showed a specific base substitution in position 5 ("A" instead on the normal "C"), all the others showed the consensus sequence, All the 18 clones of Mundo Novo so far sequenced showed the consensus sequence ("C" in position 5).

DISCUSSION

The RAPD analysis is very much in line with the suggestions reported by Smith (1997) on Cultivar identification and varietal protection and indeed the technique proved to be adequate for the characterisation of Bourbon LC.

The dendrogram resulting from the RAPD analysis does not describe a strict evolutionary relationship among varieties, rather, it describes the divergence among Cultivars in relation to the 33 RAPD here used. The main characteristics shown by the dendrogram are the following:

1) One branch of the dendrogram includes all the Bourbon LC samples and the Laurina samples, indicating their similarity.

2) Bourbon LC Somaclones 2154 and 2165 are closely related but distinct. They also differ from the Laurina donor plants.

3) Bourbon LC Somaclone 2154 is more closely related to the Laurina plants than the Somaclone 2165.

4) There is little or no variation between the Bourbon LC 2154 reference plant 171 and the mix of ten Bourbon LC plants (positive control).

5) All the different Cultivars are on different dendrogram branches.

The Subtractive PCR succeeded in identifying specific *BamHI* restriction sites which are not present in Caturra and Mundo Novo. Notably the Bourbon specific restriction sites were found in repeated DNA sequences, both the ribosomal DNA and the plastid DNA are supposed to be present in multiple copies in each cell. Multiple copies of the same DNA sequence allows for a certain degree of mutation without effecting the viability. We think that more Bourbon LC specific restriction sites can be found by changing the restriction enzyme. Moreover this techniques allows for further technical developments for easy testing of large numbers of plants.

Very much in line with the results of the subtractive PCR, the sequencing of ITS1 and 2 showed sequence variation amongst different cultivars of this repetitive DNA. Single base substitution are very stable mutation and, very likely, they will be maintained for many generation and in very large numbers of progenies. Most probably they represent the most effective characterisation of a cultivar. Moreover, once that the base mutation has been found, easy tests for large population analysis can be developed.

On the bases of the data here reported, we can conclude that the Cultivar *C. arabica* var. Bourbon LC is effectively distinct from the other cultivars tested and that the different molecular approaches could enlighten very distinct elements.

ACKNOWLEDGEMENTS.

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SUMMARY

Three different strategies for the characterisation of the cultivar Bourbon LC, a natural somatic variant from variety Laurina, are reported. 1) Random Amplified Polymorphic DNA (RAPD) analysis. From 33 RAPD primers (Operon Technologies Inc.), 292 bands were obtained, 186 of which were either present or absent in different *C.a.* varieties. The multivariate analysis of the electrophoretic profiles allowed for the construction of a dendrogram showing high similarity but distinctness between Bourbon LC and Laurina and adequate distance of the Bourbon LC/Laurina group from the other varieties. 2) Subtractive PCR. Bourbon LC DNA was digested by *BamHI* and following the addition of adapters and competitive amplification (driver DNA: Caturra and Mundo Novo), two specific clones were obtained. Both clones are delimited by *BamHI* sites, specific for Bourbon LC. 3) Sequence variations of the Internally Transcribed Spacers of the ribosomal genes, ITS1 and ITS2. Bourbon LC ITS1 was sequenced and the spanning of the region defined by comparison to other vegetal species. Bourbon LC and Caturra ITS 2 were cloned and 50 clones sequenced. About 20% of the Bourbon LC clones showed a sequence variation in position 5 in which the normal C was substituted by an A.

The RAPD approach allowed for a general characterisation of Bourbon LC, while the two other experimental approaches allowed for a very specific characterisation of this new coffee cultivar.

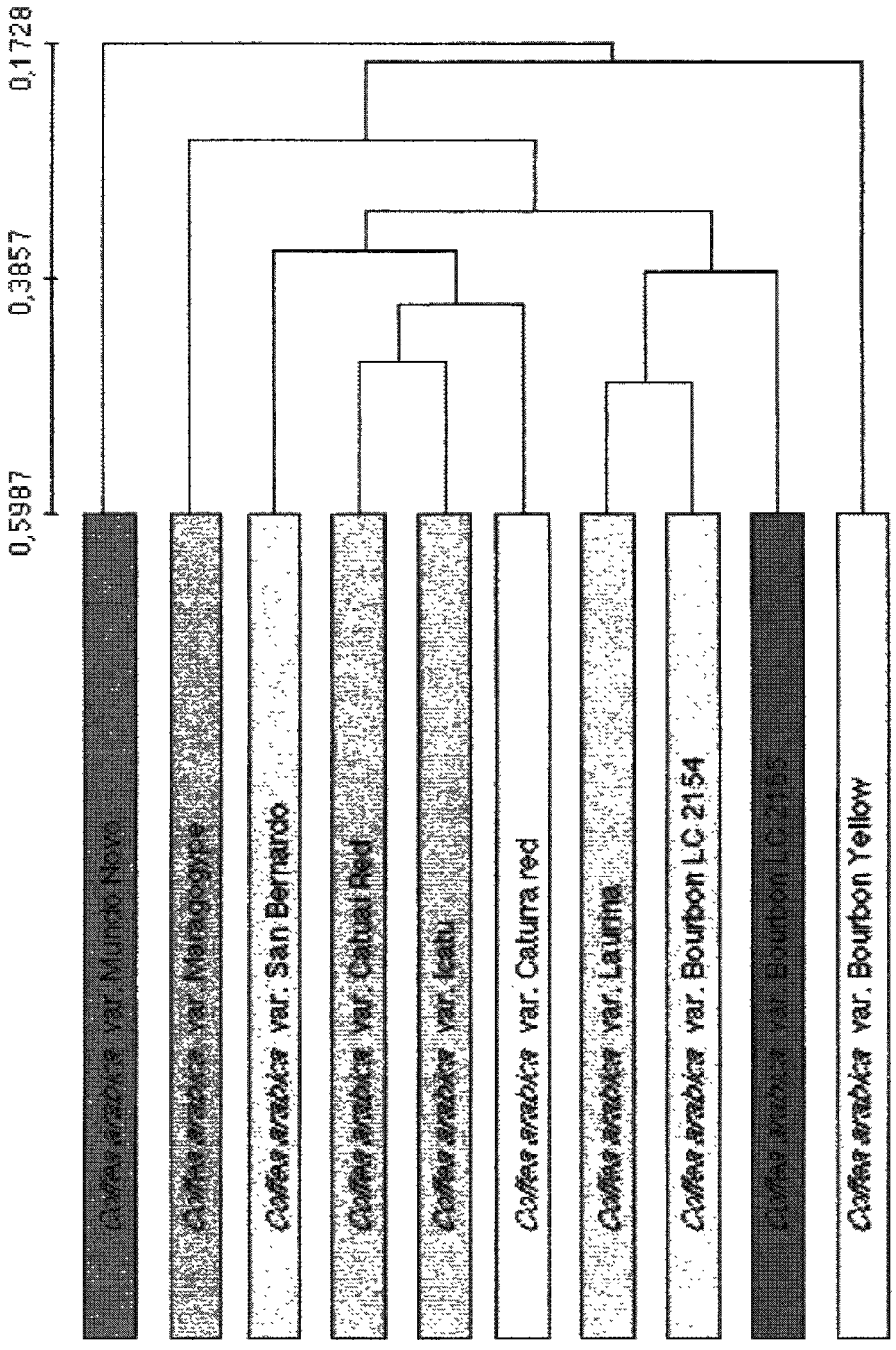

Fig.1 Characterisation of Bourbon LC DNA by Multivariate Data Analysis with 33 RAPD.

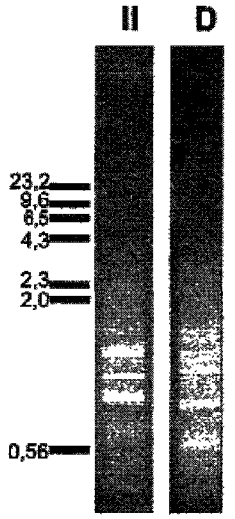

Fig. 2. Subtractive PCR of Bourbon LC versus Caturra and Mundo Novo. Lane II: agarose gel electrophoresis of the DNA molecules obtained after the second cycle of subtractive hybridisation.. Lane D: driver amplicons (Caturra and Mundo Novo). Numbers indicates the molecular weight markers in Kbp.

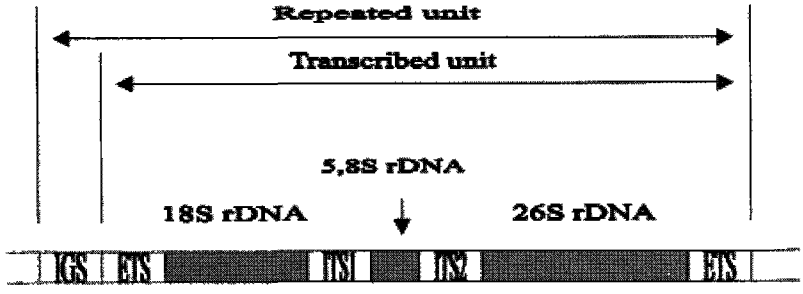

Fig.3. Structure of the ribosomal gene. IGS: Intergenic Spacer; ETS: External Transcribed Spacer; ITS: Internal Transcribed Spacer; rDNA: ribosomal DNA.

Cloning and Characterization of Fruit-Expressed ACC Synthase and ACC Oxidase from Coffee

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The major problem that limits the effectiveness of mechanical harvesting of coffee is the lack of uniform ripening of fruit. Losses of cherry during mechanical harvesting can approach 30% as compared to hand harvesting methods that select only the ripe cherry. This problem is exasperated by the lack of an abscission zone in coffee fruit. The pull force for green coffee and cherry is similar, resulting in a large amount of green coffee being prematurely harvested when mechanical harvesters are utilized. These immature beans are of inferior quality and, if not removed, will decrease the overall quality of the coffee produced.

With increasing costs and scarcity of labor, mechanical harvesting is being increasingly utilized. If coffee fruit ripening could be synchronized both the yield and quality of mechanically harvested coffee would improve. An additional benefit could be the ability to control the ripening process to maximize machinery, labor and other resources.

Certain fruits, designated climacteric fruits, require ethylene for the final stages of ripening. In these fruits the ability to synthesize ethylene is under tight genetic control: the genes responsible for ethylene production remain turned off until the fruit has reached a certain stage of development. These genes are then switched on and a rapid burst of ethylene is produced that initiates the final stages of ripening. It has been shown in tomato (Oller et al. 1991; Gray et al. 1992) and a few other climacteric fruits that if ethylene synthesis is inhibited, the fruits remain at the mature green stage and do not undergo the final stage of ripening. Application of exogenous ethylene will then initiate the completion of the ripening process.

Previous to this research it was not known if coffee was a climacteric fruit. Due to the small size of the fruit and the lack of uniform ripening it was not possible to measure ethylene evolution from ripening coffee fruit. However, application of exogenous ethylene does hasten the ripening of immature fruit making it probable that coffee is a climacteric fruit (Mitchell 1988).

Ethylene is synthesized in a two-step reaction from *s*-adenosylmethionine (Yang and Hoffman 1984). *S*-adenosylmethionine is converted into 1-amino-cyclopropane-1-carboxylic acid (ACC) by ACC synthase. ACC is then broken down to give ethylene by ACC oxidase (Fig. 1).

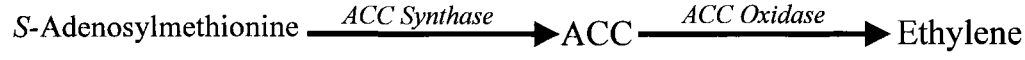

Figure 1. Biosynthetic Pathway for Ethylene.

In climacteric fruits the amount of both ACC synthase and ACC oxidase rise dramatically just before the burst of ethylene synthesis. The mRNAs for these enzymes also increase indicating that the burst of ethylene biosynthesis is the result of gene activation.

We have isolated ACC synthase and ACC oxidase cDNAs from ripening coffee fruit and found that their mRNA levels increase dramatically during the ripening process indicating that coffee is indeed a climacteric fruit.

Methods

Fruits of *Coffea arabica* var. Typica were harvested at various stages of development from the University of Hawai'i' Waimanalo Research Station and from the Hawaii Agricultural Research Station at Kunea. For Southern blotting DNA was extracted from young leaf tissue of *C. arabica* var. Typica or *C. canaphora* from the Hawaii Agricultural Research Station at Kunea.

RNA was isolated from coffee fruit using the method of Levi et al. (1992). Messenger RNA was isolated using the PolyATract® mRNA Isolation System IV (Promega Corp.). The cDNA library was constructed using a ZAP-cDNA Synthesis Kit (Stratagene).

Segments of fruit-expressed ACC synthase and ACC oxidase mRNAs were amplified by reverse transcriptase polymerase chain reaction (RT-PCR) using degenerate primers to highly conserved regions of known ACC synthase and ACC oxidase genes as previously described (Neupane et al., 1997). The RT-PCR products were radiolabeled using the Prime-a-Gene Kit (Promega Corporation) and used to screen the cDNA library. cDNAs containing at least the entire coding region of both ACC synthase and ACC oxidase were obtained.

Northern blotting was done using 25 µg of total RNA isolated from fruit at various stages of development. The RNA was fractionated in 1% agarose-formaldehyde gels, transferred to nylon membranes (MSI Micron Separation) and hybridized as described by Fourney et al. (1988). The probes were produced by PCR using primers complementary to the ends of the ACC synthase and ACC oxidase sequences and labeled as described above.

DNA was isolated using the method of Doyle and Doyle (1990). Five µg of coffee DNA was digested with either *Acc*I, *Eco*RI or *Hin*dIII using conditions recommended by the manufacturer (Promega Corporation). The digested DNA was separated on 0.8% agarose gels and transferred to HybondN$^+$ membranes (Amersham International) by capillary transfer in 0.4 M NaOH as recommended by the manufacturer. Prehybridization and hybridization were done in 5 X SSPE, 5 X Denhardt's solution, 0.5% SDS and 100 µg/ml denatured herring sperm DNA at 65°. ACC synthase and ACC oxidase hybridization probes were produced by PCR as described above.

Results and Discussion

Several clones were obtained from a cDNA library constructed using mRNA isolated from fruit at various stages of development surrounding the mature green stage. This library was screened with ACC synthase and ACC oxidase-specific probes generated by PCR as described above. An ACC synthase cDNA of 2040 bases was isolated that had an open reading frame of 488 amino acids. The coffee fruit-expressed ACC synthase has from 58% to 68% identity to other ACC synthase genes. The deduced amino acid sequence from this cDNA is from 51% to 68% identical to other ACC synthase proteins. In addition, regions highly conserved in other ACC synthases are present in this cDNA.

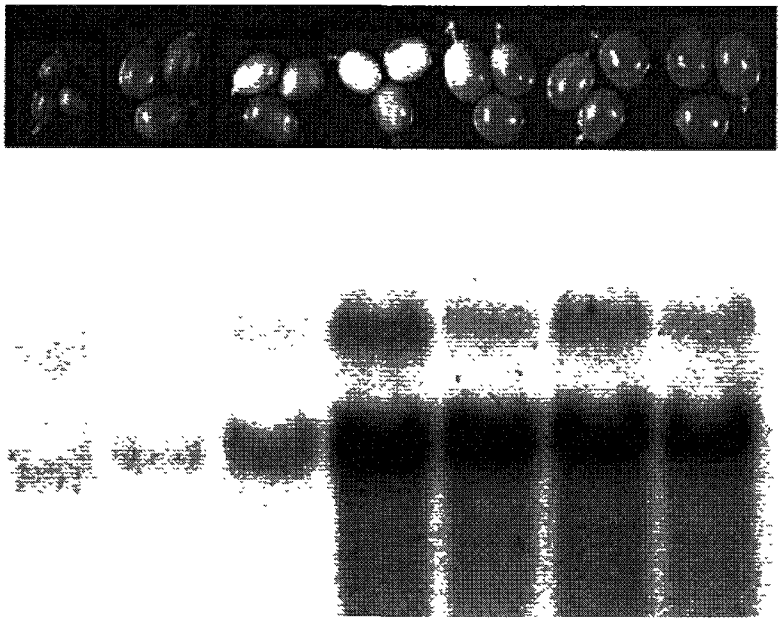

Figure 2. Northern Analysis of ACC synthase and ACC oxidase during fruit ripening. Fruit stages (from left to right) are immature green, mature green, color break, 25% red, 50% red, 75% red, and 100% red.

An ACC oxidase cDNA of 1320 bases was isolated and contains a 318 amino acid open reading frame. This clone shows from 50% to 83% identity to other ACC oxidase genes and the deduced ACC oxidase sequence is from 33% to 87% identical to other ACC oxidases. Again, certain highly conserved ACC oxidase consensus sequences are present in the coffee ACC oxidase sequence.

The pattern of gene expression was determined by Northern blotting. RNA from various stages of fruit development from immature green to fully ripe were simultaneously probed using the cloned ACC synthase and ACC oxidase cDNA sequences. As shown in figure 2, the message levels of both ACC synthase and ACC oxidase rise dramatically at about color break. The level of ACC oxidase is considerable above that of ACC synthase. This has also been found in most other climacteric fruits. The pattern of ACC synthase and ACC oxidase expression that we have observed is consistent with coffee being a climacteric fruit.

The coffee fruit-expressed ACC synthase gene appears to be a low copy gene and is highly conserved between *C. arabica* and *C. canaphora* (robusta) (Fig. 3A). The restriction pattern was identical between *C. arabica* and *C. canaphora* for all three restrictions used, *Acc*I, *Eco*RI and *Hin*dIII. The multiple bands seen in the *Eco*RI digest result from the presence of an *Eco*RI site in the coding region as determined from the sequence of the ACC synthase cDNA.

The coffee fruit-expressed ACC oxidase genes are a small gene family. This is most evident in the *Hin*dIII digest (Fig. 3B) where multiple high molecular weight bands are observed. Again, the restriction pattern for *C. arabica* and *C. canaphora* is identical suggestion that these genes have also been highly conserved.

If coffee is a climacteric fruit, it may be possible to synchronize ripening by inhibiting ethylene production during fruit maturation. This should arrest fruit development at the mature

green stage. Less developed fruit would continue to develop so that most or all of the fruit would reach the mature green stage and remain there until exogenous ethylene was applied. The

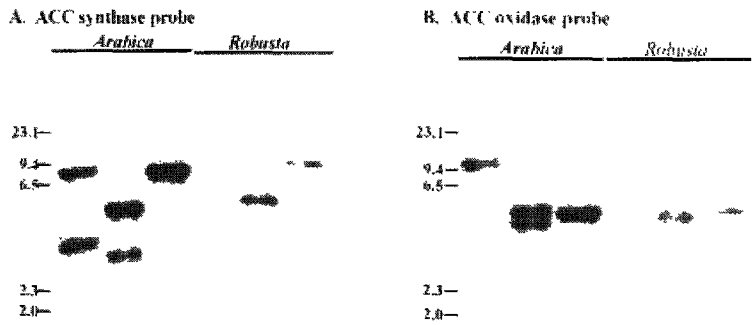

Figure 3. Southern blot analysis of ACC synthase and ACC oxidase fruit-expressed genes in *C. arabica* and *C. canaphora* (robusta). Five µg of *C. arabica* or *C. canaphora* DNA were digested separately with *Acc*I, *Eco*RI or *Hin*dIII, separated on an 0.8% agarose gel and probed with the fruit-expressed ACC synthase cDNA (A) or the fruit-expressed ACC oxidase cDNA (B).

fruit could then be ripened in a synchronous manner by the application of an ethylene-releasing compound such as ethephon. It has been shown in tomato that antisense technology can be used to inhibition of ACC synthase (Oller et al. 1991) and ACC oxidase (Picton et al. 1993) and delay or inhibit ripening. Normal ripening will occur when exogenous ethylene is applied. We are now using this same approach to produce coffee plants that will have controlled ripening. Antisense coffee ACC synthase and ACC oxidase cDNAs have been inserted into pBI121, a standard binary transformation vector used with *Agrobacterium* transformation, in antisense. The CMV 35S promoter is driving the antisense expression. Coffee leaf tissue has been transformed and is now being regenerated and characterized. We anticipate field tests of transgenic plants to commence in 2000.

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CLONING AND CHARACTERIZATION OF XANTHOSINE-N7-METHYLTRANSFERASE, THE FIRST ENZYME OF THE CAFFEINE BIOSYNTHETIC PATHWAY

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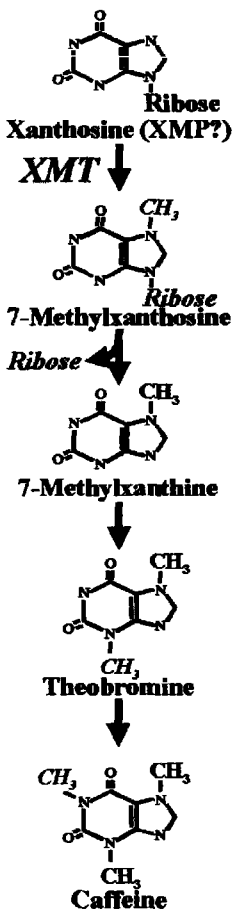

Caffeine is a major pharmacologically active compound in coffee, tea, cola, cacao and a number of other plants that many consumers wish to avoid. Despite the fact that in the United States about one-fourth of the coffee consumed has been decaffeinated, consumers are concerned about the potential health risks of the chemicals used in the decaffeination process and the loss of cup quality. If sufficient information was available on the caffeine biosynthetic pathway and the genes that control the biosynthesis of caffeine, biotechnology could be used to eliminate caffeine from the coffee plant. Coffee plants that grow caffeine-free would eliminate the need for the decaffeination process. We now report the isolation of the gene that encodes the first enzyme of the caffeine biosynthetic pathway, xanthosine-N^7-methyltransferase.

Caffeine, a tri-methyl xanthine, is synthesized by the sequential methylation of the xanthine ring. The initial substrate, xanthosine, is methylated at the N^7 position by xanthosine-N^7-methyltransferase (XMT in fig. 1) with *s*-adenosylmethionine serving as the methyl donor. After cleavage of the ribose from 7-methylxanthosine to give 7-methylxanthine, the xanthine ring is methylated at the N^3 position to give theobromine again with *s*-adenosylmethionine as the methyl donor. A final methylation at the N^1 position gives caffeine. The details of this pathway have been recently reviewed (Crozier et al. 1997) and are shown in figure 1. There is a recent report that XMP can also serve as the initial substrate, however, 7-methylXMP has not been identified in coffee tissue.

We now report the purification and partial amino acid sequence determination of xanthosine-N^7-methyltransferase from coffee leaves and the cloning of a xanthosine-N^7-methyltransferase cDNA.

Figure 1. Caffeine Biosynthetic Pathway

MATERIALS AND METHODS

Plant material

Xanthosine-N^7-mehtyltransferase and RNA for the cDNA library were extracted from young leaf tissue of *Coffea arabica* var. Typica grown at the University of Hawaii Waimanalo Research Station, O'ahu Hawaii.

Purification of Xanthosine-N^7-methyltransferase

Leaf tissue was frozen in liquid nitrogen immediately after harvest in the field. Approximately 150 g of frozen leaf tissue was ground to a fine powder in a coffee grinder. The powdered leaf tissue was extracted with 2 l of ice cold 80% acetone containing 10 mM thiourea and 12.5 mM β-mercaptoethanol. After filtration, the tissue was washed with an additional 2 l of acetone. The extract was air dried and lyophilized for at least 48 hours.

The lyophilized extract was suspended in 400 ml of 0.1 M PIPES (pH 7.10), 0.5 mM Na_2EDTA, 5% (w/v) ascorbic acid, 5 mM phenylmethylsufonly fluoride, 5 mM DTT, 10 mM threonine, 1% PEG (20 kDa) and 15 g polyvinylpolypyrrolidone. After homogenization for 10 min in a blender, the debris were removed by centrifugation at 23,000 X *g* for 30 min at 0°. The supernatant was brought to 40% $(NH_4)SO_4$ and applied to a 40 ml Macro-Prep methyl hydrophobic interaction chromatography column (BioRad). The column was washed with 1.7 M $(NH_4)SO_4$ containing 20 mM 1,3-bis[tris-(hydroxymethyl)-methylamino]propane (pH 6.8) and 5 mM DTT until a stable base line at OD_{280} was obtained. The xanthosine-N^7-mehtyltransferase activity was eluted with 10 mM tris (pH 7.0) containing 5 mM DTT and 1 mM $MgCl_2$ and applied to a 100 ml Cibacron Blue F3GA column (BioRad). After washing in the same buffer, the enzyme was eluted in 1.5 M NaCl, made 1.7 M by the addition of solid $(NH_4)SO_4$ and loaded onto a XK26/20 phenyl-Sepharose column (Phamacia). The column was equilibrated and washed with 1.7 M $(NH_4)SO_4$, 20 mM 1,3-bis[tris-(hydroxymethyl)methylamino]propane (pH 7.0) and 5 mM DTT. The column was eluted with a linear gradient of the wash buffer versus 10 mM tris containing 5 mM DTT and 1 mM $MgCl_2$. The active fractions were pooled and applied to an ATP-agarose column (Sigma) at 4°. This column was washed with 10 mM tris containing 5 mM DTT and 1 mM $MgCl_2$ and eluted with this buffer containing 100 μM xanthosine. The active fractions were pooled and concentrated using a Centriprep-10 micro-concentrator (Amicon), diluted to 15 ml with 25 mM bis[2-hydroxyethyl]iminotris[hydroxymethyl]methane (pH6.0) and 9% betaine. The sample was loaded onto a Mono-P FPLC column and eluted with PolyBuffer 74 (Pharmacia) diluted 10-fold with 9% betaine.

Gel electrophoresis

One-dimensional polyacrylamide gel electrophoresis was carried out using 12.5% polyacrylamide, 0.8% bis-acrylamide SDS gels as described by Laemmli (1970). Two-dimensional gel electrophoresis was as described by Moisyadi and Harrington (1989).

Assay of methyltransferase

The standard assay consists of 100 μl of 50 mM tricine (pH 7.0), 1.2 mM xanthosine, 5 mM DTT, 7.5 μM *s*-adenosyl-L-[methyl-^{14}C]-methionine (60 Ci/mole: DuPont NEN), 1 mM EDTA and 50 μl of enzyme sample. The reaction was for 1 hr at 25 °. The reaction was terminated by removing three 30 μl aliquots and adding 8 μl of 0.6 M $HclO_4$ to each. After centrifugation to remove insoluble material, 19 μl of each was mixed with 1 μl of 33 mM 7-methylxanthosine and chromatographed on Whatman No. 1

paper developed with *n*-butanol:acetic acid:H_2O (4:1:1, v/v/v). The region containing the 7-methylxanthosine was identified by its florescence under UV light, excised and counted in a liquid scintillation counter. The identity of 7-methylxanthosine was confirmed by mass spectroscopy after acid hydrolysis.

Cloning of xanthosine-N^7-methyltransferase cDNA

The xanthosine-N^7-methyltransferase purified as above was subjected to polyacrylamide gel electrophoresis and the region containing the xanthosine-N^7-methyltransferase was excised after staining with Coomassie Blue. The xanthosine-N^7-methyltransferase was digested in the gel with Lys-C overnight, recovered and the peptide fragments fractionated by HPLC. Three of the digestion fragments were subjected to automated Edman degradation and yielded two unique sequences (two fragments were overlapping). Degenerate oligonucleotides were synthesized to each unique peptide sequence using regions of least degeneracy. These oligonucleotides were used as primers to amplify a segment of the xanthosine-N^7-methyltransferase mRNA using reverse transcriptase polymerase chain reaction. A 750 base-pair product was produced. After gel purification, this PCR product was labeled by random priming and used to screen a coffee leaf cDNA library by plaque hybridization. The cDNA library was constructed using mRNA isolated from young leaf tissue using the methods of Bugos et al. (1995). Poly(A) RNA was isolated using the polyATract II system and methods recommended by the manufacturer (Promega). Four µg of poly(A) RNA was used to construct the cDNA library using the ZAP-cDNA synthesis kit and methods recommended by the manufacturer (Stratagene). Approximately 2 X 10^5 clones were screened by plaque hybridization. The hybridization was carried out for 18 hours at 65° in 6XSSPE containing 0.5% SDS and 100 µg/ml denatured herring sperm DNA.

RESULTS AND DISCUSSION

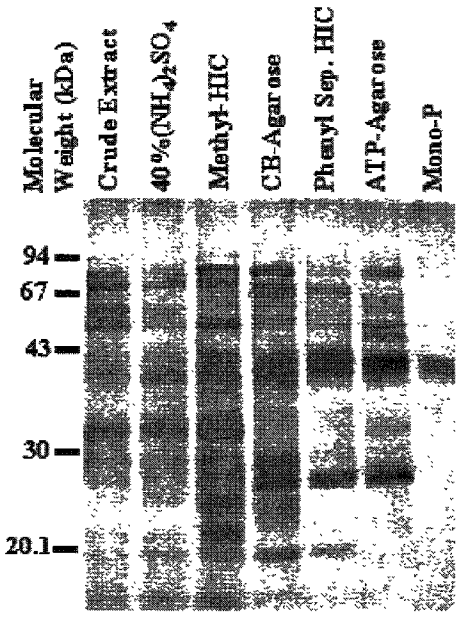

Figure 2. Polyacrylamide Gel of Various Fractions during Purification.

We have purified and characterized xanthosine-N^7-methyltransferase the first enzyme of the caffeine biosynthetic pathway. Figure 2 shows a polyacrylamide gel of the protein extract after various purification steps. Figure 3 shows a two-dimensional gel of the purified xanthosine-N^7-methyltransferase. In the most purified preparations there are four peptides separable by pI and size. The two different pH classes can be enriched by high-resolution Mono-P chromatography and each fraction exhibits xanthosine-N^7-methyltransferase activity. Coffee xanthosine-N^7-methyltransferase has a pH optimum of 7.0 and a temperature optimum of 25°. The K_m's in respect to xanthosine and *s*-adenosylmethionine are 22 µM and 15 µM, respectively (Moisyadi et al. 1998). Eleven cDNA clones complementary to the 750 base-pair PCR product were isolated from the leaf cDNA library. The largest of these contained an open reading frame encoding a 371 amino acid protein of 41 kDa. The predicted amino acid sequence of this open reading frame contains the amino acid sequences obtained from the partial sequence identifying this clone as coding for the isolated xantho-sine-N^7-methyltransferase.

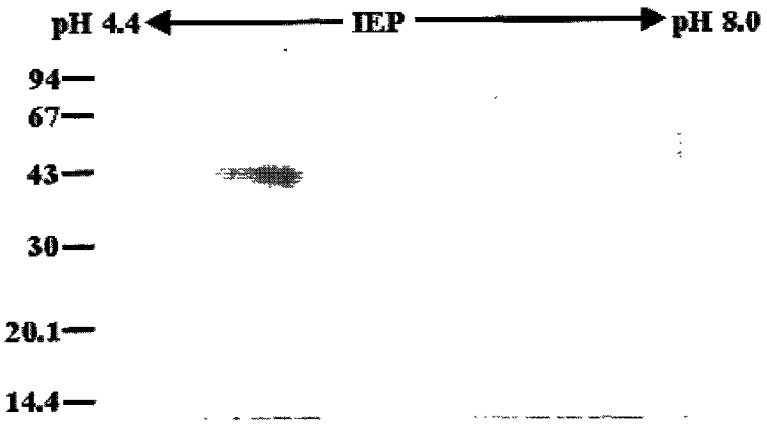

Figure 3. Two-dimensional Gel of Purified Xanthosine-N7 -methyltransferase.

Furthermore, the calculated pI of this protein is 5.8 which is similar the pH at which the protein is eluted from the Mono-P column. The xanthosine-N^7-methyltransferase shows no significant similarity over its entire length to any sequence currently in GeneBank. There are, however, certain motifs common to other methyltransferases.

A number of DNA N-methyl-transferases have been sequenced and some analyzed by site directed mutagenesis. Several consensus sequences have been identified, although most homologies are weak. One of the most conserved consensus sequence is the (S/N/D)PP (Y/F/W) motif is present in the coffee xanthosine-N^7-methyltransferase (Fig. 4).

```
  1  MAFVARQWFL LSILNVVVVC FLKPFALGEQ QVPCYFIFGD SQDDNGNNNH
 51  LNTTARANYP SPYGIDFPEG? TGRFTNGRNH ADFIGELLGF DSYIPPFANT
101  KGRDITKGIN YASGASGILD QTGRHLGDLP SFNEQLHNHE RAISRIVRLI
151  GNRSATKEYL AKCLYTVALG NNDYINNYLL PEYYPTSHLY TPRHFASLLI
201  RHYSQQLRTL YRLGARKIAV FGLGWLGCIP AELSTDGNCV DSINEEVLLF
251  NDKLKPLVDE LNTELSGAQF LYVDVIATNL NNLSTPAEIT TGNAPCCNVS
301  AAVAGGQCIP GQIPCSNRNQ YYFWDDFHPS EVVNFAYSRL AYSALSSLLD
351  ADPLAIGGLT GKNCHDKVKL Q*
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Catalytic binding motif and adenosine binding site in N^6 methyltransferases.

S-adenosylmethionine binding site.

Figure 4. Sequence of Coffee Xanthosine-N^7-methyltransferase.

The (S/N/D) PP(Y/F/W) motif is present in the coffee xanthosine-N^7-methyltransferase at position 60, except that the preceding amino is Y and not S, N or D. Several adenosine-N6-metnyltransferases have the sequence GI immediately after the PPY sequence. This is also found in the coffee xanthosine-N^7-methyltransferase. Roth et al. (1998) identified this motif as the adenine-binding and catalytic site of the *Eco*RV adenine-N^6-methyltransferase by mutational analysis. Perhaps xanthosine, also a purine, required a slightly different active site configuration than adenosine. There is also a sequence with homology to motif II identified as the *s*-adenosylmethionine binding site by Roth et al. (1998). These purine N-methyltransferase structural motifs present in the coffee xanthosine-N^7-methyltransferase are consistent with its function as an N-methyltransferase.

The coffee xanthosine-N^7-methyltransferase cDNA was inserted into a modified binary plasmid pBI121. The GUS gene of the normal pBI121 was removed and replaced with the xanthosine-N^7-methyltransferase cDNA in antisense orientation. This plasmid was transformed into *Agrobacterium* and coffee leaves were infected. After selection on either kanamycin or G418, plants were regenerated using the somatic embryo regeneration system described by Sondahl and Sharpe (1997). Plants have been regenerated and are now being characterized for transgene structure and suppression of caffeine synthesis.

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GENETICALLY MODIFIED COFFEE TREES FOR RESISTANCE TO COFFEE LEAF MINER. ANALYSIS OF GENE EXPRESSION, RESISTANCE TO INSECTS AND AGRONOMIC VALUE

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Abstract

The *cry*1Ac gene of *Bacillus thuringiensis* has been successfully used for transformation of coffee species (*Coffea canephora* and *Coffea arabica*) to confer resistance to the coffee leaf miner (*Perileucoptera coffeella* and other *Leucoptera* spp.). Somatic embryos and embryogenic cells were co-cultivated with the LBA4404 strain of *Agrobacterium tumefaciens* containing the *cry*1Ac gene, a reporter gene and a gene conferring resistance to an herbicide used as a selective agent. More than 100 transformed plants from independent transformation events have been obtained for each coffee genotype. Correct integration of the genes was analysed. The expression of the *cry*1Ac gene was studied by western blotting analyses, the insecticide protein was detected in most of transgenic trees. Some plants have demonstrated substantial resistance to leaf miner in bioassays with the insects. Agronomic conformity of the transgenic plants will be evaluated in field plantings in French Guyana, and a suitable insect management will be implemented. These plants could represent a good opportunity to observe a gene expression in a perennial crop and the management of resistance against an endocarpic insect with a protein from *B.thuringiensis*.

Résumé

Le gène de *Bacillus thuringiensis cry*1Ac a été introduit par transformation génétique dans des génotypes des deux espèces de caféier (*Coffea canephora* et *Coffea arabica*), afin de leur conférer la résistance à la mineuse des feuilles du caféier (*Perileucoptera coffeella* et autres *Leucoptera* spp.). Des embryons somatiques et des cellules embryogènes ont été cocultivés avec la souche LBA 4404 de *Agrobacterium tumefaciens* contenant le gène *cry*1Ac, un gène rapporteur et un gène de résistance à un herbicide, utilisé comme agent sélectif. Plus de 100 plantes transformées issues d'événements indépendants ont été obtenus pour deux des quatre génotypes utilisés, une trentaine pour les autres. L'intégration correcte des gènes a été analysée. L'expression du gène *cry*1Ac a été étudiée par western blotting, la protéine insecticide a été détectée dans la plupart des plantes transformées. Certaines plantes s'avèrent très résistantes aux attaques des insectes dans les bio-essais. L'essai au champ qui sera implanté en 2000 en Guyane française permettra d'évaluer la conformité agronomique des plantes transformées et leur résistance aux insectes en conditions naturelles. Ces plantes représentent une bonne opportunité de suivre l'expression des gènes *Bacillus thuringiensis* et la gestion de la résistance contre un insecte de type mineur pour une plante pérenne tropicale.

INTRODUCTION

Coffee is an extremely important agricultural crop with more than 6.5 million tons of green beans produced every year on about 11 million hectares. In terms of economic importance on the international markets, it is second only to oil and contributes more than 9,000 M US $. One of the major pests threatening this production is the leaf miner *Perileucoptera* spp., which has an economically important impact in East Africa and Brazil

(Guerreiro et al. 1990). This insect pest is characterised by a strict endocarpic larval stage, which makes useless sprayable formulations of chemical or biological pesticides (Crowe 1964). Systemic chemical pesticides are usually harmful and implementing an environmentally friendly way of controlling this pest would be a preferable option. Since insecticidal proteins do not work, like chemicals, as contact or external poisons but instead as internal poisons, the insecticidal protein must be eaten by the pest and the only strategy available would therefore be the transgenic approach. The use of *Bacillus thuringiensis* genes to transform plants for protection to insect is currently the most reliable strategy (Estruch et al. 1997; Schuler et al. 1998) and previous investigations were therefore conducted to determine the susceptibility of *Perileucoptera* sp. to *B. thuringiensis* insecticidal proteins and identify the candidate genes for transformation of coffee (Guerreiro et al. 1998). To date, six different endotoxins of *B. thuringiensis* have been tested against coffee leaf miner (Guerreiro et al. 1998), and the toxin expressed by the *cry*1Ac gene, widely used to confer resistance to lepidopterae (Dandekar et al. 1998), has been demonstrated to be the most effective.

In addition to pest control, conventional breeding of woody species is a low efficiency and time consuming process due to their long life cycles. Genetic engineering could alleviate these problems by incorporating known genes into elite genetic backgrounds.

The first genetic transformation of coffee cells reported (Barton et al. 1991) was by protoplast electroporation. Genetic transformation with *Agrobacterium sp.* has also been reported (Feng et al. 1992; Freire et al. 1994). Regeneration of transgenic coffee trees was first obtained by transformation of somatic embryos *via Agrobacterium rhizogenes* (Spiral and Pétiard 1993; Spiral et al. 1993; Sugiyama et al. 1995), and more recently *via Agrobacterium tumefaciens* (Leroy et al. 1997). Although somatic embryogenesis is still a tedious process for coffee species (Yasuda et al. 1985; Berthouly and Michaux-Ferrière 1996) regeneration is easy obtained. Thus somatic embryos are of great interest as primary explant for genetic transformation (Fisk and Dandekar 1993).

In this article we report the first transformation of coffee using *Agrobacterium tumefaciens* for expression of an agronomic trait. In the present work, a synthetic *cry*1Ac gene has been introduced into four coffee genotypes from the two cultivated species *C. arabica* and *C. canephora*. The data reveals the correct integration and expression of the *cry*1Ac gene within the coffee genome. Furthermore there was a good correlation between *cry*1Ac gene expression and insect bioassays performed on transformed plants.

MATERIALS AND METHODS

Plant material

Four genotypes were used for these experiments, two from *Coffea canephora* and two from *Coffea arabica*. The *C. canephora* genotypes (126 and 197) were selected clones of good agronomic value, a Catimor (8661-4) and an F_1 hybrid (Et29 X Ca5) were used as *C. arabica* genotypes.

Transformation vector

The *Agrobacterium tumefaciens* disarmed strain LBA4404 was used for transformation, with the pBin19 plasmid. Three genes were introduced: (i) the *uidA* bacterial gene isolated from *E. coli* coding for β glucuronidase, with an additional intron for specific expression in plants. The gene is controlled by the cauliflower mosaic virus (CaMV) promoter 35S, and the 35S terminator, (ii) the *csr1-1* gene isolated from *Arabidopsis thaliana* (Brasileiro et al. 1992) conferring resistance to the herbicide chlorsulfuron. This herbicide was used for selection of transformed cells. It is also controlled by the CaMV promoter 35S with a duplicated enhancer sequence, and by the *csr1-1* terminator; (iii) the *cry*1Ac gene from *B. thuringiensis* in a modified form synthesised at the University of Ottawa (Sardana et al. 1996). After preliminary studies on coffee cells demonstrating its efficiency in transient expression (Van Boxtel et al. 1995), the EF1α promoter from *Arabidopsis thaliana* (Curie et al. 1991) was chosen, together with a promoter enhancer sequence Ω' derived from tobacco mosaic virus and the nopaline synthase terminator.

Transformation protocol
Leaf explants were cultured on semi-solid medium, with 5 µM benzylaminopurine (BAP) and 0.09 M sucrose for *C. canephora* and Catimor genotypes, and successively on the two media defined for the F_1 hybrid genotype (Berthouly and Michaux-Ferrière 1996). Afterwards, they were subcultured every five weeks for three to five months until somatic embryos or embryogenic callus appeared at the edge of the explants. Somatic embryos were harvested at the torpedo stage, and then wounded with a scalpel; embryogenic cells were harvested without wounding. They were soaked in a 0.9% NaCl solution for two hours with bacteria at a DO_{600nm} of 0.3-0.5. The explants were then co-cultivated in the dark on semi-solid MS medium (Murashige and Skoog 1962) without hormones for three days. They were then rinsed in liquid MS medium supplemented with cefotaxim (1 g/l) for three to five hours. Afterwards, they were cultivated on semi-solid medium with 5 µM BAP, 0.09 M sucrose, supplemented with cefotaxim (400 mg/l) under low light conditions (16 hours per day).

After a period of 21 to 28 days, they were transferred to a selective MS medium supplemented with 400 mg/l of cefotaxim and 80 µg/l of chlorsulfuron. Due to a decrease of herbicide concentration with time, the embryos or cells were transferred to new selective medium every four weeks. After regeneration from calli, the transformed embryos were sub-cultured on the germination semi-solid MS medium with Morel vitamins, 1 µM BAP and 0.03 M sucrose. The rooting medium was identical but without BAP.

GUS histochemical assay
Calli, shoots, leaves or roots were incubated overnight at 37°C in the classical medium modified by the use of phosphate buffer (0.2 M) pH7. Methanol (20% v/v) was used to eliminate any eventual endogenous expression of the non integrated GUS gene.

Molecular analyses
Plants that presented a positive reaction to the GUS histochemical test were used for total DNA extraction. Extractions were made from plantlets cultivated *in vitro* and also from greenhouses cultivated plants.

Seven pairs of primers were chosen in order to analyse the quality of the DNA and the structure of the integrated T-DNA by PCR amplification. These primers defined various sequence domains of the plasmid. Another pair of primers was used to test the presence of residual bacteria by amplification of *virD2* gene from the virulence plasmid. Amplifications were performed on 25 ng of genomic DNA

Total DNA (5 µg) was digested with *Ssp*I, *Bgl*II and *Sca*I (10U/µg) for Southern blotting analyses. Standard methodology was used to separate DNA on a 0.7% agarose gel in TRIS-borate-EDTA buffer, then transfer it to a Hybond N+ membrane (Appligene) and hybridise with α^{32}P-labelled DNA probes.

Western blot hybridisation and bioassays
Proteins were extracted from about one gram of fresh leaves, and the detection of the insecticidal protein was performed by western blotting (Rogers et al. 1991) using a rabbit polyclonal antiserum raised against the Cry1Ac protein previously purified in our laboratories (dilution of 1/1200 v/v). A secondary goat anti-rabbit antiserum alkaline phosphatase conjugate (Sigma) was then used for final detection, at a dilution of 1/1000 (v/v).

Bioassays were performed using two leaf miner species from Tanzania (*Leucoptera caffeina* and *L. meyricki*). Plants to be tested were put in cages for 24 hours to let the adult insects lay eggs on the leaves. Fifteen days later, an overall score is attributed to the plants, according the following scale: 0, death of larvae after hatching, no galleries observed; 1, galleries shorter than 3 cm, death of the larvae usually before one week; 2, galleries larger than 3 cm before the death of the larvae; 3, living larvae on the leaves, possible development of pupae; 4, complete development of the larvae with formation of pupae.

RESULTS AND DISCUSSION

Transformation and regeneration

Approximately 20 experiments were completed involving between 1,000 and 10,000 somatic embryos or 10 to 50 grams of embryogenic cells each. After three to six months on a selective medium with 80 µg/l of chlorsulfuron, calli or embryos appeared on the edge of the necrotic explants. Such calli occurred on approximately 1% of explants. For the Catimor genotype, small groups of embryos appeared on 0 to 5% of the primary explants, with considerable variability. On the F_1 *C. arabica* genotype, less than 0.1% of the explants produced calli. The transformation process has been improved with further experimentation. Nevertheless, it remains difficult to obtain transformed calli or embryos for each experiment; most of the results were obtained from two transformation assays for the *C. canephora* and Catimor genotypes.

For the *C. canephora* and the F_1 *C. arabica* genotypes, isolated calli were then cultivated on the same selective medium. After a few weeks of culture somatic embryos were observed. The rate of calli giving rise to embryos is lower than 30% for *C. canephora*, and lower than 10% for the F_1 *C. arabica* genotype. When the embryos were sub-cultured on the germination medium (three to six weeks) more than 80% of them germinated correctly. Plantlets were then sub-cultured on the rooting medium for one to three months before transfer to the greenhouse. For the Catimor genotype, groups of between one and ten transformed embryos grew directly on primary explants. More than 50% of these embryos were converted into plantlets.

The histochemical GUS assay was performed on calli and plantlets. A transformed stained callus is shown in Figure 2B. Approximately 50 % of calli or embryos on selective medium exhibited a blue staining indicating *uidA* gene expression. However, a high rate of calli or embryos recovered from selective medium failed to show positive GUS activity. Increasing the chlorsulfuron concentration did not alleviate this problem and drastically decreased the rate of transformation events. This high error rate leads us to use GUS test as secondary screening of transformed calli or plantlets.

According to the protocol described, more than 300 transformation events were obtained with Catimor, more than 200 with *C. canephora* 126 and only 30 with the F_1 *C. arabica* and *C. canephora* 197 genotypes. Between 20 and 60 plantlets were generated from each of 120 out of the 200 calli obtained from the *C. canephora* genotype 126. For the Catimor genotype, one to ten plantlets were obtained from each group of embryos from 100 transformation events.

Molecular characterisation of transformed plants

Six PCR amplifications were performed on each of 51 plants from independent events obtained with *C. canephora*. The two PCR amplifications with primers CHLOR and BtSynt designed to amplify the T-DNA genes *csr1-1* and *cry*1Ac, were positive except for two plants. Nevertheless, a correct hybridisation was observed by Southern blotting for these two plants. This result could be explained by internal recombinations inside the T-DNA. The control plasmid and also untransformed plants behaved as expected for all amplifications.

Southern blotting was used to evaluate the number of T-DNA copies integrated into the plant genome. Among the 51 events studied, 35 plants (69%) present one T-DNA copy, eight (16%) two copies, two (4%) three copies, but one (2%) plant has also shown four copies and one (2%) five copies. These results are consistent with observations made in various dicotyledonous plants including tobacco, petunia, tomato and sunflower (Zambryski 1988).

Protein expression and bioassays

Expression of the *cry*1Ac gene was tested by western analyses on proteins extracted from 23 transformed plantlets. In 18 of them, the polyclonal antibody (Table 1) detected protein. An expected signal of 66 kD

molecular weight was observed with purified Cry1Ac protein and with leaf extract from transformed plants. However in the other five transformed plants, although the *cry*1Ac gene was detected by PCR and Southern blotting, the protein was not detected by western analyses. This may be due either to a level of protein below the detection threshold of the antibody, which is about 0.1% of total protein in this assay, or to a lack of expression of the protein. It could be also explained by a recombination event within the T-DNA, which has altered the structure of the *cry*1Ac gene. Bioassays were performed on these 23 plants. The resistance/susceptibility of the plants was estimated with overall score according to the symptoms observed two weeks after the release of adult insects, and by the number of pupae counted one week later (Table 1). The control plants, as expected, have shown a high susceptibility, with overall score higher than 3.5 and more than 20 pupae produced for most of them. When plants are ranked according to their overall score in bioassays (Table 1), two main observations can be done. Firstly, in all plants with a score below or equal to 2 (14 events), the Cry1Ac protein was detected, and the height of plants is diversified, from 5 cm (plant 21) to 28 cm (plant 24). Thus a good correlation does exist between the presence of the insecticidal protein and the resistance, expressed by the lacking of pupae on the plants.

Table 1 Results of western analyses and bioassays on plants from 23 independent transformation events. Overall score and number of pupae are an average value from 1 to 3 plants observed for each event

Plant	Presence of Cry1Ac protein[a]	Overall score in bioassays	Number of pupae Per plant	Height of plant (cm)	Plant	Presence of Cry1Ac protein[a]	Overall score in bioassays	Number of pupae Per plant	Height of plant (cm)
45	+	0.0	0	12	**31**	+	1.7	0	25
13	+	0.2	0	18	**75**	+	2.0	0	16
21	+	0.5	0	5	**59**	-	2.1	2	25
63	+	0.5	0	10	**23**	+	2.5	2	22
18	+	0.5	0	10	**7**	+	2.5	20	25
65	+	0.6	0	8	**9**	-	2.5	3	25
62	+	0.9	0	10	**46**	+	3.0	23	24
6	+	1.0	0	10	**22**	-	3.0	1	20
44	+	1.0	0	12	**76**	-	3.1	20	30
16	+	1.0	0	27	**8**	+	3.4	24	26
24	+	1.0	0	28	**10**	-	3.8	13	29
48	+	1.5	0	10					

[a] + : protein detected -: protein not detected.

Secondly, for the other transformation events, whether the Cry1Ac protein was detected or not, the score of the bioassays is not correlated with the level of resistance expressed by the average number of pupae per plant after 15 days. Three plants with a score of 2.1 (plant 59), 2.5 (plant 9) and 3.0 (plant 22) and where the Bt protein was not detected have a low number of pupae (1 to 3). On the contrary, three plants where the protein was detected are highly susceptible with 20 to 24 pupae per plant, and scores 2.5 (plant 7), 3.0 (plant 46) and 3.4 (plant 8). We can hypothesis that below a given threshold, for the presence of the Cry1Ac protein, the plant is more or less susceptible, and that this threshold is at the limit of the immunological detection. However, it has been noticed that all plants of this group are higher than 20 cm.

Moreover, bioassays were performed on 26 other transformed plants from *C. canephora* 126. Global results on bioassays are the following for the 49 plants tested:

- 20 were highly resistant without development of larvae and pupae
- 20 were slightly susceptible with development of some larvae without pupae
- 9 were susceptible, because a complete development of the insects was observed.

CONCLUSIONS

In this work, we report for the first time the regeneration of transgenic coffee plants containing a *B. thuringiensis* gene, which has been integrated into the coffee genome. As previously foreseen (Guerreiro et al. 1998), the *cry*1Ac gene conferred resistance to leaf miner as demonstrated by the bioassays. A correlation between the detection of the insecticidal protein in leaf extracts from transformed plants and resistance to leaf

miner was observed in most cases. However, the observations suggested that, in some cases, the level of resistance and development stage could be related. This aspect will be checked carefully when the plants are transferred in 1999 to French Guyana, for a field trial. Suitable insect management will be implemented in parallels with monitoring the expression of the *cry*1Ac gene throughout the five-year experiment.

Up to now, all the results dealing with the evaluation of the efficiency of a B. thuringiensis gene in agricultural condition have been obtained on annual crops. Thus, the observations that we intend to carry out on coffee will be very valuable for the future development of a B. t. strategy on perennial crops. Besides the stability of gene expression, it must be borne in mind that a targeted insect can develop resistance to toxins from *B. thuringiensis* (Rousch 1997; Gould 1998; Tabashnik et al. 1998). Therefore, the use of genes with different resistance mechanisms is ideally required to maintain a B. t. strategy. Thus the *cry*1B gene could be used in order to obtain a cumulative effect with *cry*1Ac (Guerreiro et al. 1998), since the gut receptors are different for the two proteins. Furthermore, integrated pest management, including trapping, using parasitoides, nematodes or entomophagous fungi, and adapted agricultural practices (optimising of herbicides and other pesticides uses), also to be considered. This is particularly relevant for a perennial crop like coffee.

Analyses on pollen dissemination will be implemented throughout the five-year experiment, to study how the flow of transgenes might need to be controlled. To analyse this flow, non transgenic coffee trees will be planted around the trial, at different distances (50 to 1000 metres), and their flowers will act as traps for any genetically marked pollen. Presence of transgenes in seeds will be tested by herbicide applications on the growing seeds.

Transformation of coffee for resistance to the coffee leaf miner is a first step in the process of creating insect-resistant transgenic coffee plants. Although economically important, the coffee leaf miner is restricted to Brazil and East Africa and deliverables of this work will benefit only part of the coffee producers. Another obligate endocarpic pest, the coffee berry borer *Hypothenemus hampei*, is present worldwide and is considered as the most devastating and economically important insect pest of coffee. The next step could therefore be the development of coffee plants resistant to the coffee berry borer.

The same approach has been applied to other coffee genotypes, and more than 100 plants from two *C. arabica* accessions, corresponding to independent transformation events, are already available. It is intended that genetically modified plants from both species, *C. canephora* and *C. arabica*, will be soon produced. The results presented in this work open the way to new opportunities to improve the coffee species, not only for other agronomic traits but also for those of technological interest. With regards these perspectives, the recent cloning of a coffee seed specific promoter (Marraccini et al. 1999) is an important contribution for further developments.

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IN VITRO CULTURE OF IMMATURE EMBRYOS OF *Coffea arabica* cv CATIMOR

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Summary

In the present study, different culture media associated with seed cold pre-treatment were experimented in order to optimise the *in vitro* culture of immature embryos of *Coffea arabica* cv Catimor. Coffee seeds were maintained at 4°C in darkness for 9 weeks. Coffee embryos were recovered every week and cultured in different culture media, under light and dark conditions. Seed sterilization was undertaken with mixed sterilization procedures, which had a rate of 100% of success. The results demonstrated that the loss of embryo viability was not related to light conditions but it was a consequence of the seed cold pre-treatment. It was observed a 5% loss of embryos in the first 3 weeks, more or less 50% till 6th week and 75-79% in the end of the experimental period. Embryo development was evaluated by root and leaf formation as well as by hypocotyls length. The best results were observed in culture media with full or half strength MS mineral salts supplemented with Adams vitamins, 1 mg.l^{-1} IAA and 1 mg.l^{-1} Kinetin and 30 g.l^{-1} of sucrose. Leaf formation was better under light, and embryos submitted to the longest period of cold pre-treatment showed a quicker leaf formation. Hypocotyls length was higher under darkness. Nevertheless, the cold pre-treatment induced a more visible reduction of hypocotyls length in dark conditions than in light conditions. Root formation was effective both under light and dark conditions, and was not affected by the seed cold pre-treatment.

Resume

Le présent travail a consisté à optimiser la culture in vitro d'embryons immatures de *Coffea arabica* cv Catimor prélevés sur des semences de café maintenues à 4 °C et à l'obscurité pendant 9 semaines. Les semences ont été au préalable désinfectées en associant différentes techniques, ceci avec un taux de réussite de 100%. Chaque semaine, des embryons ont été prélevées et mis en culture sur différents milieux de culture avec et sans lumière. Les résultats montrent que la perte de viabilité des embryons n'est pas liée à l'éclairement mais au pré-traitement par le froid des semences. On a observé un taux de perte de 5% au cours des 3 premières semaines et ensuite une perte de 50% jusqu'à la 6ème semaine pour atteindre 75-79% à la fin de l'expérimentation. Le développement des embryons a été évalué en observant la formation des racines et des feuilles ainsi que la longueur des hypocotyles. Les meilleurs résultats ont été obtenus sur le milieu de culture contenant des vitamines Adams, 1 mg.l^{-1} de IAA , 1 mg.l^{-1} de kinétine et 30 g.l^{-1} de sucrose. Les concentrations en sels minéraux dans les conditions de l'expérience n'a pas eu d'effets. La formation des feuilles est plus importante dans les cultures exposées à la lumière lorsque les embryons ont été soumis à la plus longue période de froid. La longueur des hypocotyles est plus grande à l'obscurité mais il semble que le pré-traitement au froid affecte leur croissance. La formation des racines n'est pas affectée par les conditons d'éclairement ni par le pré-traitement au froid des semences.

INTRODUCTION

Coffee is one of the most important agricultural commodities in the world and *Coffea arabica* L. and *Coffea canephora* Pierre ex Fröehner are the main commercial species of the genus *Coffea*. Many *in vitro* techniques have been proved successful in *Coffea* spp. *In vitro* culture of coffee embryos allowed essential knowledge in different areas, namely phytopathology, biochemistry and genetics. Seed viability, intra-specific incompatibility, hybrids multiplication, germoplasm conservation, metabolic and nutrient studies and hybrid embryos recuperation are specific problems of coffee that have been reported with positive results.

Temperature of seed preservation could be one important point to optimise seed viability and the conservation of coffee genetic resources. *C. arabica* seeds are known to be cold sensitive. The relationship between desiccation and longevity is low and its life span does not permit a long-term conservation (Couturon, 1980).

Seed viability of 20-22 weeks old immature embryos of *Coffea arabica* L. cv Catimor and the effect of a cold pre-treatment at 4°C, were studied.

MATERIAL AND METHODS

Immature fruits of *Coffea arabica* L. cv Catimor 20 or 22 weeks-old were collected. The fruits were washed in 3% Benlate solution and rinsed with water, were dried and maintained at 4°C in darkness for 9 weeks.

Every week, one sample of 24 fruits was surface sterilised with a 75% ethanol solution for 3 min. and rinsed three times in distilled-sterilised water. Afterwards the seeds were excised from the fruits. The seeds were immersed in a 7% aqueous filtered solution of calcium hypoclorite with 5 drops of Teepol and 0,1% cysteine-HCl for 20 min. in continuous agitation (Rotatory Shaker, 150 rpm). The seeds were rinsed with sterile distilled water and immersed in a 0,1% cysteine-HCl solution for 3 hours before embryo excision.

Embryos with 0,4 to 0,6±0,1 mm were excised and immediately transferred to culture tubes. The basal media were composed of MS or half strenght MS mineral salts (Murashige & Skoog, 1962), the organic constituents of B_5 (Gamborg *et al.*, 1968) medium added with 37 $mg.l^{-1}$ of cysteine-HCl or the organic constituents of Adams (Adams, 1975) medium, 30 $g.l^{-1}$ of sucrose and 0,6% of agar. These basal media were supplemented with 1 $mg.l^{-1}$ IAA and 1 $mg.l^{-1}$ BAP (Raghuramulu *et al.*,1989) or 1 $mg.l^{-1}$ IAA and 1 $mg.l^{-1}$Kinetin. The pH was adjusted to 5,7.

Cultures were cultivated in a 16h/8h light regime or under dark conditions at a thermoperiod of 16h at 28 ± 1°C and 8h at 26 ± 1°C.

The cultures initially kept in darkness were transferred to light conditions after 6-7 weeks.

RESULTS

The conditions of sterilization used gave 100% of efficiency.

The cold pre-treatment induced different responses in embryos viability. Loss of embryos viability was of 12,5-13,5% in the first 5 weeks, increasing more or less 50% in the 6^{th} week. This loss achieved 75-79% when the embryos were submitted to 7 weeks of cold. The light conditions did not interfere with

the losses of the embryos viability (Fig.1). Embryo development was evaluated by root and leaf formation and by the hypocotyls length. The culture media selected (Table 1) showed to be good for embryos development. Under light conditions the culture media A, B and C showed a low oxidation and in the dark culture medium E was more effective (Fig. 2)

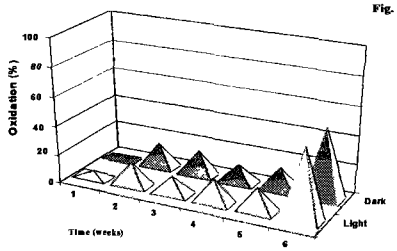
Table 1 : Composition of different culture media

Culture medium	Mineral salts	Organic constituents	Growth regulators
A	MS	Adam	BAP+IAA
B	MS	Adam	Kinetin+IAA
C	MS	B_5+Cysteine-HCl	BAP+IAA
D	MS	B_5+Cysteine-HCl	Kinetin+IAA
E	MS/2	Adam	BAP+IAA
F	MS/2	Adam	Kinetin+IAA
G	MS/2	B_5+Cysteine-HCl	BAP+IAA
H	MS/2	B_5+Cysteine-HCl	Kinetin+IAA

Table 2 : First leaf formation after different weeks of cold pre-treatment under light conditions

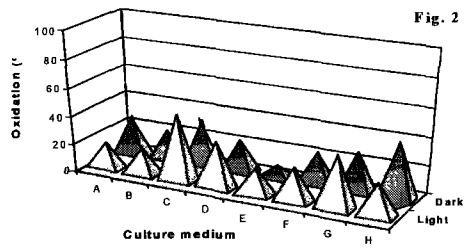
Culture medium	1	2	3	4	5	6
A	10	10	7	7	8	6
B	10	9	8	7	6	7
C	10	11	10	7	6	6
D	10	9	8	9	8	6
E	10	9	8	7	6	6
F	10	9	8	7	6	6
G	11	11	10	9	7	7
H	12	9	8	7	7	7

Fig. 1-



Percentage of oxidation of immature embryos cultivated in dark or in light conditions after different times in cold pre-treatment.

Fig. 2

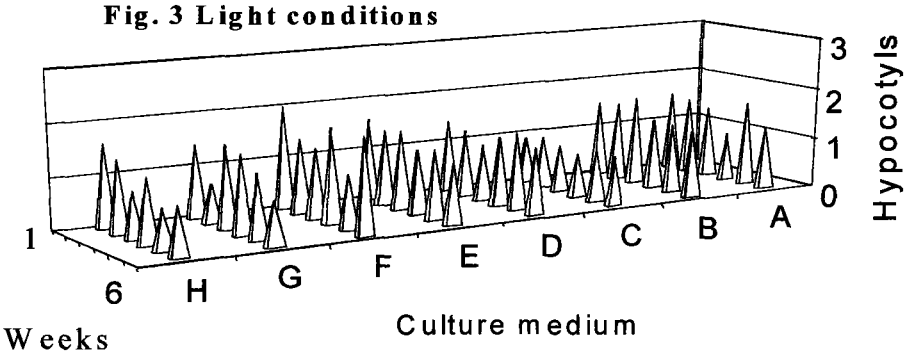


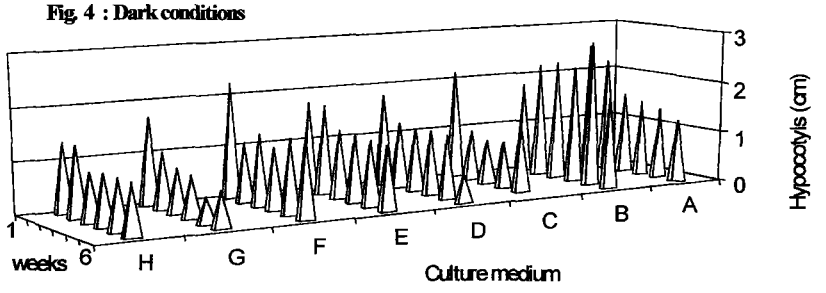
Percentage of oxidation of immature embryos cultivated in light or in dark conditions in different culture media.

Expansion of cotyledonary leaves was observed in all culture media in the first or second week. In three weeks, cotyledonary leaves were green under light. Yellow cotyledonary leaves were observed in the dark after three weeks. In light conditions leaf formation occurred between 6-12 weeks for all the culture media. It was observed that the longest cold pre-treatment period reduced leaf time formation (Table 2), irrespective of the culture medium used. The formation of the 2nd leaf pair occurred 2-3 weeks after the 1st one. The transfer of the cultures grown under dark to light conditions induced the 1st leaf pair formation three weeks later.

Hypocotyls were of green colour under light and pink under dark between the 2nd and the 3rd week. Hypocotyls grew better in A, B and F culture media, under dark conditions, and in B and F culture media, under light conditions (Fig. 3 and Fig. 4).

Hypocotyls were less affected in their development in the dark by the cold pre-treatment than under the light. Nevertheless, hypocotyl length in dark conditions seemed to be reduced with the longer time cold exposition.





The root system began with the production of a tap root. Later, it was observed the formation of 2-5 adventitious roots. The root system developed under dark conditions showed more roots than that formed under the light conditions. Root formation response was better in seeds submitted to cold pre-treatment and grown in B and F culture media under light conditions (Table 3), and in B culture medium when grown under dark conditions (Table 4).

In A and E media, the development of roots was not observed in any conditions.

Table 3 : Root formation in light conditions after the cold pre-treatment

ROOT FORMATION (Light)							
Culture Media	weeks						
	1	2	3	4	5	6	
A							
B	+	+	+	+	+	+	
C				+			
D				+	+	+	
E		+					
F	+	+	+	+			
G			+	+			
H	+	+					

Table 4 : Root formation in dark conditions after the cold pre-treatment

ROOT FORMATION (Dark)						
Culture media	weeks					
	1	2	3	4	5	6
A						
B	+	+	+	+	+	
C						
D	+		+	+		
E		+				
F	+	+	+		+	
G			+	+		
H				+		

CONCLUSIONS

Seed sterilization had a 100% rate of success Embryos viability losses increased after the 7 weeks of cold pre-treatment and did not seem affected by light conditions but rather as a consequence of seed cold pre-treatment. The best culture media for the immature embryos culture was full or half-strength MS mineral salts supplemented with Adams organic constituents, 1 mg.l^{-1} Kinetin, 1 mg.l^{-1} IAA and 30 g.l^{-1} sucrose. The leaf formation took place earlier as a consequence of a longest period of seed cold pre-treatment. The hypocotyls were longer under darkness, and in this conditions a length reduction was observed when the seed cold pre-treatment was longer. The root formation was effective both under light and dark conditions and was not influenced by the cold pre-treatment.

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POLYMORPHIC MICROSATELLITES IN *COFFEA ARABICA*

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INTRODUCTION

DNA polymorphisms are being applied on an increasingly wider scale in the field of biotechnology, as for example in variety characterisation, agronomic traits identification and marker assisted breeding programs. Some DNA polymorphisms have also been found in *Coffea arabica* through different technical approaches such as RFLP (Lashermes et al, 1996a), RAPD (Orozco-Castillo, 1994; Lashermes et al, 1996b) and AFLP (Lashermes et al, 1999). However, it has been reported that the degree of polymorphism of this species is relatively low (Paillard et al., 1993; Paillard et al., 1996). *C.a.* is an autogamous species and this reproductive strategy undoubtedly leads to a high degree of homozygosity. Furthermore, the genetic base of most coffee cultivars is rather narrow (Bertraud J.and Charrier A, 1988) thus reducing the degree of variability.

Microsatellites are highly polymorphic DNA repetitive sequences. They have been found in all animal and vegetal species so far analysed and are therefore expected to be present in *C. arabica* too. Moreover, they have a relatively high mutation rate which should increase the heterozygosity of coffee, thus compensating for the restricted genetic base. We carried out screening and selection of two genomic libraries enriched in Simple Sequence Repeats (SSR) to identify polymorphic microsatellites, in view of constructing a low density genetic map of *Coffea arabica*.

MATERIAL AND METHODS.

Samples.

The genomic libraries were constructed from DNA of a Caturra plant of the IRD collection. The polymorphism were assessed on a F2 population (Caturra x Ethipia ET30, IRD) and on a limited number of cultivars.

DNA extraction.

Particular care was taken in DNA extraction and purification when preparing the genomic libraries. The methods reported by Murray and Thompson (1980) and Orozco-Castillo et al (1994) were modified as reported by Vascotto et al, 1999).

Preparation of the genome libraries.

Two genomic libraries were prepared, one enriched in $(TG)_n$ and the other in $(ATC)_n$. The basic approach adopted for the enrichment in SSRs has already been reported by Rafalski et al. (1996),

Morgante et al. (1998). The relevant selection steps were a first enrichment through magnetic beads conjugated with biotin-strepavidin oligonucleotides complementary to TG and ATC and a second degree selection performed on the clones by colony hybridisation with TG or ATC oligonucleotides. The DNA fragments obtained following the magnetic beads step were cloned into the *EcoRI* site of λ-ZAPII (Stratagene, La Jolla, Calif.).

Sequencing and primer design.

The positive clones were sequenced using a Thermo Sequenase dye terminator cycle pre-mix kit (Amersham Pharmacia Biotec) following the producer's instructions. The reaction products were analysed on an ABI 373A sequencer. The sequences were screened for presence of SSRs and primers were designed for the positive sequences using the on-line programs Primer3 (Whitehead Institute for Biomedical Research, Cambridge, Massachusetts, USA) or *Primers!* (Williamstone Enterprises). A constant tail KS (5'-TCGAGGTCGACGGTATC-3') was added to one of the primers for each primer pair.

Amplification and analysis of the microsatellites.

To avoid using radioactive labels, we developed a three primer system: two primers were locus specific primers while the third primer was complementary to the constant tail of one of the primers and was tagged by either 6-FAM (6-carbossifluoresceine) or JOE (2',7'-dimetossi-4',5'-dicloro-6-carbossifluoresceine). Amplification conditions are reported elsewhere (Vascotto et al, 1999).The amplified fragments were run on sequencing gels in an automatic sequencer ABI 373A and alleles were identified via GENESCAN 672 (Perkin Elmer) software.

RESULTS.

Both genomic libraries contained a large number of clones carrying SSR. Thus the selection procedures proved to have been very effective. Table I reports the numbers of clones analysed in the various successive steps of selection. The clones presently screened constitute approximately 20% of the libraries.

Table I

	N.	%
Clones screened	7.800	
Positive clones	692	9%
Clones sequenced	349	50%
Clones containing SSRs	249	71%
Primer designed	46	18%
Clones containing polymorphic microsatellites	13	25%

Some of the 249 SSR containing clones are still being analysed for primer design, hopefully they will provide more polymorphisms. The 46 primer analysed so far gave the following results: 14 gave no amplification product, 5 were aspecific, 2 gave an unexpected product, 12 were monomorphic and the remaining 13 showed polymorphic bands.

The screening for polymorphism was carried out on a limited number of cultivars and on a F_2 population (12 plants) and accordingly a large number of different alleles was not expected. Nevertheless in our screening population we found from 2 to 5 different alleles. Normally each single plant tested had either one or two alleles, presumably they were either homo or heterozygotes. Only locus E12-3CTG showed 3-4 alleles in the same plant. The result for the locus C2-2CATC can be seen in fig.1 where the parental plant Caturra is heterozygote, plants F_2-1 is apparently homozygote for the 209 bp allele and plant F_2-18 carries two alleles one of which is common to the Caturra progenitor.

DISCUSSION

As clearly shown by the sequencing, the approaches adopted here to generate two genomic libraries enriched in microsatellites were very effective. On the bases of the polymorphisms so far identified we can estimate that the two libraries contain at least 200 polymorphic microsatellites.

The second point of interest is the number of alleles found in each plant. As *C. arabica* is tetraploid and we could expect our primers to recognise the homologous locus on both the two pairs of ancestral homologous chromosomes. This is most probably the case for the locus E12-3CTG, as we

obtained 3- 4 alleles in the same plant and the E12-3CTG primer definitely amplifies multiple loci. The remaining primer pairs are apparently specific for only one of the ancestral genomes.

Finally, we wish to point out that, as shown by the analysis of the F_2 segregating population, microsatellites in *Coffea arabica* behave as in any other species: they are codominant and can be easily traced through a family together with possible useful associated traits.

ACKNOWLEDGEMENTS.

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SUMMARY.

We are currently screening two genomic libraries enriched for the sequences $(TG)_n$ and $(ATC)_n$. The identification of microsatellites is based on the following strategy: a) sequencing of the DNA inserts; b) design of specific primers for those clones containing Simple Sequence Repeats (SSRs); c) primer test on a panel of cultivars and on an F_2 population. Until now we sequenced about 350 clones, 71% contained a microsatellite. Of the 46 primer pairs designed,13 gave polymorphic bands. As far as the allele distribution is concerned, we found loci with 2, 3 or 4 alleles. Each single plant carried only one or two alleles, presumably they were either homozygotes or heterozygotes. One single locus showed a peculiar behaviour: all the samples so far analysed carried either 3 or 4 alleles.

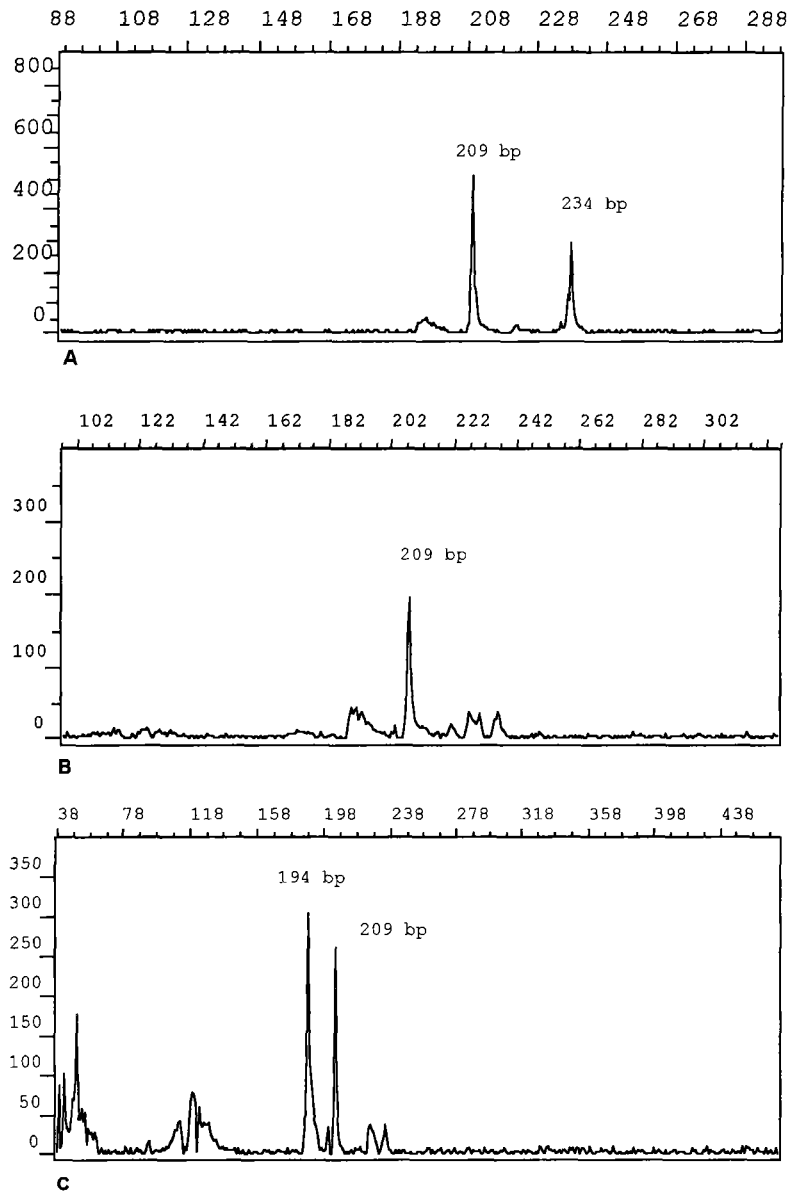

Fig. 1 Electropherograms of the amplification products of locus C2-2CATC: Pane (**A**) Caturra progenitor plant. Two examples of the F_2 segregating population are reported in the panes (**B**) and (**C**). The numbers close to the peaks refer to the allele expressed in bp.

PREMIERE EVALUATION D'HYBRIDES NATURELS ENTRE *Coffea canephora* ET *Coffea arabica* DE NOUVELLE-CALEDONIE

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Résumé

Coffea. arabica est la première espèce de caféier introduite en Nouvelle-Calédonie en 1856. En raison de l'apparition de la rouille orangée à partir de 1908, elle fut cependant progressivement remplacée par l'espèce *Coffea canephora*. Il en résulta des plantations mixtes qui favorisèrent les croisements spontanés entre les deux espèces, phénomène rare mais déjà observé dans d'autres pays comme l'Indonésie (Hybride de Timor). Les conditions climatiques locales ainsi que le désintérêt des producteurs pour la caféiculture furent également propices à l'apparition d'hybrides interspécifiques spontanés en Nouvelle-Calédonie.

Compte tenu de l'intérêt scientifique que peut présenter ce type de matériel végétal, le Cirad commença à le collecter dès 1985 avant de réaliser, en collaboration avec l'Ird (ex-Orstom), une prospection en 1991. Ce programme aboutit en 1993 à la création d'une collection regroupant de nombreux génotypes afin de les évaluer aux plans agronomique et biologique.

Les observations réalisées par le Cirad de 1995 à 1998 ont porté sur 84 génotypes. Les mesures agronomiques ont concerné la croissance, le développement végétatif, l'encombrement et la caractérisation foliaire des hybrides. Les observations biologiques ont porté sur le niveau de ploïdie, la fertilité mâle et la fertilité femelle. Enfin, des observations complémentaires ont été réalisées afin de préciser notamment le potentiel de production et les caractéristiques technologiques des génotypes les plus productifs.

L'étude du cytotype de ces hybrides a permis une première classification de ce matériel végétal. Les résultats obtenus montrent que le niveau de ploïdie influence fortement la plupart des variables étudiées, qu'elles soient agronomiques, biologiques ou technologiques.

Summary

Coffea arabica was the first coffee species to be introduced into New Caledonia in 1856, but with the appearance of leaf rust in 1908, it was gradually replaced by the *Coffea canephora* species. The result was mixed plantations that facilitated wild crosses between the two species, which is a rare phenomenon, though it has already been seen in other countries, such as Indonesia (Timor hybrid). Local climatic conditions, along with a lack of farmer interest in coffee cultivation were also propitious to the appearance of wild interspecific hybrids in New Caledonia.

Given the scientific interest that such planting material might offer, Cirad began collecting it in 1985, prior to undertaking a survey in 1991, in conjunction with Orstom. In 1993, this programme culminated in the creation of a collection containing numerous genotypes, with a view to carrying out agronomic and biological assessments.

The observations carried out by Cirad from 1995 to 1998 concentrated on 84 genotypes. The agronomic measurements covered growth, vegetative development, canopy size and foliar characterization of the

hybrids. The biological observations covered the degree of ploidy, male and female fertility. Lastly, additional observations were undertaken, primarily to determine the yield potential and technological characteristics of the highest-yielding genotypes.

The study of the cytotype of these hybrids enabled an initial classification of the material. The results obtained show that the degree of ploidy strongly affects most of the variables studied, be they agronomic, biological or technological.

Communication

INTRODUCTION

Coffea arabica est la première espèce de caféier introduite en Nouvelle-Calédonie en 1856. En raison de l'apparition de la rouille orangée à partir de 1908, elle fut cependant progressivement remplacée par l'espèce *Coffea canephora* (Saussol, 1967). Il en résulta des plantations mixtes qui favorisèrent les croisements spontanés entre les deux espèces, phénomène rare mais déjà observé dans d'autres pays comme l'Indonésie (Hybride de Timor).

Les conditions climatiques locales caractérisées par une saison sèche et fraîche en particulier sur la côte Ouest, l'humidité permanente dans les vallées de la chaîne centrale, ainsi que le désintérêt des producteurs pour la caféiculture furent également propices à l'apparition d'hybrides interspécifiques spontanés en Nouvelle-Calédonie. La conjonction de ces trois facteurs permit la croissance et la reproduction des hybrides naturels, dont les caractéristiques (résistance à la rouille, grande vigueur mais faible production) n'avaient pas échappé aux producteurs qui les appelèrent «cafés métis».

Compte tenu de l'intérêt scientifique que pouvait présenter ce type de matériel végétal, le Cirad commença à le collecter dès 1985 avant de réaliser, en collaboration avec l'Ird (ex-Orstom), une prospection en 1991. Ce programme aboutit en 1993 à la création d'une collection regroupant de nombreux génotypes, afin de les évaluer aux plans agronomique, biologique et technologique.

MATERIEL ET METHODES

Les observations réalisées par le Cirad de 1995 à 1998 ont porté sur 84 hybrides interspécifiques naturels de caféiers cultivés, récoltés dans d'anciennes plantations abandonnées où *Coffea arabica* se trouve en mélange avec *Coffea canephora* (Charmetant et Le Pierres, 1991). Ils sont issus de plusieurs zones géographiques situées principalement dans la région centrale de la Nouvelle-Calédonie. Multipliés par bouturage, ils ont été plantés en collection début 1993.
Ils sont comparés à 5 témoins : une variété de caféiers Sarchimor (1668), trois variétés de caféiers Catimor (F5, F6 et 1702), et un clone de *Coffea canephora*.

Le dispositif expérimental comprend une ligne de 4 à 6 pieds par génotype. La densité de plantation des caféiers est de 2 500 plants par ha (2 m x 2 m). Ils sont conduits sans ombrage, en croissance libre, à raison de 2 caules par pied.

Variables étudiées

- Niveau de ploïdie : Il a été apprécié par la technique de cytométrie en flux (Ollitrault et Michaux-Ferrière, 1992).
- Variables agronomiques : L'accroissement du diamètre au collet entre 1 et 2 ans et la hauteur des plants à 2 ans ont été mesurés.
- Caractérisation foliaire : La surface et la forme (L/l) des feuilles ont été calculées à partir d'un échantillon de 30 feuilles par génotype.

- Variables biologiques :
  - fertilité femelle : calcul du coefficient de remplissage à partir d'une centaine de cerises immatures par génotype (De Reffye, 1974).
  - fertilité mâle : calcul de la viabilité pollinique à partir du comptage et de la classification d'une trentaine de grains de pollen par génotype, après coloration au bleu d'Alexander (Jahier, 1992).

- Potentiel de production : Une notation individuelle de production a été réalisée pendant 3 années (de 1996 à 1998) afin d'évaluer sur pied la production en cerises des génotypes. Cette notation s'est échelonnée de 0 (aucun fruit sur l'arbre) à 5 (arbre très chargé).

- Résistance à la rouille orangée : L'objectif de ces observations, conduites en collaboration avec l'Ird, était de permettre un premier contrôle de la résistance des hybrides naturels à la race d'*Hemileia vastatrix* présente en Nouvelle-Calédonie (Boccas et *al.*, 1985). Trois boutures plagiotropes par hybride ont été placées en milieu confiné et l'inoculation des feuilles a été réalisée par frottage de leur face inférieure avec des feuilles présentant des taches actives de la rouille orangée, fraîchement récoltées sur des parcelles de caféiers Arabica. Après inoculation, le matériel végétal a été maintenu 4 jours dans l'obscurité totale et dans une humidité saturante afin de favoriser la germination des urédospores. La lecture des réactions a été réalisée six semaines plus tard et a permis de confirmer la résistance (R ' absence de sporulations sur les 3 boutures) ou la sensibilité des génotypes testés (S ' présence de sporulations sur au moins une bouture). Les résultats ont été comparés à ceux des témoins sensibles et résistants.

- Variables chimiques et technologiques : Les teneurs de composés chimiques des grains des génotypes les plus productifs ont été mesurées (caféine, trigonelline, acides chlorogéniques, saccharose). La granulométrie et le poids de 100 fèves ont été également observés.

Analyse statistique

Des analyses en composantes principales ont été réalisées pour visualiser les dépendances entre les variables quantitatives étudiées. Par ailleurs, une discrimination entre les types 3C et 4C a été effectuée globalement avec des analyses discriminantes et par variable (analyse de variance) afin de détecter les caractères qui permettent de mieux différencier ces deux types de caféiers. L'ensemble des analyses a été réalisé avec le logiciel statistique SAS.

Résultats

- **Niveau de ploïdie** : L'analyse du niveau de ploïdie des hybrides naturels de Nouvelle-Calédonie a permis leur classification en deux groupes distincts : les hybrides de type 3C (triploïdes) et les hybrides de type 4C (tétraploïdes) (tableau 1).

- **Variables agronomiques** : Le niveau de ploïdie influence fortement la vigueur et le développement végétatif des hybrides. L'analyse de variance met en évidence une différence hautement significative ($P < 0,0001$) entre les deux groupes d'hybrides pour l'accroissement moyen du diamètre au collet entre 1 et 2 ans et la hauteur moyenne à 2 ans. Les histogrammes de fréquences par classes (figures 1 et 2) illustrent les différences observées entre les 2 groupes d'hybrides.

Tableau 1 : Répartition des génotypes étudiés par origine géographique et par niveau de ploïdie.

Origine	Nombre de Génotypes Observés	Niveau de ploïdie		Résistance à la rouille		
		3C	4C	Résistant	Sensible	Non testé
Canala	4	2	2	2	2	-
Kongouma	3	3	-	2	-	1
Nakety	2	-	2	2	-	-
Coula	1	1	-	1	-	-
Goapin	4	1	3	1	-	3
Farino	14	3	11	11	2	1
Pouembout	1	1	-	1	-	-
Neklaï	1	1	-	-	-	1
Moindou	1	1	-	1	-	-
Ny	9	8	1	9	-	-
Neoua	1	1	-	1	-	-
Oueroupime	6	6	-	6	-	-
Konoe	11	10	1	10	-	1
Dogny	6	5	1	4	2	-
Bourail	1	1	-	1	-	-
Mechin	2	2	-	2	-	-
Pothe	10	2	8	7	-	3
Kokingone	1	1	-	1	-	-
Sarramea	6	6	-	6	-	-
Total	84	55	29	68	6	10

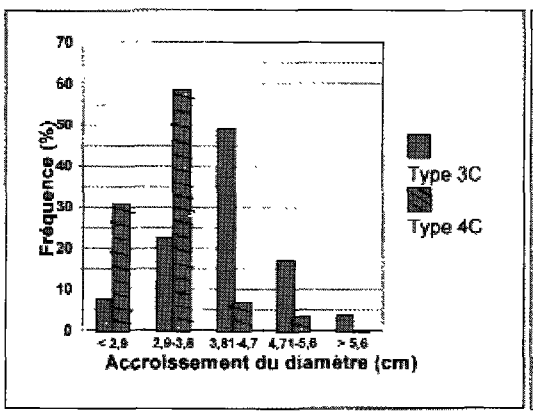

Figure 1 : Fréquence des hybrides par classe d'accroissement au collet

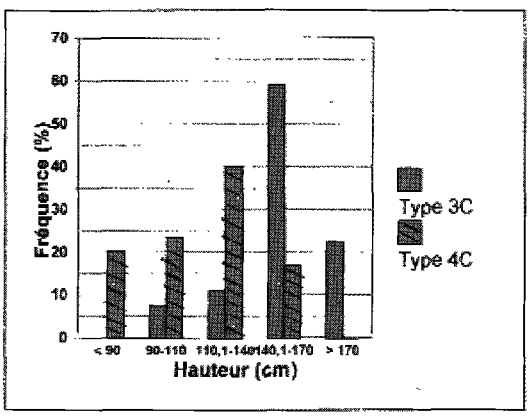

Figure 2 : Fréquence des hybrides par classe de hauteur

Quelle que soit la variable étudiée, le développement végétatif des hybrides de type 3C est supérieur à celui des autres groupes de caféiers. Le développement végétatif des hybrides de type 4C est également supérieur à celui des témoins Sarchimor/Catimor et *Coffea canephora.*

L'analyse en composantes principales (ACP), réalisée les 2 groupes d'hybrides confondus (type 3C et type 4C), montre des corrélations significatives entre ces variables agronomiques.

- **Caractérisation foliaire** : Le niveau de ploïdie influence aussi les caractéristiques foliaires des hybrides naturels. L'analyse de variance met en évidence une différence hautement significative ($P < 0,0001$) entre les 2 groupes d'hybrides pour la forme de leurs feuilles. Les hybrides de type 3C sont ainsi caractérisés par des feuilles plus allongées que celles des hybrides de type 4C

- **Variables biologiques** : Le niveau de ploïdie influence fortement la fertilité mâle et la fertilité femelle des hybrides étudiés. L'analyse de variance met en évidence une différence hautement significative ($P < 0,0001$) entre les 2 groupes d'hybrides pour le coefficient de remplissage et la viabilité pollinique. Les histogrammes de fréquences par classes (figures 3 et 4) illustrent les différences observées entre les deux groupes d'hybrides.

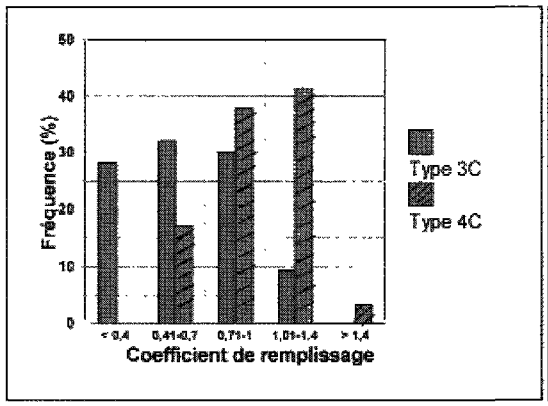

Figure 3 : Fréquence des hybrides par classe de coefficient de remplissage

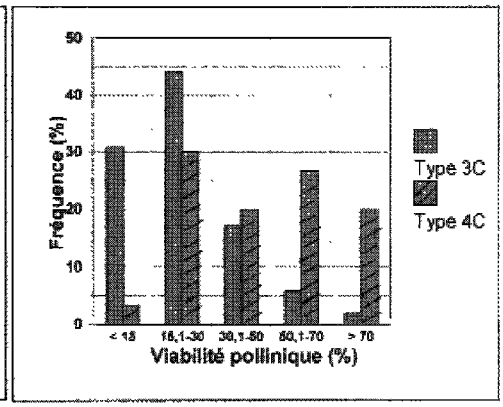

Figure 4 : Fréquence des hybrides par classe de viabilité pollinique

Les hybrides de type 4C présentent une fertilité femelle et une fertilité mâle supérieures à celles des hybrides de type 3Coffea Elles demeurent cependant inférieures à celles des témoins Sarchimor/Catimor et *Coffea canephora*.
L'ACP réalisée les 2 groupes d'hybrides confondus (type 3C et type 4C), montre des corrélations significatives entre ces variables.

- Résistance à la rouille orangée :

Sur l'ensemble du matériel végétal testé, 74 hybrides ont pu faire l'objet d'une notation (tableau 1). La majorité d'entre eux (68) ont confirmé leur résistance à la race *d'Hemileia vastatrix* présente en Nouvelle-Calédonie, dont 22 hybrides de type 4C (Jagoret, 1997).

- Potentiel de production

Le niveau de ploïdie influence également le potentiel de production des hybrides naturels. L'analyse de variance met en évidence une différence hautement significative ($P < 0,0001$) entre les 2 groupes d'hybrides pour la note moyenne de production. Les notes moyennes de production des hybrides de type 4C sont dans l'ensemble supérieures à celles des hybrides de type 3Coffea Elles demeurent cependant inférieures à celle des témoins Sarchimor/Catimor.

- Variables chimiques et technologiques

De nombreux hybrides présentent un taux de caféine inférieur à celui des cafés Arabica, ou relativement proche (Jagoret, 1998). Le niveau de ploïdie influence les teneurs en caféine et en trigonelline. L'analyse de variance met, d'ailleurs, en évidence une différence hautement significative ($P < 0,0001$) entre les 2 groupes d'hybrides pour ces 2 variables.

DISCUSSION-CONCLUSION

L'évaluation conduite par le Cirad de 1993 à 1998 en Nouvelle-Calédonie a permis de préciser, à travers l'étude de plusieurs variables, principalement agronomiques et biologiques, la connaissance des caractéristiques des hybrides interspécifiques naturels collectés dans ce pays, et de montrer leur grande diversité.

Les deux-tiers des génotypes étudiés sont des hybrides de type 3C qui descendent principalement de croisements de première génération entre *Coffea arabica* et *Coffea canephora*, compte tenu de la faible probabilité de rétrocroisements en raison de leur fertilité limitée. Les autres hybrides sont de type 4C issus de croisements directs entre *Coffea arabica* et *Coffea canephora* rendus possible par des températures assez basses permettant une non-réduction des gamètes. Ils peuvent également provenir, soit de rétrocroisements sur *Coffea arabica*, soit d'autofécondations. Dans tous les cas, leur existence confirme que ce type de croisement entre les 2 espèces de caféiers n'est pas rare lorsque les conditions de milieu leur sont favorables.

La classification de ce matériel végétal en deux groupes distincts (hybrides de type 3C et hybrides de type 4C) demeure cependant arbitraire compte tenu des limites de la technique utilisée . La cytométrie en flux ne permet en effet que le comptage de cellules présentant une intensité de fluorescence donnée, la quantité d'ADN contenue dans les cellules étant proportionnelle à cette dernière. Les résultats obtenus doivent donc être confirmés par des mesures de comptage de chromosomes afin d'éliminer les biais dus aux différences de taille des chromosomes de *Coffea arabica* et *Coffea canephora*, et de mieux identifier les hybrides aneuploïdes (triploïdes ou tétraploïdes avec plus ou moins quelques chromosomes).

L'étude réalisée met également en évidence l'influence prépondérante du niveau de ploïdie sur la fertilité mâle et la fertilité femelle des hybrides naturels. Les hybrides de type 4C s'avèrent en effet significativement plus fertiles et plus productifs que les hybrides de type 3C, malgré une vigueur légèrement inférieure. Ils sont toutefois moins performants que les témoins de *Coffea arabica* et de *Coffea canephora* auxquels ils ont été comparés, ce qui exclut toute utilisation directe de ce type de matériel végétal.

En revanche, compte tenu de leur résistance à la *Hemileia vastatrix*, les hybrides interspécifiques de Nouvelle-Calédonie peuvent présenter un intérêt à moyen terme dans le cadre de programmes d'amélioration de *Coffea arabica* par transfert de leur résistance à la rouille. Ils permettraient ainsi d'élargir la base génétique des variétés de caféiers actuellement diffusées dans les pays producteurs. Cette opportunité doit être néanmoins validée auparavant par des tests complémentaires visant à confirmer leur résistance, d'une part aux autres races de rouille sévissant dans le monde, et d'autre part, à d'autres maladies (anthracnose) ou parasites (nématodes).

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CREATION AND SELECTION OF *Coffea arabica* HYBRIDS IN TANZANIA

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ABSTRACT

Arabica coffee improvement in Tanzania was started in the 1930's. Homogeneity trials were carried within the existing populations of Bourbon and Kent varieties. Selection was based on individual tree yields. Regularity of yields, out-turn, weight of beans and density as well as cup quality. As a result, the now traditional varieties N39, KP162, KP423 and H66 were selected and distributed to growers by 1960. However, all these varieties are susceptible to Coffee Berry Disease (CBD) and Coffee Leaf Rust (CLR).Hybridisation was undertaken to introduce resistance to CBD and CLR, mainly using the Rume Sudan and Geisha varieties respectively. Hibrido de Timor CIFC 1343 was used extensively as a source of resistance to both diseases. CLR and CBD resistance was successfully transferred into traditional varieties. A large number of hybrid materials, most of which are complex crosses has been generated. In these, sixteen hybrids have been selected and propagated through cuttings to be evaluated in a 24 sites multilocational trial in the main coffee growing areas of the country. Selection has been based on the mean values of the progenies for disease resistance, yields and cup quality. First release of the best hybrids is envisaged for the year 2001. The main results of this work are presented and discussed.

RESUME

L'amélioration de *Coffea arabica* débuta en Tanzanie dans les années 1930 par des essais d'homogénéité dans les variétés Bourbon et Kent. La sélection était basée sur la valeur individuelle des arbres, le rendement à l'usinage, et les qualités physiques et organoleptiques. Il en résulta la distribution des variétés N39, KP162 et 423, H66 à partir de 1960. Ces variétés sont sensibles à la rouille orangée (CLR) et l'anthracnose des baies (CBD). Le programme d'amélioration s'est donc attaché à introduire des résistances dans ces variétés. Le programme de croisement utilise les variétés Geisha, Rume Sudan et l'Hybride de Timor CIFC 1343 pour introgresser plusieurs facteurs de résistance. Un grand nombre d'hybrides plus ou moins complexes ont ainsi été créés. Ils présentent des niveaux de résistance aux maladies appréciables. Après une sélection sur les moyennes, seize hybrides ont été multipliés par boutures et implantés dans un essai multilocal de 24 sites, dans les principales régions caféières du pays. On a tenu compte, dans la sélection, de la résistance aux maladies, de la productivité et de la qualité à la tasse. La distribution de ces hybrides est envisagée à partir de 2001. Les principaux résultats de ce programme sont présentés et discutés.

INTRODUCTION

Coffee is the leading foreign exchange earner in Tanzania. With an annual average value of US$ 115 million over the last seven years, it accounts for 20% of the total domestic exports (Anon., 1999). There are in the country about 420,000 small scale coffee growers who depend on coffee for their income and social welfare. Arabica coffee accounts for 80% of the total coffee produced in Tanzania. Nearly all the coffee produced is exported and only less than 5% is used locally. The high quality of the

arabica coffee from Tanzania is recognised throughout the world and this is reflected in the inclusion of Tanzania and Kenya with Colombia in the top class of mild coffees in the world market.
Arabica coffee improvement in the country was started in the 1930□s. Homogeneity trials were carried within the existing populations of Bourbon and Kent varieties. Selection was based on individual tree yields, regularity of yields, out-turn, weight of beans, and density, as well as cup quality. As a result the now traditional varieties N39, KP162, KP423 and H66 were selected and distributed to the growers by 1960. However, all these varieties are susceptible to the two main diseases, namely Coffee Berry Disease (CBD, *Colletotrichum kahawae*) and Leaf Rust (CLR, *Hemileia vastatrix*).
Profitable production depends largely in the growers□ ability to control the two diseases, especially CBD. Yield losses attributed to CBD may vary between 30% and 60% depending on individual farm management, weather conditions, and accessibility to fungicides which are sold at prohibitive prices (Ngulu *et al.*, 1998).

From a national perspective, the control of CBD and CLR which dominates the pesticide market in Tanzania, decreases the profit margin of coffee growers at the same time drain the country's meagre foreign exchange earnings through importation of fungicides. This has enforced the need to haste the breeding programme for resistance to CBD and CLR.
This paper reports on recent attempts being made to develop resistant arabica coffee varieties.

MATERIALS AND METHODS

Development of *C. arabica* hybrids

The breeding material used in this work has descended from crosses that were made between 1952 and 1983.

(i) 1952-53: The commercial cultivars N39, KP423 and H66 were crossed to Geisha and Amphillo, both of Ethiopian origin. Geisha was used as donor for CLR resistance and vigour (Fernie, 1961).
(ii) 1963-64: With the spread of CBD to Tanzania from neighbouring country, new sources of resistance were sought. Rume Sudan and Hibrido de Timor CIFC1343 (HDT) were found to be resistant to CBD. HDT was also resistant to CLR. Other origins identified to resist CLR were Kaffa and Illubabor, both from Ethiopia. a number of crosses were made involving the 1952-53 hybrids, N39, KP423 with Rume Sudan, HDT, and Kaffa.
(iii) 1972-73: Selected 1963-64 F1 hybrids were back-crossed to their respective parents to improve bean and cup quality while maintaining disease resistance. New cultivars like SL28 and SL34 were crossed to the hybrids.
(iv) 1982-83: A large hybridisation programme was carried in order to combine the high level of disease resistance expressed by the hybrids consisting Rume Sudan and HDT as parents. New crosses with Illubabor and a cross with Caturra and HDT were also made.

Field observations

Seedlings resulting from hybridisation programmes (i), (ii) and (iii) above were planted out in different observation plots in single rows of varying number of plants. Data on yields and disease assessments were recorded on an individual tree basis.

A total of 58 crosses (both simple and multiple crosses from the 1982-83 hybridisation programme) were planted in three trials in April 1985. The seedlings were not pre-selected for CBD resistance using the hypocotyl test. The first trial was set in a randomised complete block design with four replicates. Forty entries were included with 10 trees per entry in single rows. The commercial variety N39, both seeds and cuttings was included as a check. The second trial was set in a randomised design with three replicates, 14 entries, and each 10 trees in single rows. The third trial had only two replicates, nine entries, and each 10 trees in single rows. The unshaded, capped, multiple two stem trees were managed

according to recommended practice in Tanzania with a spacing of 2.74 x 2.74 m (9' x 9'). No fungicides were applied. The first four years (1987-90) yields had been recorded as totals per row of 10 trees. From 1991 to 1998 data on yield and disease assessments were recorded on an individual tree basis.

Coffee yields are normally given as weight of clean coffee, i.e. sun-dried beans, using a standard average ratio of fresh ripe fruit to clean coffee of 6:1 (Cannell, 1973). However, this ratio may vary greatly in such hybrids and further investigation is thus needed. Therefore yields are presented as kg cherry (= fresh ripe fruits)per tree per annum. Disease assessments were normally done under natural field infection.

RESULTS AND DISCUSSION

The analyses of variance on mean yields revealed different levels of significant differences between the hybrid genotypes (Tables 1, 2, 3). This is a predictable result since the genotypes evaluated belong to different cultivars with distinct origins, some with unproven agronomic characteristics, inferior bean size and poor cup quality. The results indicate a general trend towards biennial production of the hybrids. This could be partially due to heterogeneity in the years, characterised by long and erratic drought and short rainfall periods, extended heavy rainfall periods (e.g. 'El Nino'), the inherent heterogeneity of the hybrid genotypes and to a lesser extend the large size of Trial 1. Such a large plot may doubtless introduce environmental variation, thus reducing experimental precision.

However, most of the hybrids have given mean yields ranging from one to two tons of clean coffee per hectare per year compared to the commercial variety, N39, which has given yields that are below one ton per hectare (Tables 1, 2, 3). It is interesting to note the superiority of the progenies from crosses with Illubabor which have given higher mean yields that are above 1300 kg clean coffee per hectare. The HDT x Illubabor progeny (Table 1) is particularly outstanding. This emphasises the influence of Illubabor on the progeny yields which reflects high vegetative vigour.

Disease assessments which were carried between 1992 and 1995 have indicated very low average CBD and CLR infection of these hybrids. The 1992 and 1995 years were not conclusive for CBD assessments as the weather did not allow natural disease pressure. This was reflected in the high mean cherry production of the susceptible variety N39 (control) which has compared well with other hybrids. However, both CBD and CLR resistance in the hybrids varied amongst individual trees. Not all plants obtained in crosses with Rume Sudan and Hibrido de Timor were completely resistant to CBD and CLR respectively. This may suggest that not all Rume Sudan and HDT trees used in these crosses were homozygous for CBD and CLR resistance as it was found by Van der Vossen and Walyaro (1980).

Selection of resistant varieties

Observation on yields, CBD and CLR resistance as well as bean and cup quality were carried out before 1994 in trials 1 - 3 and also in two trials of hybrid clones, altogether containing about 76 different hybrids. This permitted the selection of 16 hybrid progenies (Table 4). These include eight simple crosses and eight complex crosses (including three to five different parents). Fourteen progenies have HDT and six have Rume Sudan as one of the parents respectively. Only one cross has neither HDT nor Rume Sudan parentage i.e. Kaffa x Kent. Table 4 shows that the hybrids selected within these trials display average resistance to CBD and CLR, and up to 50% above the control N39 variety. Liquoring tests carried out to date indicate that the results were not satisfactory as the normally high quality N39 variety have scored below its expected level of 2-3. The average quality data show that most of the hybrids have scored between 4 and 6 which together with bean characteristics are commercially acceptable.
It may be worthwhile to analyse the quality data separately for the years in which the control variety has scored at top level. The sixteen selected crosses are currently being evaluated in 24 multilocational trial sites in all agroecological zones where arabica coffee is grown in the country. Data being collected include yield, disease resistance, vigour, yield components, bean and cup quality.

Table 1: Group 1, Lyamungu /Yields 1991 – 1997, best entries
Mean kg cherries per cross per tree

Selected Cross	Entry	Cross	1991	1992	1993	1994	1995	1996	1997	% control	kg cc/ha/year
	1	*(N39xHDT) x Illubabor*	*1.7*	*15.0*	*9.0*	*9.1*	*11.9*	*5.3*	*8.3*	*281%*	*1 915*
SC1	**2**	**RS x HDT**	**0.5**	**6.6**	**2.1**	**6.2**	**4.6**	**3.2**	**6.2**	**136%**	**926**
	3	Kaffa x KP423	3.6	11.3	6.7	9.5	7.6	5.5	3.9	224%	1 524
	7	Kaffa1 x HDT	1.2	8.6	5.8	7.5	7.0	3.1	8.6	194%	1 324
	8	*RS x Illubabor*	*0.9*	*12.3*	*4.8*	*9.7*	*5.8*	*7.1*	*7.3*	*223%*	*1 521*
	9	*HDT x Illubabor*	*1.2*	*9.3*	*5.9*	*8.2*	*9.3*	*6.7*	*8.1*	*227%*	*1 547*
	11	(N39 x Kaffa1) x RS	0.5	9.8	4.1	8.3	7.0	6.7	7.8	205%	1 396
	12	*Illubabor x (BMJ x S6C.)*	*3.4*	*13.6*	*7.5*	*9.2*	*10.6*	*8.6*	*10.9*	*296%*	*2 021*
	13	HDT x (N39 x HDT)	0.1	7.4	3.9	10.0	7.3	5.7	6.5	190%	1 295
	15	*HDT x Illubabor*	*0.7*	*13.9*	*6.4*	*9.7*	*12.4*	*7.2*	*15.1*	*304%*	*2 073*
SC2	**18**	**Kaffa2 x KP423**	**1.0**	**10.0**	**4.6**	**8.4**	**4.4**	**4.3**	**5.1**	**176%**	**1 198**
	21	(N39xHDT) x Catimor	0.9	10.4	3.8	9.4	4.5	9.0	5.3	201%	1 370
	23	((N39xOP729) xHDT)x(N39xHDT)	0.6	11.1	3.1	10.8	5.3	7.7	9.9	226%	1 538
SC3	**25**	**(N39 x HDT) x RS**	**0.4**	**12.9**	**3.8**	**10.0**	**6.6**	**7.4**	**8.3**	**230%**	**1 566**
	26	(N39 x Kaffa1) x HDT	0.8	7.4	4.2	6.9	7.7	4.8	7.5	183%	1 246
	27	Catimor x KP423	0.8	10.6	4.7	9.1	5.2	7.6	5.5	202%	1 377
SC4	**28**	**Kaffa2 x((N39 x Geisha)x HDT)**	**0.3**	**7.7**	**2.0**	**8.7**	**4.4**	**3.7**	**5.3**	**149%**	**1 014**
	30	(N39 x HDT) x KP423	1.0	8.7	2.9	9.4	5.7	5.7	6.6	186%	1 270
	32	(N39 x HDT) x Kaffa2	1.2	10.7	5.1	8.1	7.2	4.9	8.5	213%	1 454
	33	HDT x HDT	0.3	8.7	3.6	8.6	8.2	5.3	10.5	210%	1 434
	36	(BMJ x S6C.) x KP423	1.1	12.8	2.3	11.8	5.2	8.0	3.2	207%	1 410
	38	(N39 x Kaffa1) x KP423	1.4	11.3	3.2	9.8	5.7	5.4	5.9	199%	1 354
N39	**39**	**N39 (control)**	**0.7**	**9.2**	**1.7**	**7.7**	**3.6**	**1.4**	**0.2**	**113%**	**773**
N39	**40**	**N39 (control)**	**0.5**	**5.9**	**1.9**	**4.7**	**3.8**	**0.8**	**1.1**	**87%**	**590**
		Mean	**0.8**	**9.3**	**3.8**	**7.9**	**6.0**	**5.1**	**6.1**	**182%**	**1 238**

Analysis of variance on cumulative yields 1991-97: significant (p=0.05)

Table 2: Group 2, LyamunguYields 1991 – 1997, best entries
Mean kg cherries per cross per tree

Selected Cross	Entry	Cross	1991	1992	1993	1994	1995	1996	1997	% control	kg cc /ha/year
SC5	*3*	***(N39xHDT) x Illubabor***	***0.9***	***12.8***	***2.9***	***11.3***	***7.7***	***6.3***	***4.4***	***225%***	***1 467***
SC6	7	**RS x HDT**	**0.2**	**12.8**	**1.8**	**12.3**	**2.6**	**8.2**	**4.5**	**207%**	**1 346**
SC7	12	**(N39 x Kaffa1) x (RS x HDT)**	**0.8**	**11.1**	**1.7**	**8.6**	**5.3**	**6.1**	**6.7**	**196%**	**1 280**
	5	*Illubabor x KP423*	*0.9*	*11.8*	*3.7*	*8.2*	*5.8*	*7.0*	*2.8*	*196%*	*1 275*
	10	(CaturraxHDT) x (KP423xHDT)	0.7	8.6	0.4	9.2	2.6	7.7	5.2	*167%*	1 088
	6	(N39xHDT) x (RSxG.)	0.6	10.1	2.3	4.5	6.0	3.1	6.1	*158%*	1 031
	9	Kaffa2 x (RSxHDT)	0.5	8.0	2.2	7.6	4.0	3.7	4.3	*148%*	963
N39	14	**Control**	**0.5**	**8.0**	**0.6**	**7.4**	**2.3**	**1.1**	**0.8**	**100%**	**652**
		Mean	**0.5**	**8.1**	**1.6**	**7.3**	**3.9**	**4.6**	**3.8**	***146%***	**951**

Analysis of variance on cumulative yields 1991-97: significant (p=0.05)

Table 3: Group 3, Lyamungu
Mean kg cherries per cross per tree

Selected cross	Entry	Cross	1991	1992	1993	1994	1995	1996	1997	% control	kg cc /ha/year
	5	*Kaffa x HDT*	*0.4*	*15.6*	*2.2*	*12.0*	*5.5*	*9.3*	*4.6*	*316%*	*1 568*
	2	*(N39xKaffa1) x Illubabor*	*2.6*	*14.0*	*4.4*	*7.8*	*5.9*	*4.2*	*5.9*	*286%*	*1 421*
SC8	1	**((N39 x OP729)x HDT)x Illubabor**	**0.5**	**10.5**	**2.4**	**9.7**	**6.7**	**5.0**	**9.5**	**283%**	**1 405**
	4	*(N39 x Kaffa1)x(RS x Geisha)*	*1.0*	*9.8*	*2.4*	*7.3*	*5.0*	*3.0*	*6.8*	*225%*	*1 119*
SC10	7	**((N39xOP729)x HDT)x(H66xHDT)**	**0.5**	**7.6**	**0.4**	**7.4**	**1.4**	**3.7**	**4.3**	**162%**	**802**
SC9	6	**((N39 x OP729) x HDT) x N39**	**0.6**	**9.8**	**0.5**	**6.5**	**1.9**	**2.4**	**3.4**	**159%**	**788**
	3	(N39 x HDT) x (RS x HDT)	0.3	5.0	1.3	4.5	1.7	1.9	3.4	116%	574
	8	**N39**	**1.1**	**5.7**	**0.9**	**4.5**	**2.0**	**1.2**	**0.2**	**100%**	**496**
		Mean	**0.9**	**9.7**	**1.8**	**7.4**	**3.8**	**3.8**	**4.8**	**206%**	**1 021**

Analysis of variance on cumulative yields 1991-97: significant (p=0.05)

Table 4: Reaction to diseases, beans and liquoring data for selected crosses and control

Selected Cross	Cross	Trial	Entry	Mean yieldd 1991-97 kg cc/ha/year	Mean CBD *	Mean CLR **	Bean characteristics					Mean cup quality ***	
					1992-93 Mean%	1992-93 Mean%	%AA	%A	%B	%PB	%R	1994-95	1998
SC1	*Rume Sudan x HDT*	*1*	*2*	*926*	*2.2*	*1.5*	*55*	*29*	*9*	*4*	*4*	*5+*	*7*
SC2	Kaffa2 x KP423	1	18	1 198	3.6	2.8	32	29	32	3	4	5+	6
SC3	(N39 x HDT) x Rume Sudan	1	25	1 566	0.7	0.0	30	38	21	8	3	5+	5+
SC4	Kaffa2 x((N39 x Geisha)x HDT)	1	28	1 014	0.3	0.0	56	18	13	8	6	6	5+
SC5	*(N39xHDT) x Illubabor*	2	3	*1 467*	2.2	0.7	26	44	21	5	4	6+	6+
SC6	*Rume Sudan x Hibrido de Timor*	*2*	*7*	*1 346*	*0.4*	*3.2*	*27*	*32*	*27*	*10*	*4*	*6+*	*7+*
SC7	(N39 x Kaffa1) x (RS x HDT)	2	12	1 280	0.5	2.7	43	21	28	3	5	6+	6+
SC8	((N39 x (N39xGeisha))x HDT)x Illubabor	3	4	1405	1.1	0.0	46	30	17	5	2	6+	5+
SC9	((N39 x (N39xGeisha)) x HDT) x N39	3	6	788	1.9	0.3	49	21	11	13	5	5+	4+
SC10	((N39x(N39xGeisha))x HDT)x(H66xHDT)	3	7	802	0.0	0.0	43	24	19	11	2	6+	5+
SC11	N39 x Hibrido de Timor	4	1	1732	3.8	0.0	45	29	14	10	3	6	5+
SC12	(HDTxN39)x((N39x(N39xGeisha))xHDT)	4	7	1328	4.2	0.0	61	14	14	7	4	5	5+
SC13	H66 x Hibrido de Timor	4	9	1455	3.1	0.0	62	24	5	6	3	5	5+
SC14	KP423 x Hibrido de Timor	5	4	1310	3.4	0.0	33	33	8	17	8	6	5+
SC15	*Rume Sudan x Hibrido de Timor*	*5*	*5*	*1449*	*0.4*	*0.0*	*46*	*28*	*13*	*10*	*3*	*5*	*8+*
SC16	N39 x Rume Sudan	4	2	1376	4.8	15.9	45	29	11	12	3	6	4+
N39	*Control*	*1*	*40*	*681*	*34.7*	*51.6*	*43*	*25*	*20*	*5*	*6*	*5+*	*3+*

* % cherries infected. Key: <5%: Resistant, 6-20% Partially Resistant, 21-40% Moderately Susceptible, 40-100% Susceptible
** % leaves infected. Key: <5% Resistant, 6-25% Partially Resistant, 26-60% Moderately Susceptible, 61-100% Susceptible
*** 2 Good, 3 Fair to good, 4 Fully fair, 5 Fair (FAQ), 6 About fair, 7 Poor to fair, 7 Poor

CONCLUSION

An important result is the development of new superior hybrids of immediate commercial value to the coffee industry. The hybrid vigour is likely to be responsible for the high yields. In order to take a full advantage of this characteristic it is necessary to select high yielding individual trees which could be multiplied through tissue culture techniques (Berthouly *et al.*, 1995, Berthouly & Michaux-Ferrière, 1996).

Field resistance to CBD and CLR displayed by these hybrids is also encouraging. It is now intended to screen individual plants for CBD and CLR resistance using both field and laboratory techniques (Kilambo *al.*, 1999). Collaborative work has been initiated between CIRAD and Lyamungu.

Quality depends on bean size and the liquor and is the most important factor that determines the desirability by the consumer and thus the price. Unfortunately breeding for disease resistance normally also affects most of quality characteristics. The quality results indicate that most of the hybrids produced a very good bean size and a commercially acceptable cup quality, although there is room for improvement.

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ETUDE DES PARAMETRES GENETIQUES DE LA PRODUCTION ET DE CARACTERES ASSOCIES A PARTIR D'UN PLAN DE CROISEMENT DIALLELE CHEZ *COFFEA ARABICA*

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Introduction

L'amélioration de *Coffea arabica*, espèce autogame, a traditionnellement été basée sur la sélection de lignées issues de plusieurs générations d'autofécondation (Carvalho, 1965). Cette pratique a permis de sélectionner des variétés fixées, adaptées à des zones écologiques particulières et produisant du café généralement de bonne qualité (Van der Vossen *et al*, 1981). Le café représente aujourd'hui l'une des principales denrées agro-alimentaires échangées de part le monde, mais les fluctuations des cours pénalisent la culture intensive nécessitant de nombreux intrants. La sélection de variétés productives et résistantes aux divers pressions parasitaires ou climatiques est devenue l'un des objectifs prioritaires pour de nombreux pays producteurs. Afin de répondre à cet objectif, certains programmes de recherche se sont orientés vers la création d'hybrides pouvant associer une bonne vigueur à une plus grande résistance aux aléas que les lignées traditionnellement cultivées (Bertrand *et al*, 1998). Par ailleurs, la création d'hybrides F1 peut permettre une sélection de nouvelles lignées dérivables, après plusieurs cycles d'autofécondation. Afin de mener à bien de telles sélections, il est nécessaire d'estimer les paramètres génétiques relatifs aux principaux caractères d'intérêt agronomique.
Au Cameroun, la sélection de la variété Java a permis un gain génétique appréciable par rapport à la variété Jamaïque traditionnellement cultivée (Bouharmont, 1994). Cependant, la création d'hybrides a été envisagée pour les prochaines étapes de l'amélioration génétique. Afin d'étudier la performance de certains hybrides et d'envisager de nouvelles sorties variétales, un demi-diallèle 7x7, avec autofécondations, a été mis en place en 1986 dans la station de Foumbot de l'Institut de Recherche Agronomique pour le Développement (IRAD).
La transmission de différents caractères agronomiques et morphologiques est étudiée et une estimation des principaux paramètres génétiques est présentée. L'intérêt des hybides par rapport aux lignées est discuté à partir des résultats obtenus dans cet essai.

Matériel et méthodes

Matériel végétal
Les caféiers observés sont issus d'un plan de croisements diallèle, avec les autofécondations et sans les croisements réciproques. Avec 7 géniteurs, le nombre de croisements observés est donc de 7x8/2 = 28, dont 7 autofécondations et 21 croisements hybrides.

Les 7 géniteurs sont issus de la collection d'arabica du Cameroun :
Ja1 : variété Java, sélectionnée et cultivée au Cameroun pour sa productivité et sa résistance à l'antracnose des baies (CBD),
Ca5 : variété Caturra, sélectionnée au Brésil pour son potentiel de production et son port nain, mais sensible à la rouille et au CBD,
Et3, *Et7* et *Et56* : lignées spontanées, collectée en Ethiopie (Charrier, 1978), notées résistance au CBD,
Dg10 : variété issue d'une ancienne sélection massale effectuée à Dschang au Cameroun,
Il3 : lignée semi-spontanée de Illubabor (Ethiopie), introduite d'une collection du Rwanda (Sylvain, 1955).

Chacun des arbres des 28 croisements ont été plantés en 1986 suivant un dispositif en randomisation totale. Le nombre de répétitions (arbres plantés) est de 20 par croisement, sauf pour 3 croisements : *Ja1* x *Et3* (11 caféiers), *Ja1* x *Il3* (7 caféiers) et *Dg10* x *Et56* (10 caféiers). L'écartement est de 2 m entre les lignes et de 1 m entre les arbres d'une même ligne.

Méthodes
Les productions par arbre ont été mesurées durant les 4 premières années de production, de 1989 à 1992 ; il s'agit du poids de cerises mures récoltées, exprimé en kg.
Les autres observations ont été réalisées en 1990. Le diamètre au collet, le nombre de branches primaires de chaque pied, la hauteur des arbres, le nombre de noeuds fructifères à différents étages, le nombre de rameaux morts ont été notés. Un récapitulatif des différentes variables obervées est indiqué dans le tableau 1.

Tableau 1 : Listes des caractères observés par caféier

noms des variables	caractères observés
y89, y90, y91, y92	productions annuelles, exprimées en kg de cerises
y4	cumul des productions par arbre sur 4 années (kg)
diam	diamètre des tiges en cm
haut	hauteur des caféiers en cm
nprim	nombre de branches primaires
nram	nombre de rameaux morts
nfr4	nombre de noeuds fructifères sur la 4ème paire de primaires en partant du sommet
nfr11	nombre de noeuds fructifères sur la 11ème paire de primaires en partant du sommet

Une comparaison des lignées autofécondées et des hybrides F1 est réalisée pour ces différents caractères, éventuellement après transformation des variables si les hypothèses implicites de l'analyse de variance ne sont pas vérifiées (normalité des résidus, homoscédaticité).

Les analyses diallèles sont ensuite effectuées sur les individus issus de croisements hybrides. Le modèle de Griffing, adapté aux dispositifs déséquilibrés (Keuls et Garretsen, 1977) est utilisé pour l'analyse des différentes variables :

$$P_{ijk} = \mu + g_i + g_j + s_{ij} + E_{ijk}$$

avec:

P_{ijk} : valeur phénotypique de l'arbre k du croisement ixj
g_i : aptitude générale à la combinaison du géniteur i
s_{ij} : aptitude spécifique à la combinaison du croisement ixj
E_{ijk} : erreur résiduelle de variance σ^2.

Les analyses multivariées ont été réalisées de la même façon, ce qui permet d'avoir accès aux covariances et aux corrélations entre les variables étudiées pour les différents facteurs considérés. L'application du modèle génétique permet ensuite d'estimer les héritabilités et les corrélations génétiques entre les caractères étudiés (Baradat *et al*, 1995).
Le nombre de géniteurs étant faible, les intervalles de confiance sur les héritabilités sont calculés par la méthode robuste du Jacknife (Lebart *et al*, 1979 ; Baradat *et al*, 1995), et les corrélations de rangs entre les différents caractères sont proposés pour les effets génétiques.
Les écovalences sont également estimées afin de d'évaluer la participation relative de chaque géniteur à l'intéraction (ASC) estimée pour chaque caractère (Wricke, 1962).

Résultats

Le principal caractère à améliorer est la production cumulée des 4 années. Pour ce caractère, les meilleurs individus sont les descendants hybrides du géniteur *Ca5*, avec une moyenne de 1.6 t de café marchand/an/ha, obtenue dans des conditions difficiles de culture, la moyenne de l'essai étant de 0.75 t de café marchand/an/ha.

Comparaisons des hybrides F1 aux lignées parentales
Pour le caractère de production cumulée sur 4 ans, une différence importante est constatée entre les hybrides F1 et les lignées, avec une nette supériorité des hybrides (tableau 2). L'analyse de variance est réalisée sur la variable *ly4* =Log(*y4* + 1), afin que les conditions d'application du modèle soient respectées. Il est à noter que la meilleure productivité des hybrides est détectable dès la première année de production avec la variable *y89*. Cette supériorité des hybrides concerne pratiquement tous les caractères observés, notamment les caractères de vigueur, comme le diamètre au collet, la hauteur des arbres ou le nombre de branches. Ainsi, toutes les composantes du rendements observées apparaissent plus favorables chez les hybrides.
Toutefois, la meilleure production des hybrides pourrait être due à une vigueur plus importante de ceux-ci. Les analyses des rapports du cumul de production sur différentes variables de vigueur sont donc proposées. Il apparait que le rapport production sur vigueur est toujours supérieurs pour les hybrides quelle que soit la variable de vigueur considérée (tableau 2). Les hybrides sont donc plus productifs que les lignées autofécondées.

Tableau 2 : Moyennes des lignées autofécondées et des hybrides F1

Variables	autofécondations	hybrides
ly4 ** (Log)	1.43	3.94
ly89 ** (Log)	0.73	1.77
diam **	3.02	3.70
haut **	137.82	151.71
nprim **	45.76	55.44
lnram ** (Log)	2.44	3.42
nfr4 **	17.73	22.75
nfr11 **	11.84	19.33
y4/diam **	3.95	10.12
y4/haut **	0.099	0.263
y4/nprim **	0.258	0.671

** : différence significative à 1 % ; (Log) : transformation Log (x+1) utilisée pour l'analyse de variance

Comparaisons des différentes lignées

Un classement des différentes lignées est proposé pour les caractères observés présentant des différences significatives entre lignées (tableau 3). Les nombres de rameaux morts sont significativement identiques pour les différentes lignées. La production de l'année 1989 permet de prédire le cumul des 4 années. Les variétés Caturra et Java sont les plus productives.

Tableau 3 : Moyennes des lignées et tests de Newman et Keuls (5%)

	ly4 *	*ly89* *	*diam*	*haut*	*nprim*	*nfr4*	*nfr11*	*y4/ diam*	*y4/ haut*	*y4/ nprim*
Ca5	3.15 a	1.96 a	3.16 b	111 d	55.3 a	18.4 ab	13.8 b	9.27 a	0.262 a	0.540 a
Ja1	2.91 a	1.45 a	4.09 a	163 a	56.6 a	25.4 a	25.5 a	6.74 a	0.172 b	0.485 a
Il3	1.19 b	0.73 b	3.45 ab	154 ab	52.3 a	26.3 a	12.1 b	3.08 b	0.071 c	0.209 b
Et56	0.67 b	0.22 c	2.31 c	119 cd	37.1 b	7.0 b	5.5 b	2.39 b	0.049 c	0.166 b
Dg10	0.58 b	0.11 c	2.69 bc	136 bc	33.5 b	11.2 ab	5.9 b	1.92 b	0.039 c	0.148 b
Et3	0.36 b	0.09 c	2.00 c	118 cd	33.7 b	13.2 ab	8.1 b	1.36 b	0.025 c	0.084 b
Et7	0.26 b	0.04 c	2.60 bc	148 ab	43.2 ab	16.5 ab	7.4 b	0.82 b	0.016 c	0.047 b

* moyennes des données brutes, tests sur la transformation Log(x+1)

Analyse du demi diallèle

Une forte aptitude générale à la combinaison est détectée pour les différents caractères alors que l'aptitude spécifique ne se manifeste que pour les caractères de production et de vigueur (tableau 4). Les héritabilités au sens strict et au sens large sont estimées. Les intervalles de confiance à 95 % sont donnés pour les héritabilités au sens large. Seule l'héritabilité du nombre de rameaux morts n'est pas significativement différente de 0.

Tableau 4 : Valeur des tests de Fisher associés aux AGC et aux ASC et héritabilités

Variable	AGC	ASC	h^2	h^2_L [Intervalle de confiance à 95 %]
ly4	19.65 (< 0.001)	3.97 (< 0.001)	0.260	0.396 [0.289 ; 0.503]
ly89	19.59 (< 0.001)	4.80 (< 0.001)	0.237	0.408 [0.315 ; 0.501]
diam	5.48 (< 0.001)	4.65 (< 0.001)	0.001	0.218 [0.093 ; 0.343]
haut	30.08 (< 0.001)	4.00 (< 0.001)	0.373	0.489 [0.399 ; 0.579]
nprim	5.21 (< 0.001)	2.87 (< 0.001)	0.042	0.161 [0.042 ; 0.280]
lnram	4.47 (< 0.001)	1.43 (0.138)	0.076	0.105 [-0.009 ; 0.219]
nfr4	3.73 (0.0015)	0.73 (0.725)	0.081	0.081 [0.014 ; 0.148]
nfr11	9.08 (< 0.001)	0.62 (0.847)	0.197	0.197 [0.128 ; 0.266]
y4/diam	29.29 (< 0.001)	2.51 (0.002)	0.405	0.466 [0.371 ; 0.561]
y4/haut	48.75 (< 0.001)	4.27 (< 0.001)	0.501	0.601 [0.510 ; 0.692]
y4/nprim	10.19 (< 0.001)	1.93 (0.023)	0.177	0.232 [0.091 ; 0.373]

La productivité et la hauteur ont des héritabilités élevées. Le diamètre au collet a une héritabilité au sens large importante mais une héritabilité au sens strict très faible. Ce caractère, essentièlement géré par la dominance, pourrait être lié au degré d'hétérozygotie des croisements. Les rapports du cumul de production sur les variables de vigueur ont des héritabilités très élevées, particulièrement le rapport du cumul de production sur la hauteur. Un gain génétique important peut donc être espéré pour cette variable composite.
L'application du modèle multivarié permet d'estimer les corrélations génétiques entre les différents caractères. Les corrélations de rangs pour les effets génétiques additif et de dominance sont présentées dans le tableau 5.

Tableau 5 : Corrélations de rangs, effets génétiques additifs (moitié supérieure), valeur seuil à 5 % : 0.75
effets génétiques de dominance (moitié inférieure), valeur seuil à 5 % : 0.43

	y4	*y89*	*diam*	*haut*	*nprim*	*nram*	*nfr4*	*nfr11*
y4	1	0.786	0.429	0.107	0.857	1	0.286	0.464
y89	0.800	1	0.214	0.036	0.714	0.786	0	0.179
diam	0.712	0.875	1	0.786	0.643	0.429	0	0.321
haut	0.457	0.748	0.817	1	0.179	0.107	-0.429	-0.250
nprim	0.713	0.906	0.917	0.861	1	0.857	0.429	0.679
nram	0.365	0.304	0.132	0.082	0.177	1	0.286	0.464
nfr4	0.034	-0.378	-0.300	-0.383	-0.238	0.351	1	0.536
nfr11	0.670	0.627	-0.617	0.638	0.704	0.213	0.209	1

Ces corrélations indiquent qu'une bonne prédiction de la production cumulée peut être obtenue par la première année de production et par le nombre de branches primaires qui semble être une composante essentielle du rendement.

Un classement des AGC des géniteurs est proposé pour plusieurs caractères avec des tests de comparaisons multiples de Newman et Keuls (tableau 6). De grandes différences sont constatées entre les classements des variétés et les classements des AGC. La variété Java (*Ja1*) qui apparaissait comme une variété très productive semble très médiocre en tant que géniteur. Le coefficient de corrélation de rang de Spearman entre les valeurs propres et les AGC correspondantes pour la variable *ly4* est quasiment nulle : r_s = - 0.036. La valeur propre d'une variété ne permet donc pas d'estimer sa valeur en croisement.

Tableau 6 : Comparaisons des géniteurs pour les AGC estimées - test de Newman & Keuls (5%)

	ly4	*ly89*	*diam*	*haut*	*nprim*	*lnram*	*nfr4*	*nfr11*	*y4/ diam*	*y4/ haut*	*y4/ nprim*
Ca5	0.58 a	0.48 a	-0.11 bc	-24.7 d	5.85 a	0.14 a	6.57 a	10.68 a	1.83 a	0.06 a	0.08 a
Et3	0.11 b	0.11 b	0.35 a	11.6 ab	1.76 ab	0.15 a	-1.64 b	-2.21 b	-0.01 b	0.00 b	0.02 b
Et7	-0.15 c	-0.14 c	0.15 ab	18.0 a	-0.00 bc	-0.11 b	0.52 b	-3.18 b	-0.64 b	-0.02 c	-0.03 c
Il3	-0.20 c	-0.22 c	-0.28 c	-5.00 cd	-4.32 c	-0.01ab	-0.40 b	-3.97 b	-0.30 b	-0.01 bc	-0.01bc
Dg10	-0.20 c	-0.22 c	-0.04 bc	7.08 b	-1.70 bc	-0.10 b	-2.36 b	1.53 b	-0.58 b	-0.02 c	-0.03 c
Ja1	-0.22 c	-0.22 c	-0.08 bc	-2.36 c	-2.22 bc	-0.15 b	0.89 b	-1.88 b	-0.47 b	-0.02 c	-0.03 c
Et56	-0.26 c	-0.08 c	-0.12 bc	-0.87 c	-3.09 bc	-0.08 b	-5.68 b	-4.85 b	-0.61 b	-0.02 c	-0.03 c

Les écovalences associées à chaque géniteur sont estimées pour les caractères qui présentent des ASC significatives (tableau 7). Les valeurs sont très variables et le géniteur *Ja1* présente une très forte écovalence pour l'ensemble des caractères, ce qui signifie que ce génotype participe fortement aux ASC détectées.

Tableau 7 : Ecovalences associées à chacun des géniteurs, en pourcentage

	ly4	*ly89*	*diam*	*haut*	*nprim*	*y4/ diam*	*y4/ haut*	*y4/ nprim*
Ca5	18.27	19.38	13.51	2.72	10.07	13.54	19.52	12.18
Et3	10.25	7.76	7.69	7.90	8.76	11.26	9.41	9.30
Et7	14.87	4.77	11.57	4.93	1.76	17.35	13.55	20.96
Il3	12.67	11.26	10.24	27.20	24.88	23.50	16.81	16.69
Dg10	9.23	8.99	20.30	29.74	17.34	7.60	5.80	14.94
Ja1	26.27	35.63	28.48	16.09	27.96	19.28	26.12	19.82
Et56	8.44	12.21	8.21	11.41	9.24	7.47	8.80	6.11

Conclusion

D'une façon générale, les hybrides de caféiers arabica sont plus performants que les lignées. Ce constat a déjà été fait dans d'autres pays, ce qui a conduit certains organismes de recherche a envisagé la micropropagation d'individus hybrides intéressants (Etienne *et al*, 1998). La

production cumulée sur 4 années est un caractère assez héritable qui peut être prédit par des productions annuelles antérieures, ce qui confirme des travaux antérieurs (Walyaro *et al*, 1979, Cilas *et al*, 1998). Ce caractère peut également être prédit par le nombre de branches primaires. Il n'existe aucune relation entre la valeur des lignées issues d'autofécondations et les AGC des mêmes lignées en croisements. Certaines lignées, comme les éthiopiens *Et3* et *Et7* semblent moins bien se comporter en autofécondation qu'en croisements. Inversement, la lignée sélectionné au Cameroun *Ja1* se comporte bien en tant que variété mais s'avère être un mauvais géniteur ; elle présente par ailleurs une très forte intéractivité en croisements avec les autres géniteurs. Il s'agit sûrement d'une lignée encore mal fixée qui présente un degré d'hétérozygotie non négligeable.
Les rapports des cumuls de production sur les variables de vigueur sont très héritables. Un progrès génétique important peut donc être obtenu sur ces variables composites et plus particulièrement sur le rapport du cumul de production sur la hauteur qui représente un objectif de sélection important.
Des études sur la résistance à la rouille orangée et à l'anthracnose des baies devraient être réalisées dans cette essai, l'objectif étant de sélectionner du matériel végétal productif et résistant aux principaux aléas phytosanitaires présents au Cameroun.
Par ailleurs, cette étude devrait être complétée par des mesures technologiques et sensorielles afin d'effectuer une sélection familiale sur un index combinant les qualités agronomiques et organoleptiques.

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Résumé :

Les paramètres génétiques de plusieurs caractères agronomiques et morphologiques chez *Coffea arabica* ont été estimés dans un essai diallèle situé dans la station de Foumbot de l'IRAD, dans la région ouest du Cameroun. Il s'agit d'un plan de croisements de type demi diallèle 7x7 avec les autofécondations, soit 21 croisements hybrides et 7 lignées autofécondées. Après une comparaison des deux types de matériel végétal, une estimation des héritabilités et des corrélations génétiques entre caractères est proposée à partir de l'analyse du diallèle sans les autofécondations. Il apparaît que les hybrides sont globalement supérieurs aux lignées pour la plupart des caractéristiques agonomiques et en particulier pour la production. Il n'existe pas de relation nette entre la valeur propre des lignées et les aptitudes générales à la combinaison. Le Caturra est une variété performante et un bon géniteur ; en revanche la variété Java se comporte très bien en tant que variété, mais elle s'avère être un mauvais géniteur. Certains caractères, comme le nombre de branches primaires, corrélés génétiquement à la production, pourraient être utilisés pour améliorer la prédiction de ce caractère. Une estimation des écovalences associées à chaque géniteur a permis de quantifier leur participation respective à l'Aptitude Spécifique à la Combinaison. Le géniteur *Ja1* apparaît comme le plus intéractif pour la plupart des caractères considérés, ce qui peut indiquer un niveau d'hétérozygotie élévée de cette variété.

Abstact :

Genetic analysis of different agronomic and vegetative traits was carried out of a half-diallel crossing scheme in *Coffea arabica*, located in the Foumbot experimental station of IRAD, Cameroon. The 7 x 7 half-diallel comprises 21 hybrid progenies and 7 parental selfed lines. Heritabilities and genetic correlations between the different characters were estimated. Hybrids were superior to lines for all the traits. There was no relationship between parental lines values and their general combining abilities. Caturra showed to be a high yielding variety and a good parent. On the other hand, Java, a variety selected in Cameroon, showed to be a good variety but a bad parent. Some traits, like the number of primary branches, were genetically correlated to yield and can therefore be used as a yield predictor. Ecovalences were also estimated in order to evaluate the relative participation of each parent to the Specific Combining Ability (interaction between parents). Java was the most interactive parent, which might indicate a higher level of heterozygosity of this variety.

EVALUATION OF AN ADVANCED BREEDING POPULATION OF ARABICA COFFEE

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Abstract

An advanced breeding population of *Coffea arabica* comprising 32 multiple cross families were preselected in the laboratory for resistance to coffee berry disease (CBD) and evaluated in the field for yield, quality and resistance to CBD and leaf rust. Three check cultivars (SL28, Ruiru 11 and Catimor) were included in the trial for comparison. The trial was planted in 1988 in a three-replicate completely randomized design with 8 seedlings per plot. Data were recorded over a period of five years on plot mean basis for cherry yield per tree, bean grade, liquor quality characters and field resistance to CBD and leaf rust. The test population performed as good as or better than the check varieties for most characters. The tall-statured population is true-to-type especially for characters under evaluation making its propagation easier than a hybrid variety.

Introduction

Coffee is an important export crop in Kenya earning the country upto 20% of total foreign currency revenue. It is also a major source of livelihood for upto 20% of the rural Kenyan population dependant on Agriculture. Coffee yields in Kenya are generally low. It has been observed that yields in small-holder sector average 534 kg of clean coffee per ha (2.8 kg of cherry per tree) while plantations record an average of 1064 kg of clean coffee per ha (5.6 kg of cherry per tree) compared to yields of 3.5 tonnes per ha (18.4 kg of cherry per tree) which have been achieved in some estates (Karanja, 1996). The low yields are attributed to prohibitive cost of inputs leading to poor management of farms and losses due to biotic and abiotic stress factors.

At the core of the Coffee Research Foundation's (CRF) mission and vision is to increase coffee production at a reduced unit cost and add value to the coffee product. Significant steps have been taken in the past to develop technologies which address this scenario. For instance, an Arabica coffee cultivar, Ruiru 11, developed by the CRF and released to growers in 1985 is not only resistant to coffee berry disease (CBD) and leaf rust but also combines high yield with fine quality and compact growth amenable to high density planting. By growing Ruiru 11, farmers save upto 30% of the production costs that go to the control of CBD alone, the most serious disease in Kenya. The growers also benefit from the premium prices the variety fetches from its fine quality. Additionally, farmers in the high potential coffee growing areas where land is a serious limitation can intensively grow the variety at double the plant population compared to the traditional varieties because of its compact stature thus increasing yield per unit land. The Ruiru 11 variety therefore enjoys an overwhelming adoption with a demand of about 15 million planting materials compared to the CRF production of about 5 million. The major limitation is the hybrid nature of the variety which involves artificially

crossing two parent populations to produce the seeds. This process is not only labour intensive but is also constrained by the short duration of about 4 days when the flowers can be emasculated and pollinated. The CBD resistance in Ruiru 11 also rests on a narrow genetic base of one or two dominant genes. Inheritance studies for CBD resistance has revealed upto three genes on separate loci (Van der Vossen and Walyaro, 1980).

An advanced breeding population has been developed comprising of elite selections, some of which carry all the three genes of CBD resistance in a genetically fixed form. The objective of this work was to evaluate the population for yield, quality and field resistance to CBD and leaf rust. The outstanding features of the variety as compared to other commercial cultivars namely, SL 28, Ruiru 11 and Catimor are discussed in this paper.

Materials and methods

A selection field was planted with an elite population comprising 32 genotypes of multiple cross families involving the CBD resistant donor parents such as Rume Sudan (RS), Hibrido de Timor (HT) and K7 with the high yielding, good quality and adaptable varieties such as SL28, SL34, SL4, Bourbon and N39 at Oaklands Breeding Station in Ruiru, Kenya (Table 1). Three check varieties were included in the trial for comparison.

Table 1. Families of elite population and their pedigrees

Family No.	Tree No.	Pedigree	Family No.	Tree No.	Pedigree
1	B15.895	SL28 x (SL34 x RS)HT	18	B15.1533	SL28 x (N39 x HT)(SL4 x RS)
2	B15.727	SL28 x (SL34 x RS)HT	19	B15.931	SL28 x (SL34 x RS)HT
3	B15.81	SL28 x (SL34 x RS)HT	20	B15.1070	SL28 x (N39 x HT)(SL4 x RS)
4	B15.89	SL28 x (SL34 x RS)HT	21	B15.113	SL28 x (N39 x HT)(SL4 x RS)
5	B15.92	SL28 x (SL34 x RS)HT	22	B15.1559	SL28 x (N39 x HT)(SL4 x RS)
6	B15.262	SL34 x (SL34 x RS)HT	23	B15.1525	SL28 x (N39 x HT)(SL4 x RS)
7	B15.675	SL28 x (SL34 x RS)HT	24	B15.1093	SL28 x (N39 x HT)(SL4 x RS)
8	B15.239	SL34 x (SL34 x RS)HT	25	B15.1292	SL34 x (SL34 x RS)HT
9	B15.283	SL34 x (SL34 x RS)HT	26	B15.1077	SL28 x (N39 x HT)(SL4 x RS)
10	B15.726	SL28 x (SL34 x RS)HT	27	B15.1534	SL28 x (N39 x HT)(SL4 x RS)
11	B15.638	SL28 x (SL34 x RS)HT	28	B15.97	SL28 x (RS x K7)(HT x SL34)
12	B15.728	SL28 x (SL34 x RS)HT	29	B15.98	SL28 x (RS x K7)(HT x SL34)
13	B15.624	SL28 x (SL34 x RS)HT	30	B15.96	SL28 x (RS x K7)(HT x SL34)
14	B15.1400	SL28 x (RS x SL28)(B x HT)	31	B15.136	SL28 x (RS x K7)(HT x SL34)
15	B15.1365	SL28 x (RS x SL28)(B x HT)	32	B15.1533	SL28 x (N39 x HT)(SL4 x RS)
16	B15.928	SL28 x (SL34 x RS)HT			
17	B15.902	SL28 x (SL34 x RS)HT			

The trial was planted in a three replicate completely randomized design with 8 seedlings per plot. Data were recorded over a period of five years on plot mean basis. The plot mean data was used to calculate the population mean.

The following characters were recorded:

Cherry yield per tree	-Cherry yield was obtained on plot mean basis and expressed in kg per tree.
Bean grade characters	-A bean grader was used to determine the fraction of bean sizes AA and AB, the premium coffee bean grades which were then expressed as percentage by weight as described by Walyaro (1983) and Njoroge (1992).
AA	-The fraction of heavy beans retained by number 18 (7.15 mm) screen.
AB	-The fraction of heavy beans retained by number 15 (5.95 mm) screen.
Liquor quality characters:	-Bean grades AA and AB from each family were combined and submitted to Mild Coffee Trade Association (MCTA) panel of liquorers in samples of 250g. Sensory evaluation was used for the assessment of liquor quality as follows.
Acidity	-with scores of 0 - 4 where, 0 = pointed and 4 = lacking.
Body	-with scores of 0 - 4 where, 0 = full and 4 = lacking.
Flavour	-with scores of 0 - 7 where, 0 = fine and 7 = poor.
Overall standard	-This was the overall evaluation of liquor quality based on bean and liquor quality characters.
Field evaluation of CBD	-Number of berries with active CBD lesions expressed as a percentage of total berries on two most infected

branches.

Leaf rust score -Visual score of disease severity on tree basis on a Scale of 0–10.

An analysis of variance was performed on the data using the MSTAT 4.0 statistical programme (Michigan State University). Mean separation was performed using Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$.

Results and discussion

Results in Table 2 indicate that the yield of the elite population was statistically similar to the yield of SL 28 and Catimor. SL 28 was used as the recurrent parent in the development of the elite populations. Ruiru 11 cultivar was the overall highest yielder with a mean of 4.73 kg/tree. The transgressive yield of Ruiru 11 which is higher than the test population and the Catimor variety, both of which are base populations from which Ruiru 11 parents have been selected is an indication that Ruiru 11 exhibits heterosis. On yearly basis, yield of the test population increased from year 1 reaching a maximum in year 3 (Figure 1). This may be attributed in part to the growth of the tree increasing in the number of bearing surfaces. In year 4, a drop was observed followed by an increase in year 5. The drop was due adverse weather conditions.

Table1. Mean score for yield, quality and field resistance to CBD and leaf rust in comparison to check varieties.

Trait	Elite pop.	SL28	Ruiru 11	Catimor
Yield (kg/tree)	3.46 B	3.75 AB	4.73 A	3.08 B
Bean grade				
AA	11.75 B	23.50 A	17.15 B	17.57 AB
AB	41.45 A	31.58 B	40.88 A	40.93 A
Liquor quality				
Acidity	2.08 A	1.73 A	2.11 A	2.60 A
Body	2.00 A	1.90 A	2.03 A	2.60 A
Flavour	3.59 AB	3.26 B	3.48 AB	4.33 A
Overall std.	3.83 AB	3.61 B	3.82 AB	4.33 A
% CBD	0.04 B	2.61 A	0.25 B	0.00 B
Leaf rust score	0.86 B	2.52 A	0.03 C	0.00 C

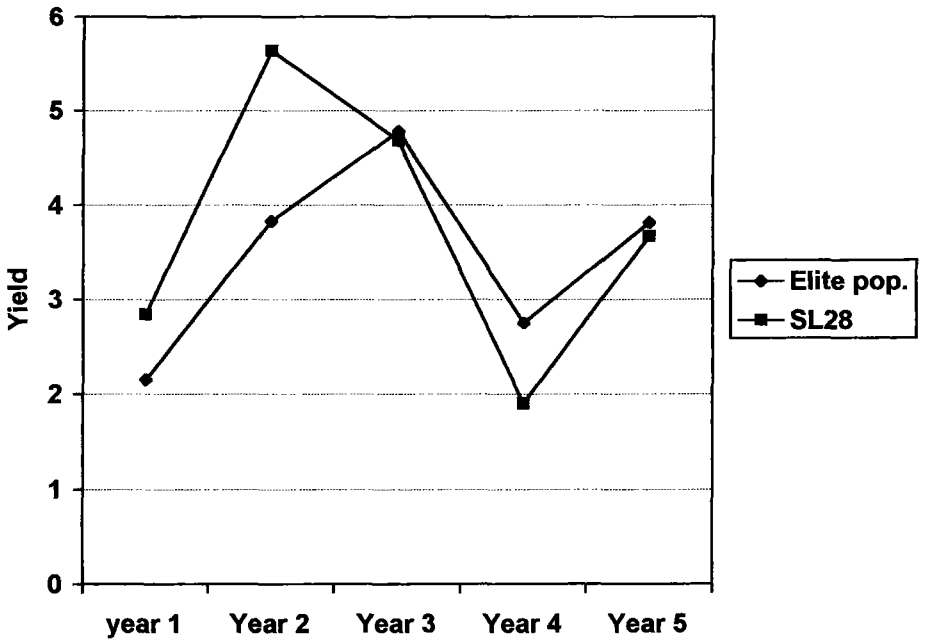

Fig. 1. Yearly variations for yield in elite population and SL28 on season basis.

Although productivity is an important trait in coffee breeding, quality is even more important because it determines the price and the ultimate income to the farmer. The Kenyan coffee quality character renown worldwide has helped put it on a competitive edge fetching attractive premium prices. The breeding programme at the Coffee Research Foundation in Kenya has been sensitive to quality demands. The bean grades AA and AB are the premium grades fetching higher prices. Although grade AA beans were significantly lower in the test population than in the traditional cultivar SL 28, the same grade of beans were not significantly different in Ruiru 11 and Catimor (Table 1). It was further observed that the elite population together with Ruiru 11 and Catimor had significantly higher grade AB beans than SL 28. The negative

correlation between bean grades AA and AB is a common phenomenon in Arabica coffee. Adams (1967) and Grafius (1978) reported that the observed correlation between traits are due to genetic linkages, pleitropic effect of genes or physiological and developmental relations. Negative relationships, when present, indicate some from of competitive inhibition. This often arises from the fact that the traits compete for the same total amount of metabolic substrates produced by the plant. The combined total of grade AA and AB beans of the test population therefore compares well with the check varieties. The yearly variations for the bean grades are presented in Figure 2. Bean grade AA exhibited minimum yearly fluctuations and therefore could be a useful criteria for selection for improved bean quality.

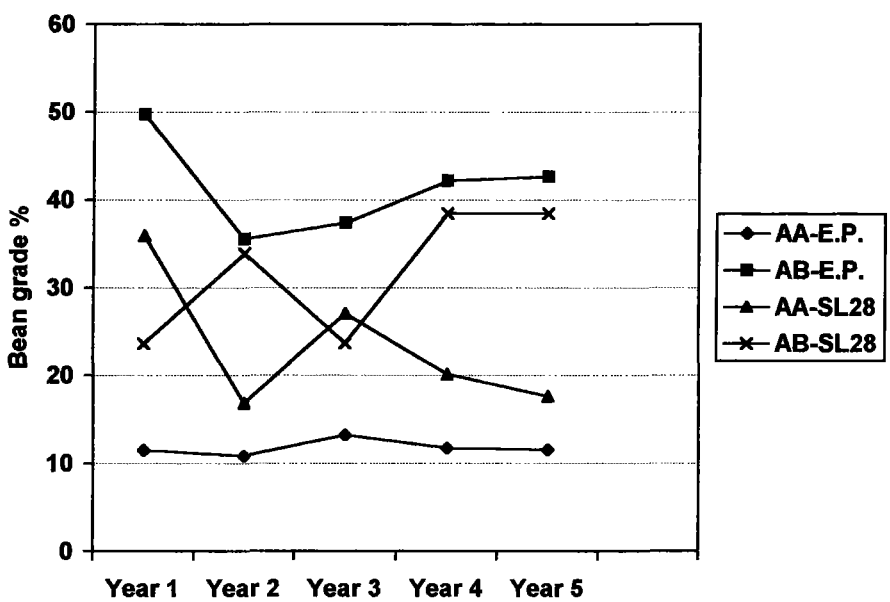

Fig. 2. Yearly variation for bean grade characters in elite population and SL28.

The ultimate price of coffee is determined by the quality of liquor. The three major attributes of liquor are acidity, body and flavour. The test population had acidity and body similar to the three check varieties (Table 1). Flavour varied significantly with the best score recorded in SL 28 which was not significantly different from the elite population and Ruiru 11. Catimor had an inferior score compared to other varieties. The fine quality SL 28 has been used in the development of the elite population and Ruiru cultivar thus imparting the good quality. Overall standard is the final classification criteria that takes into account the bean and liquor quality. It exhibited a similar trend like flavour (Table 1). SL 28 recorded the best overall standard but it was not significantly different ($P \leq 0.05$) from the elite population and Ruiru 11 except Catimor. Although all liquor quality characters exhibited yearly fluctuations as indicated in figure 3, the fluctuations for

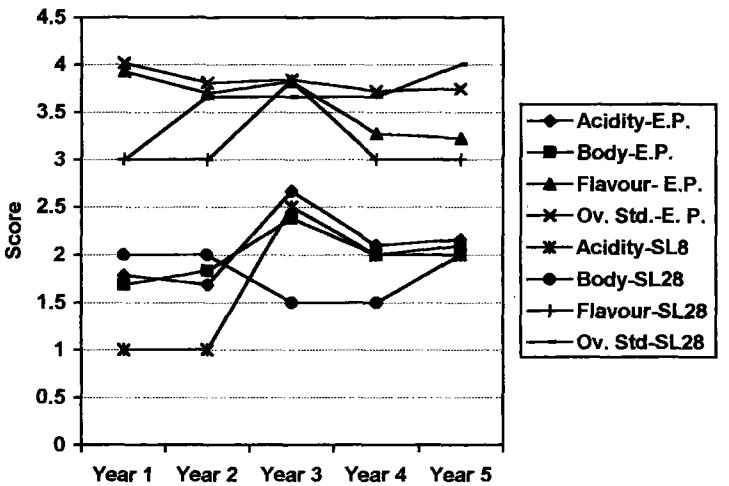

Fig. 3. Yearly variations for liquor quality characters in the elite population and SL28.

overall standard in the elite population was minimal. Selection based on overall standard could therefore lead to rapid gain as no repeated measurements are required.

Despite the outstanding quality of the SL 28 cultivar, it was susceptible to CBD and leaf rust (Table 1). Field resistance to CBD was high for the elite population, Ruiru 11 and Catimor. The three genotypes carry CBD resistance genes. Resistance to leaf rust was highest in Catimor and Ruiru 11 but intermediate in the elite population. The rust pathogen, *Hemileia vastatrix* has several physiologic races numbering 39 that have already been identified (Rodrigues Jr. et al, 1993). Resistance is conditioned by SH major genes. The genes SH_1, SH_2, SH_4 and SH_5 are of *C. arabica* origin while SH_3 is derived from natural crosses between *C. liberica* and *C. arabica.* These genes have compatible rust races. The genes of interest are those derived from *C. canephora* which confer resistance to most common races. The genes, SH_6 - SH_9 designated "group A" occur in Hibrido de Timor and its hybrid derivative Catimor. The Ruiru 11 variety also carry the genes. The rust resistance in the elite population is probably of quantitative nature. Owuor (1983) reported considerable variation for quantitative resistance especially in variety Rume Sudan and a number of genotypes from Ethiopia. The elite population carries Rume Sudan in its pedigree. The CBD scores were generally very low over the years except in the fourth year (Figure 4). It is important to note that the genotypes were pre-selected for CBD resistance in the laboratory before field planting. This confirms the conclusion that there is a high correlation between Pre-selection test based on hypocotyl seedlings and field resistant to CBD. The fourth year was a year of serious CBD epidemic as indicated by the susceptible SL28 cultivar. The yearly fluctuations for leaf rust could also be a reflection of varying disease severity in the field. In conclusion, the elite population had an outstanding performance combining field resistance to CBD and leaf rust with high productivity and fine quality. The

population is true breeding for most characters under evaluation making it easier to propagate than a hybrid cultivar. It forms an important base from which an improved cultivar could be selected.

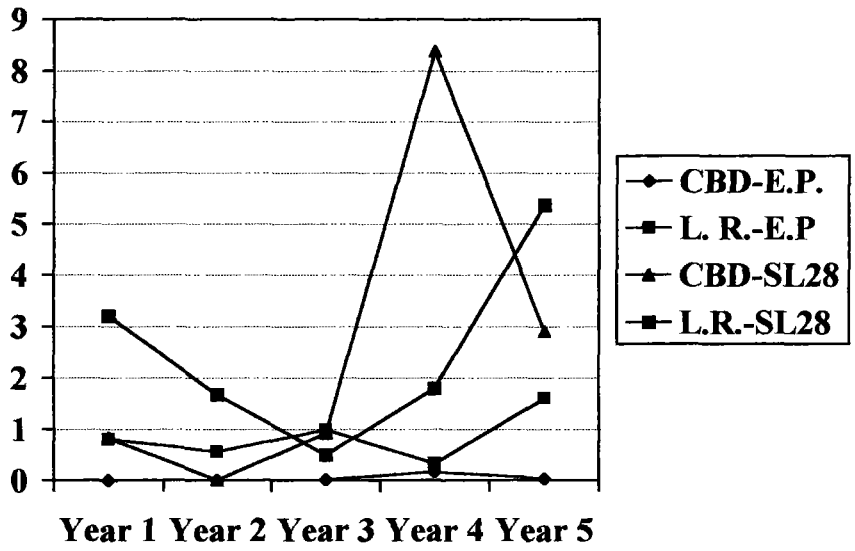

Fig. 4. Yearly variations for CBD and leaf rust score in the elite population and SL28.

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FLAVOUR : AN IDEAL SELECTION CRITERION FOR THE GENETIC IMPROVEMENT OF LIQUOR QUALITY IN ARABICA COFFEE

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Introduction

Coffee is undoubtedly the most important Agricultural commodity in the world business. It commands a turn-over of about US$ 10 billion annually, thereby making it the second most traded commodity after petroleum (Graaf, 1986; Saitoti, 1997). The commercial cultivation of coffee involves three main species: *Coffea arabica* L., *C. canephora* P. and *C. liberica* (Carvalho & Monaco, 1969). However, Arabica coffee predominates the world coffee trade due to its superior quality (Van der Vossen, 1985). Its exclusive production is nevertheless limited by a number of inherent constraints, notably disease (Van der Vossen, 1985), drought susceptibility, low tolerance to high temperatures and adverse Genotype-Environment (GE) interactions. These constraints result into an increase in the cost of coffee production and a decrease in Arabica coffee productivity.

Low productivity is of major concern, especially in the producer countries of Africa where population growth is high and pressure on Agriculturally productive land is ever increasing. In Kenya for example, the average national production for the small-scale sector is estimated at 0.5 t/ha whereas that of the large scale farmers is about 1.0 t/ha. Under these circumstances, increased production per area of land use through the adoption of improved varieties would ensure higher production of coffee on relatively reduced land area hence releasing more land for alternative uses.

In view of the above considerations, a number of breeding and selection programmes have been initiated in countries such as Cameroon (Bouharmont, 1995), Colombia (Castillo & Moreno, 1988), Ethiopia (Van der Graaff, 1981), and Kenya (Van der Vossen & Walyaro, 1981) with the objective of developing improved Arabica coffee varieties with high yields, good quality good adaptation and have resistance to the most prevalent diseases.

The coffee breeding programme initiated in Kenya in 1971 (Van der Vossen & Walyaro, 1981) was designed in light of the above considerations. Its main objectives were to breed and select for Arabica coffee varieties which combine high yields and good qualities with resistance to both coffee leaf rust and CBD. Tremendous progress has since been achieved culminating into the development of a compact type F_1 hybrid variety named Ruiru 11. Plans to release additional varieties with more robust growth habits are also in advanced stages and are discussed elsewhere in the present proceedings (Agwanda, 1999 ; Omondi, 1999).

Not withstanding the benefits of having a high yielding and disease resistance variety, quality considerations are perhaps the main overriding factors determining the success of improved varieties for commercial use. Indeed, the amount and sustainability of coffee consumption in man depends on the desirability of the coffee brew and the pleasure derived from its consumption (Charrier 1982). Liquor quality is therefore an important selection criterion in any breeding programme aimed at developing improved Arabica coffee varieties.

Most of reported work on the improvement of coffee quality however concerns the improvement of agronomic and processing practices that directly impinge on coffee quality (Cannel, 1971; Sivet, 1972; Charmetant & Leroy, 1985). Genetic improvement of liquor quality is only occasionally taken into

considerations (Vishveshwara, 1971; Suriakantha *et al.*, 1978; Walyaro, 1983; Castillo & Moreno, 1988; Owuor, 1988; Moreno *et al.*, 1995). This state of affairs is largely due to the fact that variation in quality not only depends on the genetic constitution of a given variety or species, but is also heavily influenced by non-genetic factors such as edapho-climatic conditions, cultural practices, harvesting and processing procedures, storage of parchment roasting procedures and the method of preparation of coffee brew (Charrier, 1982). The non-genetic factors are particularly important when liquor quality is considered. The lack of objective criteria for determining the value of a given variety for breeding purposes is also an important setback. Liquor quality is determined by panels of experienced coffee liquorers based on some arbitrary hedonic scale. Standardised procedures are followed during both roasting and brewing stages (AFNOR, 1991). In Kenya and Colombia, for example, liquor quality is determined on the basis of the level of acidity, body, and flavour of the brew (Devonshire, 1956; Moreno *et al.*, 1995). The three traits are known to determine, to a large extent, the liquor quality of coffee (Devonshire, 1956; Sivetz, 1972; Suriakantha *et al.*, 1978; Walyaro, 1983).

The organoleptic procedures have definite use in marketing and selection for improved varieties, particularly during the early phases of coffee breeding programmes. Their utility as a selection tool however fades away as selection progresses and homogeneity is approached. Minor differences in the overall quality differences become more difficult to perceive and to quantify, rendering the utility of data accrued from such assessments less meaningful for breeding work. A high degree of variation may also be expected from one liquorer to the next given the subjective nature of the assessment method. Faster progress should however be possible if quality traits with high fidelity of expression and hence can be determined with greater precision are identified and incorporated in the selection criteria.

The recent developments in the use of biochemical procedures (Hamphrey & Macrae, 1986; Cohen, 1993) to elucidate varietal differences in key aroma compounds, and the development of electronic noses (Bartlett *et al.*, 1993) will also provide complementary tools with which breeders could conduct more precise selection for liquor quality. However, no amount of improvements in the use of electronic noses will rival the use of the human nose in determining the final quality of a coffee brew and its desirability for human consumption (Gopel, 1997). Breeders will thus have to look for adequate ways to optimise the use of liquor evaluation based on organoleptic procedures for breeding and selection purposes. One consideration would be to identify liquor traits which could be determined by the liquorers with a greater precision, are closely related to the perceived overall liquor quality of coffee and display high heritabilities.

In this study, data collected during the curse of the coffee breeding programme in Kenya was used to evaluate the utility of three liquor quality traits, namely, acidity, body and flavour as selection criteria for the genetic improvement of the overall liquor quality in Arabica coffee.

MATERIALS AND METHODS

Test localities

Three sites representing the main coffee growing zone of Kenya were used in the study. They included Kisii, Koru and Ruiru. Kisii represented the high potential coffee growing zone in Kenya. It is located at 0° 41'S, 34° 47'E and has an elevation of 1700 m (asl). Its soils are Molic nitosols and receives a mean monthly rainfall of 156.4 mm. It has a mean maximum monthly temperature of 26.9°C and a mean monthly minimum of 12.8°C . It receives rainfall throughout most of the year, with June-July and December-January being the driest months. Koru falls within the high to medium potential coffee zone. It is situated at 0° 07'S, 35° 16'E and has an altitude of 1554 m (asl). Its soils are Eutric nitosols. It receives a mean monthly rainfall of 141.4 mm, has a mean monthly maximum temperature of 27.4°C and a mean monthly minimum of 13.7°C. Dry periods occur during September-October and December-March. The third trial site was situated at the Jacaranda farm at the Coffee Research Station, Ruiru. It is situated at 1° 06'S, 36° 45' E and has an altitude of 1603 m (msl). Its soils are Humic nitosols. It receive a mean monthly rainfall is 87.3 mm and has a mean monthly minimum temperature of 12.9°C and mean monthly maximum of 25.1°C. The site is situated in a low potential coffee zone where irrigation is used to supplement rainfall.

Plant materials

The plant materials used in this study comprised F_1 hybrids lines.

Traits recorded

Samples for assessment of liquor quality were further selected with the help of ultraviolet Sortex instrument to remove beans with mechanical or insect damages. Two samples (one per replicate), each of 250g, were taken from each family and sent blind to members of the Mild Coffee Trade Association of East Africa (MCTA) for characterisation of bean and liquor quality traits including the following: (1) Quality of roast beans (QRB) with

Liquor quality was determined on coffee samples from 4 trees per plot. Samples for assessment of liquor quality were processed using the wet method of coffee preparation. Samples for assessment of liquor quality were selected with the help of ultraviolet Sortex instrument to remove beans with mechanical or insect damages. Two samples (one per replicate), each of 250g, were taken from each family and sent blind to members of the Mild Coffee Trade Association of East Africa (MCTA) for characterisation of bean and liquor quality traits including the following:

1) Acidity of liquor - with a score of 0-4
2) Body of liquor with a score of 0-4
3) Flavour of the liquor - with a score of 0-6
4) Overall standard - the overall preference of liquor quality on basis of the above attributes, with a score of 0-6.

In the scoring system, zero represents fine liquor quality and is desirable whereas the higher score progressively indicate poorer liquor quality.

Statistical analysis

A randomised Complete Block Designe (RCBD) was used in the study. Data from individual locations were analysed with the help of MSTAT statistical software according to the model:

$$y_{ij} = \mu + F_i + r_j + \overline{E}_{ij}$$

where : y_{ij} = performance of the i^{th} family in the j^{th} replicate, μ = overall mean performance, F_i = effect due to the i^{th} family, r_j = effect due to the k^{th} replicate in environment j and $\overline{E}_{ij}$ = random error of plot means.

Association analysis

Linear correlations and Spearmans rank correlations between acidity, body, flavour and overall standard, three were calculated following standard procedures as outlined in Zar (1984).

Sensitivity Analyses

To determine the utility of the various liquor quality components as criteria for judging the overall liquor quality of Arabica coffee, the sensitivity analysis as described by Bradley and Schumann (1957) and Schumann and Bradley (1959) was used. Pair wise comparison between overall preference rating (Overall standard) and acidity, body and flavour respectively was conducted to determine the liquor quality component which would indicate the overall liquor quality of test genotypes with the greatest efficiency. The test statistic was calculated in accordance to Bradley and Schumann (1957) and Schumann and Bradley (1959).

Results and Discussion

The analysis of variance for the various liquor quality traits are shown in Table 1. Both variation due to family effects and family by assessor interaction effects were not significant. On the other hand, variation due to the effect of assessors was highly significant ($P \leq 0.001$) thus emphasising the importance of the testing panels in the determination of the liquor quality of Arabica coffee. From the breeding point of view the result indicates the possibility of a given genotype being assigned different quality ratings depending on the panel used in the evaluation. This has indeed been pointed out as one of weakness of the organoleptic procedure (Walyaro, 1983) and may reduce the efficiency of selection of improved liquor. The problem is further compounded by the low repeatability of liquor traits usually observed (Walyaro, 1983). It would therefore be of considerable interest to breeders if more objective scientific procedures were developed for use as basis of discriminating between genotypes for liquor quality. In this regard, recent developments in gas sensors (Mitrovies et al, 1997) will go along way in improving the efficiency of selection for improved liquor quality in Arabica coffee. Immediate improvements of efficiency of selection of improved quality will however depend on the ability of the breeders to identify quality traits which have high intensity of expression, are closely associated with the overall preference and can be determined with a high fidelity by liquor quality assessors.

Results shown in Table 2 indicate that the overall preference is positively correlated (P# 0.0017) to the three major components of liquor quality traits, namely, acidity, body and flavour. The highest correlations were observed between overall preference and flavour. Similarity, the Spearmans rank correlations were observed between the overall standard and flavour (Table 3). These results indicate that all the three liquor traits could be useful as indicators of the overall quality.

From the sensitivity analysis (Table 4) it was observed that the precision with which the panels could detect differences in acidity was less than the precision with which the same panels could detect the global differences in overall quality. This indicates that acidity may be inferior to the overall preference rating in discriminating between genotypes for liquor quality. The body and flavour were as efficient as the overall preference score in determining quality difference between different genotypes. Their use, especially the use of flavour may however be more advantageous since they have a definite genetic origin as opposed to the overall preference score which is reached at based on some arbitrary hedonic scale. It is therefore concluded that flavour rating could be a useful criteria for judging the worth of breeding lines for liquor quality.

Acknowledgements

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Table 1: Analysis of variance for liquor quality traits in Arabica coffee hybrids grown in Three (3) localities in Kenya

Source	Acidity				Body				Flavour				Standard			
	df	MS	F	P	df	MS	F	P	df	MS	F	P	df	MS	F	P
Reps - (Localities)	2	0.088	0.26		2	0.431	1.15		2	0.472	1.99		2	2.231	68	***
Hybrids (A)	15	0.412	1.21		25	0.207	0.55		25	0.205	0.87		25	0.362	1.10	
Assessors (B)	2	11.981	35.2	***	2	38.421	102.31	***	2	57.512	242.92	***		13.149	40.08	***
A x B	50	0.221	0.65		50	0.286	0.76		50	0.157	0.66		50	0.241	0.74	
Error	154	0.340			154	0.376			154	0.237			154	0.328		

*** P≤ 0.001

Table 2. Correlations between some quality traits in Arabica coffee hybrids

	Raw	Roast	Acidity	Body	Flavour	Standard
Raw	1.000					
Roast	0.150	1.000				
Acidity	0.123	0.008	1.000			
Body	0.087	0.107	0.495	1.000		
Flavour	0.290***	0.072	0.638***	0.578***	1.000	
Standard	0.595***	0.278***	0.393***	0.442***	0.739***	1.000

*** $P \leq 0.001$

Table 3. Spearmans rank correlations between preference note and three liquor quality for 24 Arabica coffee hybrid

	Overall Standard			
	rs	t	Probability	
Acidity	0.63	3.99	***	
Body	0.39	2.09	*	
Flavour	0.71	4.97	***	

* $P \leq 0.05$
*** $P \leq 0.001$

Table 4. Sensitivity analysis for the efficiency of three liquor traits in determining the overall liquor quality of Arabica coffee

Parameter	Acidity	P	Body	P	Flavour	P
λ_1	34.238		101.184		242.667	
λ_2	39.088		39.088		39.088	
λ	36.663		70.130		140.878	
ω	0.880		2.250	**	6.061	**
ω_0 (0.01)	2.110		1.840		1.530	
a	19.345		36.074		71.443	
b	77		77		77	

** $P \leq 0.01$

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Summary

Liquor quality is undoubtedly the most important factor that determines the suitability of coffee for human consumption. For the Colombian type Arabicas, acidity, body, flavour and preference rating (overall standard) are the main criteria used in determining the superiority of a given coffee sample for commercial use. In this paper, the four traits are evaluated for their suitability as selection criteria for the genetic improvement of the overall liquor quality in Arabica coffee. Based on correlation, repeatability and sensitivity analyses on data collected over a period of 27 years, it is concluded that flavour rating is the best selection criterion for the genetic improvement of liquor quality in Arabica coffee. The trait shows high genetic correlations with preference, is easy to determine organoleptically and has a high relative sensitivity in discriminating among different coffee genotypes.

Key Words : Arabica coffee, liquor quality, genetic improvement, selection criteria

INCREASING ROBUSTA PRODUCTION IN BRAZIL THE POTENTIAL OF 200 THOUSAND HECTARES IN SÃO PAULO STATE *

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SUMMARY

Indonesia, Vietnam, Brazil, Ivory Coast and Uganda are the largest producers of robusta coffee in the world. In Brazil, robusta is grown in States other than São Paulo. For many years, experimental and observation plots have been set up by the Instituto Agronômico in order to ascertain the potential for cultivating robustas in this State provided that 50% of the instant coffee industries are located in West of the State and there are 200 thousands ha with edapho-climatic aptitude for growing robusta. Nevertheless, in this region there is a widespread occurrence of nematodes as *Meloidogyne incognita, M. paranaensis, M. exigua, Pratylenchus spp* and also of leaf rust disease, *Hemileia vastatrix*. Recently the bacteria *Xylella fastidiosa* has been considered as a possible additional agent collaborating for the bankruptcy of arabica coffee cultivation and the consequent impoverishment of farmers in this vast region. Selection for vigor, yield, bean size and type, leaf rust and nematode resistances, resulted in the release of Apoatã (IAC 2258) cultivar of *C. canephora* which is specially suited as rootstock for all arabica cultivars. The observation of many seed plots of IAC 2258 in several localities of this region as well as controlled experiments in Campinas, Mococa, Ribeirão Preto, Pindorama and Votuporanga indicated several other lines with high yields, rate of dry fruits/green bean up to 65%, variable earliness and large flat seeds up to 90%. The characteristics of the selected lines and clones are discussed in this paper. So far, promising lines are: IAC 66-1, IAC 66-3, IAC 67-9, IAC 68-10, IAC 69-7, IAC 70-1, Guarini (IAC 1598-1), Robusta col 10, IAC 640, IAC 1645-5, IAC 1647-1, IAC 1647-7, IAC 1655-7 IAC 2290 and IAC 2291. A few hundred clones are currently under evaluation. The low altitudes, hot climate, poor and eroded soils infested with nematodes limit many agriculturally attractive options of this marginal region for producing arabica coffee. Thus, the success of this enterprise could have a high social impact and may represent an economically valuable solution for this former arabica producing region of São Paulo.

POSTER

INTRODUCTION

Very few crops have such a recent history of domestication and impressive economic importance as coffee cultivation in the world. The first introduction of coffee plants in Brazil dates back to only 272 years ago (11, 13, 16). The supremacy of the country's production and exportation is largely based on the species *Coffea arabica*. However the robusta coffee (*C. canephora*) which accounts for 30% of the world trade is also a quite valuable agribusiness, being Brazil the second largest producer with 850 million plants and 4.7 million bags (60kg) per year. There is an increasing demand in the international market for robusta coffee. Over 90% of the robusta in Brazil is grown in the States of Espírito Santo and Rondônia located from 1000 to 4000km away from the instant coffee manufacturers of São Paulo State that buy 50% of the production.

Although the price of robustas is usually 20% less than arabicas its production cost is considerably lower. Compared with arabicas, robustas are, as the species name suggests, sturdier, with vigorous

roots, more resistant to diseases and drought. In most cultivars the berries stay firmly attached to the branches even after ripening.

ROBUSTA COFFEE IN SÃO PAULO – THE EXPERIENCE OF IAC

The São Paulo State (SP) is traditionally a producer of arabicas. Historically the main coffee rush in Brazil had São Paulo as the major base of production, latter spreading out to the Southern and Northern States. Presently, coffee cultivation is facing a steady decline in a vast region of SP (FIGURE 1) due to the erosion of its sand soils, hot climate and a widespread infestation of nematodes in arabica fields, already suffering with heavy attacks of rust and leaf miner. General impoverishment due to limited agricultural options, is the situation of most coffee farmers, the majority of them small holders, in this extensive region that comprises about 200,000 hectares.

In order to withstand the adverse condition of the above mentioned region, investigations were carried out at the Instituto Agronômico de Campinas (IAC) in order to identify sources of resistance to the nematodes *Meloidogyne exigua* (8, 9), races of *M. incognita* (6, 10), *M. paranaensis* and *Pratylenchus*. Once multiple resistance was not readily available among *C. arabica* lines, an overall survey of germplasm was realized in order to develop resistant rootstock cultivars.

Among a number of diploid species tested, *C. canephora* was soon identified as the most promising one as rootstock. Hence, its several accessions and lines were more intensively studied. Basically, the most important lines were selected among acessions of cultivar robusta of a collection established in the 30'^{s} with seeds bred in Indonesia, also among plants of the cultivar Conilon introduced in the 30'^{s} from Espírito Santo State and from

FIGURE 1. Climatic zonning indicating apt regions for Robusta cultivation in São Paulo State, Brazil. Mean annual temperatures 22-26oC, water deficiency < 150 mm, Cwa and Aw climates. A. Altitudes < 500m, 51oW 21oS; B. Altitudes < 200 m, 48oW 24o30'S (Camargo *et al.*, 1999).

a series of accessions secured in the 70'^{s} belonging to IICA of Turrialba, Costa Rica.

Initial screenings were performed in protected greenhouses with nematode infested soils from different areas. Progenies from promising lines were planted in infested fields and recurrently subjected to cycles of single plant selections.

Elite plants cuttings of different fields were also made and special plots of them were set up, thus promoting, by the natural cross pollination (3, 4), the ensuing recombination and concentration of

genes for resistance. Alternatively, in some plots, unselected plants were strategically cut back prior flowering and seeds from remaining plants were used for new cycles of selection. These selection cycles were made with different populations of nematodes for which, tests with differential hosts revealed, not rarely, a different race of *M. incognita* or even the occurrence of other species in the same population. Although this complex situation precluded , except for a few cases, fine studies on the genetics of coffee resistance genes/alleles correspondent to specific nematode race/species it nevertheless, broadened as the selection cycles progressed, the spectrum of resistance among elected plants and their progenies. For instance, selections still in progress, have identified single plants resistant to all the four races of *M. incognita*, in addition to *M. exigua*, *M. paranaensis*, and *Pratylenchus*.

On the course of this programme the selections also took into account the type of resistance to leaf rust (*Hemileia vastatrix*), shape, size and outurn of beans, percentage of peaberry, besides general vigour and yield of the plants. The highest yielding lines, regardless the level of nematode resistance, were also tested in competition trials at several regions of SP State such as Campinas, Mococa, Ribeirão Preto, Pindorama, Adamantina and Votuporanga. Trials were also set up in the State of Rondônia (15) allowing for an evaluation at a more typical region cultivating robustas.

A specially interesting line (T 3561) was identified among the accessions from Turrialba. Initial greenhouse tests showed that it was highly resistante to *M. exigua* and 70% of the plants were also tolerant to one population of *M. incognita* and about 50% of them to other populations of the same species. In further tests this line proved to be also a reliable source of resistance to *M. paranaensis* (formerly *M. incognita* race 5). Four cycles of selection in greenhouse and infested fields considerably increased the percentage of resistant plants in areas infested with different nematode populations. The progenies of more advanced selections were released as a rootstock cultivar, IAC 2258, and named Apoatã, meaning 'strong root'. It is congenial with and induce good agronomic performance in different arabica cultivars grafted onto it. It is also suitable as a rootstock for other robustas.

Seed plots of Apoatã rootstock were then rapidly established by growers, cooperatives and State Extension Service over the Western São Paulo. Years of observation of these plots of Apoatã revealed an outstanding adaptation to these areas with lusty plants producing copious harvests in many places. This fact drove the attention of the IAC researchers suggesting the possibility of exploiting it, not only as a rootstock, but also as a robusta cultivar for bean production (2, 7).

Besides the evident social impact stimulating such an enterprise, the technical aspects of it also strongly support the idea. The climate in the West region of São Paulo is marginal to arabicas, but quite apt for cultivation of robustas (FIG 1) (2) where altitude is below 500m, annual mean temperature is between 22-26°C and water deficiency is less than 150mm. Such conditions are similar to the ones found in the native habitats of *C. canephora* in Africa (3). This potential area for growing robustas in São Paulo State comprises 198 cities in the Western region in addition to another 14 in the Southeast. Most farmers have expertise in coffee cultivation and still keep appropriate infrastructures in the farms. The cultivation of robusta in this area may represent a valuable economic option and an opportunity of social amelioration of the region.

CULTIVARS AND LINES

During the investigations, chiefly regarding selection for nematode and rust resistance within *C. canephora*, lines were also selected on the basis of yield and bean type. Nematode susceptible lines can be used either as cultivars in non infested areas or grafted onto robusta cultivar Apoatã. The main characteristics of the selected cultivars (FIG 2) are outlined.

Conilon – It is usually a highly variable cultivar. Subjected to single plant selections it has shown individuals with lower levels of rust and leaf miner (*Perileucoptera coffeella*) attack. This suggests the occurrence of horizontal resistance to both rust and leaf miner among its plants. General characteristics of this cultivar grown in São Paulo is shown in FIGURE 3.

Figure 2. Some characteristics of *C. canephora* cultivars grown in São Paulo State for 10-13 years.

Characteristic	Cultivar			
	Conilon	Guarini	Robusta	Apoatã
Average yield kg/plant	1.0	1.3	1.1 – 1.3	1.2
Plant height (m)	3.0 – 4.0	3.0 – 4.4	2.4 – 4.3	4.5
Days to ripe	290 - 320	320	320	320
% flat beans	68.4 – 81.6	65.0 – 95.0	67.6 – 94.1	90.0
Weight of 1000 seeds (g)	8.2 – 10.2	13.1	13.9	14.1
Bean grade	13 – 15	15 – 18	16 – 19	11 – 19
Soluble solids (%)	24 – 29	28.3	27.8 – 29.3	31.4
Caffeine content (%)	1.76 – 2.37	1.66	1.43 – 1.81	1.70

Figure 3. Comparative data of selected clones of cultivar Conilon assayed for 13 years in São Paulo.

Clones	Yield Kg/plant	Vigor	Rust	Height M	Bean grade	Maturity	% flat beans	Soluble solids
IAC 66-1	4.3	9	R	3.9	15.4	M – L	66	27.9
IAC 66-3	3.2	9	R	4.2	16.4	M	80	28.2
IAC 67-9	2.4	8	MR	4.5	14.0	M	--	--
IAC 68-10	1.8	8	MR	4.3	16.2	E	51	--
IAC 69-7	1.8	7	R	4.5	14.1	E	77	--
IAC 70-1	2.8	8	R	4.3	15.2	M	81	29.3

Selected lines from it (FIG 4) differ as to yield, vigor, rust reaction, and maturity. They have, in general, small beans with a brownish silver skin quite adherent depreciating its appearance. Caffeine content varies from 1, 7 to 2,3%. It shows high resistance to *M. exigua* and varies as to other species and races.

Figure 4. Selected lines of *C. canephora* cultivars developed by IAC.

CULTIVAR	LINES
Conilon	IAC 66-1; IAC 66-3, IAC 67-9; IAC 68-10; IAC 69-7; IAC 70-170-1
Guarini	IAC 1598-1
Robusta	IAC 640; IAC 37; IAC 1645-5; IAC 1647-1; IAC 1647-7; IAC 1655-7; IAC col 10
Apoatã	IAC 2258

Guarini – It has larger seeds than Conilon. Some plants are resistant to all tested races of leaf rust. It is highly resistant to the nematode *M. exigua* and also, to a certain degree, to *M. incognita and M. paranaensis*. Root system is quite vigorous and yields are generally good. At present, only one line (FIG 4) is recommended, but several other clones are under investigation.

Robusta – This cultivar, introduced a long time ago in the IAC collection represent an important source of variability for rust (12) and nematode resistance introgressed into *C. arabica*. Some plants display resistance to all races of rust. It is highly resistant to *M. exigua* and some progenies are also resistant to some populations of *M. incognita* and *M. paranaensis*. Since it is variable and not throughly tested for nematodes, it is recommended to be grafted onto Apoatã if grown in soils infested with nematodes other than *M. exigua*. There are several lines of them (FIG 4) with high yields and resistance to rust. It is a late cultivar (FIG 2), with beans larger than Conilon

with nematodes other than *M. exigua*. There are several lines of them (FIG 4) with high yields and resistance to rust. It is a late cultivar (FIG 2), with beans larger than Conilon

Apoatã – Derived from several cycles of both greenhouse and field selection of a Turrialba (T 3561) accession, Apoatã (IAC 2258) is resistant to *M. exigua*, highly resistant to *M. incognita* races and *M. paranaensis*. Since nematode populations are very complex and not completely understood, some susceptible plants may occasionally occur. Also it is resistant to leaf rust. It is a late cultivar producing heavy crops of large (grade 19) flat (90%) beans. Cup quality is considered to be neutral, as other robustas (14).

CONCLUDING REMARKS

Although the prospects of cultivating robustas in São Paulo State is very promising and there is a number of selections available, the extent of the potential land is extremely large. This implies that more intensive experimentation is needed in order to establish commercial large scale fields. These and other cultivars and lines should be assessed in competition trials over many more localities, coupled with studies of appropriate cultivation systems and joint cooperative work with the instant coffee manufacturers. The organoleptic qualities of the beans so produced must be fully evaluated. Clonal evaluations (5) and production of hybrid cultivars should be emphasized in the selection programmes. Largely negleted in the studies of *C. canephora*, breeding for quality and the use of interspecific hybridization exploiting the natural variability of selected species may substancially contribute for the future improvement of robusta coffee.

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COFFEE CULTIVARS IN BRAZIL

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SUMMARY

A thoughtful choice of cultivars associated with appropriate farming management is one of the most important components assuring the success of coffee cultivation. Research on genetics and coffee breeding at the Instituto Agronômico de Campinas, SP, started 67 years ago and led to the development of tens of cultivars and lines and the accumulation of considerable knowledge on their characteristics and behavior in the diversified growing regions of Brazil. More than 90% of the 4 billion coffee trees presently cultivated in Brazil derive directly from this program. This contribution spans as well to 5 other main research institutes where breeding and regional selection started after 1970. The overall result of these programs is the present distinct recommended cultivars and lines. This provides the growers with a range of options to meet particular requirements of climate, farming technologies and final products. Among arabica cultivars, all are seed propagated and can be grouped into: a) tall, susceptible to leaf rust, b) tall, resistant to leaf rust, c) short, susceptible to leaf rust, d) short, resistant to leaf rust, e) with distinct cup quality. Among robustas, available cultivars are a) seed propagated, b) clonal propagated and c) nematode resistant rootstock. In each group there are several lines, in a total of 70. The main characteristics of them such as potential yield, size and type of beans, color of fruits, earliness, resistances, recommended spacing, general adaptativeness, reaction to pruning and distinguishable attributes are summarized in this paper.

POSTER

INTRODUCTION

In 1932, the Instituto Agronômico de Campinas (IAC) in Brazil, laid the foundations of a sound project on genetics and coffee breeding anticipating the demands of farmers and foreseeing the continuous economic importance of coffee cultivation in Brazil. Basically this programme involves intensive efforts for the establishment and maintenance of a large collection of species, varieties and lines of coffee, studies on evolution, citology, basic genetics, biology of reproduction and breeding techniques associated with specific research on phytopathology, entomology and nematology. In these 67 years of uninterrupted investigations, a number of lines were developed (9), tested, and presently recommended for growing in the diversified coffee regions of the country. Over 90% of the 4 billion coffee trees in Brazil belong to cultivars developed by the IAC. Some of those are planted in other countries as well, like Caturra Vermelho, Caturra Amarelo, Catuai and Mundo Novo. Several other cultivars, although not recommended anymore, were quite important in the past. The development of short stature, high yielding, durable cultivars such as Catuai Vermelho and Catuai Amarelo modified production systems, increased profitability of farmers and created valuable opportunity for growing a cash crop in former non-productive lands like the Brazilian Cerrados. Tall cultivars like Acaiá being mechanically harvested are also suited to that region. Tracing back to a 1950 cross of *Coffea canephora x C. arabica*, the new lines of Icatu Vermelho, Icatu Amarelo and Icatu Precoce (2,3), released in 1992, are resistant to leaf rust (*Hemilea vastatrix*) providing considerable savings for the

This paper is a tribute to C.A. Krug, A. Carvalho and L.C. Monaco, milestones of coffee breeding in IAC.

grower (5), lower rates of environmental pollution, and less health risks for growers and consumers. Icatu lines have cup quality as good as the standard Catuai and Mundo Novo. Some lines however, due to an additional backcross to Bourbon, have even better quality. Short stature cultivars such as Obatã and Tupi are resistant to rust and have been recommended since 1996 for close spacings and mechanized harvest.

The widespread occurrence of nematodes chiefly, *Meloidogyne* species and races in São Paulo State led to the selection of the resistant Apoatã cultivar of *C. canephora* as a rootstock for all recommended arabicas and robustas. This made viable the return of arabica cultivation in nematode infested soils of several States of Brazil and abroad. Moreover, cultivation of robusta itself in 200.000 ha of São Paulo is also a potential benefit of this cultivar that bears large flat beans on high yielding trees resistant to rust.

Other research centers in Brazil started breeding programs and regional selections after 1970. This was the case of IAPAR, EPAMIG, EMCAPA, MARA-PROCAFÉ, UFV and UFLA. Only EMCAPA has a strong focus in the breeding of robustas, mainly for State of Espirito Santo. Regional, open pollinated selections and vigorous clones of Conilon have been released and are recommend for planting in low altitudes, where robustas are grown.

COFFEE CULTIVARS

C. arabica is a tetraploid ($2n$=44), autogamous species and normally is not propagated from cuttings or mature grafts. Thus breeding strategies were directed toward the development of homozygous cultivars breeding true from seeds (12). In most cases, pedigree selection preceeded by hybridization was the breeding method used. Developed cultivars can be grouped into: a) tall, susceptible to leaf rust; b) tall, resistant to leaf rust; c) short stature, susceptible to leaf rust; d) short, resistant to rust and e) with distinct quality attributes.

C. canephora is a diploid ($2n$=22) self-incompatible species, reproducing by cross pollination. Usually, lines and recommended cultivars display considerable degree of variation allowing further selections to be made. Since it roots promptly and orthotropic shoots are easily induced by bending the branches, individual plant selections can be easily cloned and plots are thus established from them, provided donor plants are compatible. Therefore, among robustas, available cultivars are either seed or clone propagated. Nematode resistant lines are, however, only seed propagated since coffee grafts are done soon after germination of both rootstock and scion. Among arabica and robusta cultivars there are several lines that reflect selections for some distinct plant type, regional adaptation or other characteristic. These provide the growers with a range of options to meet particular requirements of climate, farming practices and final products. The general characteristic of them are summarized in this paper (FIG 1,2).

TALL CULTIVARS SUSCEPTIBLE TO LEAF RUST

Bourbon Amarelo – Although old Bourbon Vermelho fields are found in Brazil, it is not highly recommended since the selected yellow version of this cultivar, Bourbon Amarelo, yields 40% more (1). Its lines were released in 1945 after extensive experimentation dating back to 1930, when C. A. Krug found in Pederneiras, SP, a yellow coffee thought to be a natural cross of Bourbon Vermelho and Amarelo de Botucatu. Bourbon Amarelo represents earliest cultivar, maturing one month or more before Mundo Novo. For this reason it is recommended for high altitudes or to spread out the harvesting time, although it yields 50% less than Mundo Novo or Catuai. There is a consensus that this cup quality is superior to Mundo Novo or Catuai.

Mundo Novo – This cultivar was found in Urupes, SP in 1943 and selected up to 1952 (5). It is, probably, a natural cross of Sumatra and Bourbon Vermelho. At the time it was released produced 80% more than the original unselected M. Novo plants, 50% more than Bourbon Amarelo, 95% more than Bourbon Vermelho and 240% more than Typica, in three different regions. The plants are

Figure 1. General characteristics of some cultivars released by IAC. Correspondent lines are shown in FIG. 2.

Cultivar	Color Young shoots	Plant Stature	Fruit color	Maturity uniformity	Bean grade	Rust resistance	yield	Obs.
Bourbon Vermelho	Green	tall	red	early, u	16	S	low	Good cup quality for v..high altitudes
Bourbon Amarelo	Green	tall	yellow	v. eraly, u	16	S	aver. *	Good cup quality - * see text
Ibairi	Bronze	tall	red	med, u	13	S	low	Excellent cup quality
Mundo Novo	G.B	tall	red	medium	17	S	high	IAC388-6 and –17 have long lateral branches
Acaiá	Bronze	tall *	red	med-early, u	18-19	S	high	Dense plantings – mech. Harvesting - * short lateral branches
Catuai Vermelho	Green	short	red	late	16	S	high	Dense plantings – mech harvest- windy areas
Catuai Amarelo	Green	short	yellow	late	16	S	high	Dense plantings – windy areas- mech harvest
Icatu Vermelho	B,G	tall *	red	m-late **	17-18	R ***	high	* vigorous ** fruits tightly attached *** horizontal
Icatu Amarelo	B,G	tall *	yellow	m-late **	17-18	R ***	high	* vigorous ** fruits tightly attached *** horizontal
Icatu Precoce	B,G	tall	yellow	early *	16	HR	high	** fruits tightly attached
Obatã	Green	short *	red	late	17-18	R	high	* vogorous - well branched - fertile soils/for dense plantings – windy areas
Tupi	Bronze	short	red	early	17-18	R	high	For dense plantings - windy areas/fertily soils
Apoatã	Bronze	v. tall	red	v. late	17-18	R	high	Nematode resistant *C. canephora* rootstock. – S. solids 31,2% - caffeine 1,7%
Robusta	Bronze	v. tall	l. red	v. late	17	R	high	S. solids 26,3 – 31,4% - caffeine 1,4% - 1,8%
Guarini	Bronze	v. tall	l. red	v. late	16-17	R	high	S. solids 27,5 – 28,3% - caffeine 1,7%
Conilon	Bronze	v. tall	l. red	early-medium	14-16	S	average	H. resistance to leaf miner- S.solids 27,0% - 29,9%. – Caffeine 1,7 – 2,40%
Laurina	Green	v. short	red	medium	14	V.S	v. low	0,6% caffeine

vigorous, adapted to a wide range of environmental situations, bearing heavy crops during many years (11). Some original test plots are still in production. Several lines have been selected. Among them, some show better adaptation to specific regions, while others display longer lateral branches. It is generally recommended for wide spacings or for use with systematic pruning.

Acaiá – It is a selection of Mundo Novo, prefix IAC 474, characterized by larger seeds than Mundo Novo and shorter lateral branches. With good adaptability and yields it is recommended also for mechanical harvest at closer spacings than Mundo Novo.
Acaiá Cerrado – It is a line of Acaiá with good adaptation to some regions of Minas Gerais State. It was released by EPAMIG. General characteristics are similar to Acaiá, mainly to the line IAC 474-1.

Figure 2. Presently recommended lines of *C. arabica* and *C. canephora* cultivars released by IAC.

CULTIVAR	LINES
Bourbon Vermelho	
Bourbon Amarelo	
Mundo Novo	IAC 379-19; IAC 376-4; IAC 382-14; IAC 388-6; IAC 388-17; IAC 464-12; IAC 515-20; IAC 501-5; IAC 502-1; IAC 467-11; IAC 480-6
Acaiá	IAC 474-1; IAC 474-4; IAC 474-6; IAC 474-7; IAC 474-19; IAC 474-20
Catuaí Vermelho	IAC H2077-2-5-15; IAC H2077-2-5-24; IAC H2077-2-5-44; IAC H2077-2-5-72; IAC H2077-2-5-81; IAC H2077-2-5-99; IAC H2077-2-5-144
Catuaí Amarelo	IAC H2077-2-5-17; IAC H2077-2-5-32; IAC H2077-2-5-39; IAC H2077-2-5-47; IAC H2077-2-5-62; IAC H2077-2-5-74; IAC H2077-2-5-86; IAC H2077-2-5-100
Icatu Vermelho	IAC 2941; IAC 2942; IAC 2945; IAC 4040; IAC 4041; IAC 4042; IAC 4043; IAC 4045; IAC 4046; IAC 4228
Icatu Amarelo	IAC 2944; IAC 3686; IAC 2907
Icatu Precoce	IAC 3282
Obatã	IAC 1669-20
Tupi	IAC 1669-33
Apoatã (porta-enxertos)	IAC 2258

TALL CULTIVARS RESISTANT TO LEAF RUST

The rust resistance of the tall cultivars was originated from the cross of an artificial tetraploid *C. canephora* (2*n*=44) and a normal Bourbon Vermelho of *C. arabica* (2*n*=44) performed in 1950 at IAC followed by selfings and backcrosses to Mundo Novo and/or Bourbon Amarelo. These populations were analyzed at Centro Internationacional das Ferrugens do Cafeeiro (CIFC) in Portugal and extensively tested in many regions in Brazil for rust resistance, yield, uniformity and other agronomic characteristics (2,13). Rust resistance can be of vertical or horizontal type characterized by small pustules and reduced leaf fall. Some lines are also source of nematode and/or CBD resistance. Generally this plants are quite vigorous, leafy, with abundant secondary branches and strong roots. Fruits usually stay on the branches longer and are attached more firmly than in regular cultivars.
Icatu Vermelho – Plant height and diameter are similar to Mundo Novo. Rust resistance varies from total immunity to horizontal resistance of different degrees. The cherries are red and strongly held on the branches. It produces 20% of peaberry, but almost no elephant beans. With wide adaptability it has a vigorous growth and excellent regrowth after pruning. Yields and cup quality are similar to Mundo Novo.

Icatu Amarelo – It was derived by selections in a field of Icatu Vermelho, segregating for fruit color, possibly due to a natural cross occurred in 1970 with Bourbon Amarelo or Mundo Novo Amarelo. Except for fruit color, these lines are very similar in morphology and agronomic behavior those of Icatu Vermelho. Although both have been cultivated in altitudes as high as 1300 meters, which is a high-grown crop condition at São Paulo latitude, it can grow well in low lands below 400m, marginal to arabica. This characteristic was probably inherited from *C. canephora.*

Icatu Precoce – Selected from a Icatu population segregating for yellow fruits, this cultivar has horizontal rust resistance. In some places, according to the prevailing race, some plants can develop normal infection. The trees are smaller than Icatu Amarelo, leafy, requiring fertile, well prepared soils and more frequent fertilizations. In such situations it is a good yielder, producing an early crop tightly hold on the branches with beans similar to Bourbon Amarelo, thus smaller than Mundo Novo. The most distinctive attribute of this cultivar is its consistent good cup quality. According to some roasters, it is specially suited for espressos.

SHORT STATURE CULTIVARS SUSCEPTIBLE TO RUST

Short stature cultivars, derived from crosses of high yielding tall cultivars with the short stature Caturra Amarelo which carries the dominant allele *Ct* for short internodes and compact plant type (7,15).

Catuai Vemelho – It is a short, vigorous cultivar, adapted to different regions and producing high yields and a late crop. The allele *Ct* interacts quite intensively with the environment resulting in plants taller than 3 meters high at high altitudes. Released in the 60's it was derived by pedigree selection from the cross between Caturra Amarelo and Mundo Novo, done in 1949. Production starts in the 2nd field year. The plants are compact and represent a good option for close spacing systems.

Catuai Amarelo – Developed concurrently with Catuai Vermelho, this cultivar has similar attributes, except for been homozygous for the yellow fruit xantocarpa factor (*xc xc*). Similarly to the Vermelho counterpart it is a late variety with a somewhat desuniform maturity. At very high altitudes, above 1200m, ripening occurs too late, almost at the flowering time, fact that precludes its cultivation in this situation. Beans are larger than Bourbon and it produces up to 90% of flat beans. In some years, low rates of fertilization leads to an increased amount of elephant beans. Several lines tested in close spacings, with heavy fertilizer application, have produced high yields for many years.

Catuai Rubi – The Catuai Rubi is a selection at EPAMIG (10) of a cross made at IAC in 1961 between Catuai Vermelho and Mundo Novo. It has very good vigour, probably due to the backcross to Mundo Novo. It seems to have the plagiotropic branches more spread out, resembling the line Catuai Vermelho, H 2077-2-5-44. Accordingly, it seems a little earlier and with more uniform ripening than other lines of Catuai Vermelho. The yield is similar to the – 44 line

Topázio – Similar to the above, Topázio is a short stature cultivar, selected by EPAMIG in lines derived from a backcross of Catuai Amarelo to Mundo Novo done by IAC in 1961. It has good yields and vigour, yellow fruits and uniform ripening.

SHORT STATURE CULTIVARS RESISTANT TO RUST

The development of short stature rust resistant cultivars relied on the use of the dwarfing gene *Ct* (Caturra) originally present in the cultivar Caturra Amarelo studied at IAC, later transferred to the cultivar Catuai, or also on *Vs* (Vila Sarchi) which is an allele at the *Ct* locus, originally present in the corresponding cultivar from Costa Rica. The source of rust resistance traces back to *C. canephora*, rescued either from cultivar Icatu, which is a backcross pedigree derivative cultivar of the cross carryed out at IAC *C. arabica* x *C. canephora*, or from Timor

Hybrid, a natural backcross segregating population of similar origin found in Timor Island by researchers of CIFC. Crosses of Vila Sarchi from IAC collection with Timor Hybrid were done at CIFC in Portugal and sent to IAC in Campinas, where they were further advanced.

Tupi – F_2 seeds from the F_1 hybrid H361-4 (Vila Sarchi x Timor Hybrid) seeds were planted in 1972 at IAC. Pedigree selections were carried out up to F_6 resulting the short stature rust resistant cultivar Tupi. Yield potential is similar to Catuai Vermelho (FIG. 3) but it is earlier, the seeds are larger and the plants are shorter than Catuai or Obatã . The compact size and rust resistance make it specially recommended for close spacings.

Obatã – It has a similar origin to Tupi except that a natural cross of a selected F_2 plant of H361-4 occurred with Catuai Vermelho and was used as a control in the selection field. F_2 of this BC2 and subsequent selfing generations were advanced by the pedigree method, giving rising to this high yielding rust resistant short stature cultivar. In some regions, it yields more than Catuai Vermelho, mainly when the first six crops are compared (FIG. 3). The large beans are similar to Tupi and maturity, in some places, is even later than Catuai. It is also highly recommended for dense plantings.

IAPAR 59 – The origin of this cultivars is the same of Obatã (16). In 1975 F_3 seeds of a selected plant were sent to IAPAR and subjected to pedigree selection. It has very good yield potencial. The plants are shorter and earlier than Catuai Vermelho, less vigorous, requiring more fertile soils and more frequent fertilizer application. The seeds are similar to Catuai. It is recommended for close spacings with pruning after the 5th crop.

Catimor – It represents descents of F_3 and F_4 generation of Caturra Vermelho x Timor Hybrid (CIFC 831/1) with an excellent level of rust resistance (4). It has been selected in several places under different growing conditions. Extensive tests in São Paulo, conducted by IAC, revealed just a few promising progenies amongst a majority of ill adapted lines that die back after four or five good crops. In Costa Rica and Colombia, at higher altitudes, milder climates, less acid soils, similar lines of Catimor are yielding well. In Brazil, Catimor lines are still under more detailed evaluations being not recommended for commercial plantings.

Catucai Vermelho – From a test plot of Icatu at MARA-PROCAFÉ in Londrina, Paraná State, it was established a Icatu field in Rio de Janeiro State in 1986, where several short stature plants were spotted and lines were derived for further evaluation (18). The selected plants were presumably hybrids of Icatu and Catuai Amarelo-66. Catucai Vermelho has red fruits, good vigor under heavy fertilization, short stature and resistance to rust. Some progenies segregate for tall, short, general architeture of the plants and color of young shoots. Beans and yield are similar to Catuai. It came to be commercial in Minas Gerais State but is still under experimentation in São Paulo.

Catucai Amarelo – With origin and general characteristics identical to Catucai Vermelho it differs from the above with respect to the yellow color of mature fruits.

Figure 3. Agronomic performance of short stature cultivars after 6 years of consecutive harvests.

	Mean yield Kg/ha green coffee	Mean vigour evaluation	Mean height m	Mean canopy diameter m	Rust resistance
Obatã (1669-20)	2250	7.0	1.45	1.57	R
Tupi (IAC 1669-33)	2209	6.5	1.27	1.45	R
Catuaí Vermelho (IAC H. 2077-2-5-99)	2060	7.0	1.35	1.38	S
Catuaí Amarelo (IAC H. 2077-2-5-62	2186	7.6	1.42	1.50	S

CULTIVARS WITH SPECIAL QUALITY ATRIBUTES

Laurina – This cultivar was introduced a long time ago from Reunion Island (17), where it was known as Bourbon Pointy or Smyrna, and was considered there as a coffee of good quality. Plants are small, conical, densely branched, with short internodes, small leaves and pointed small beans. Plants are extremely susceptible to rust and have reduced yields. Its characteristics are determined by a pair of recessive alleles (*lr*) in Bourbon background. The distinctive feature regarding to its beverage is the low caffeine content. While normal arabicas have 1,2% of caffeine, Laurina have only one half (0,6%). Selections are in progress in order to increase yield and transfer rust resistance to this cultivar.

Bourbon Vermelho – The different lines of Bourbon Vermelho were released in late 30's and represent the first outcome of a selection program of IAC from a variable introduction from Reunion Island. There is a consensus that it produces a fine beverage, provided good growing, harvesting and processing conditions. It is not highly recommended because of lower yields, but maturation is uniform and vigor is reasonable. However, it is starting to be planted again at closer spacing, high altitudes aiming at special gourmet markets.

Bourbon Amarelo – Similarly to Bourbon Vermelho, this cultivar has superior cup quality. Selected progenies were released in 1945, after intensive regional yield tests, leading to lines 40% more productive than Bourbon Vermelho.

Ibairi – This cultivar, that in the Brazilian native language means "sweet small cherry", was selected from a cross of the original mokka mutant of genetic constitution *tt lrlr momo* with the Typica variety of genotype *TT LrLr MoMo*, followed by a backcross to Bourbon (*tt LrLr MoMo*) (6). Ibairi cultivar (*tt LrLr momo*) contrarily to the dwarf original mokka, has normal caffeine content and plant size similar to Bourbon , but with short internodes and small leaves, with salient domatias. The small, roundish dark green beans produce, after roasting, a fine, intense aroma. The beverage, with excellent flavour is full bodied, being considerably better than any other arabica so far assayed under comparable conditions in Campinas. It is not commercially grown in Brazil due to the reduced size of beans and relatively low yields compared to Mundo Novo or Catuai. Grown in small scale in Hawaii, it has confirmed there, the superior quality potential compared to Typica.

Icatu Precoce – Already mentioned in previous sections, the Icatu Precoce deserves a special attention for its good cup quality mainly in gourmet espresso preparations. Its earliness and quality attributes may be due to an additional backcross to the Bourbon cultivar. Other Icatu lines have only the first cross of Robusta with Bourbon and subsequent backcrosses to Mundo Novo or Catuai. Thus, they have aproximately one third of the genome of Bourbon. The Icatu Precoce has about 50% or even more of the Bourbon genome if one considers that the selections were made toward plants with smaller leaves, open branch type, and earliness, resulting also in smaller seeds, characteristics all from the Bourbon backcrossed genitor. Other sister lines of the same lineage developed are under investigation for superior quality.

CULTIVARS OF *C. canephora*

Although Brazil's main cultivated coffees are arabicas, the robustas are also important, provided almost 5 million bags are yearly produced. Grown in low lands with hot climates it is usually sold by inferior prices. However, the robusta crop requires less fertilizers, fruits do not fall on the ground, holding well on the branches, sprays are minimal and yields are quite high reducing considerably the production cost.

The species is self incompatible, thus outcrossed. This explains much of the physiological and morphological variation for plant and bean type found in commercial fields. Breeding methods are mostly single plant selections and hybridization followed by mass selections. More recently, clonal propagation of selected trees has been used on commercial scale.

Two distinct group of robustas are found under cultivation. The Guinea group, introduced from a region spanning from Guinea to Gana, and the Congo group from the basin of Congo River. Most of the robusta grown in Brazil belongs to the Conilon cultivar (a corruption of the botanical name kouillou) of the Guinea group with smaller beans and slightly higher caffeine content.

Conilon – This cultivar is widely planted at Espírito Santo State, north of Rio de Janeiro (8,19,20). Quite variable, it has been subjected to seed and clonal selections by EMCAPA. The representative of Campinas collection came originally from Indonesia in the 30's. Plants are very tall, up to 5m heigh, bearing lots of flowers and inflorescenses per leaf axil. As other robustas, mesocarp and endocarp are thinner and less sweet than arabicas. Percentage of flat beans is about 70% only. The beans are small with brownish silver skin firmly adherent and is highly susceptible to the bean borer (*Hypothenemus hampey*). It is susceptible to rust although at variable level.

Guarini – It is a IAC selection resistant to rust with larger seeds, higher percentage of flat beans than Conilon, maturing a month later. The vigorous root system is resistant to the nematode *Meloidogyne exigua* and to a few races of *M. incognita*. Yields are higher than Conilon.

Robusta – It is the standard type for *C. canephora*. Several accessions from Indonesia were studied and lines selected for rust and nematode resistance. One of the selected trees was used in the original cross with Bourbon to derive the cultivar Icatu. Later than Conilon, it is similar to Guarini regarded to seed and fruit characteristics, rust and nematode resistance.

Apoatã– The variability present in the original acession 2258 from Turrialba, Costa Rica, allowed for the progress of several cycles of single plant selection followed by mass selection for nematode and rust resistances as well as bean characteristics (14). Meaning "strong root", Apoatã is specially recommended for roostock of both arabica and robusta cultivars in areas infested with *M. exigua*, *M. incognita* or *M. paranaensis*. As many races of such parasites occurs and new species are frequently descrited, susceptible plants are often found. Grafted Catuai and Mundo Novo cultivated in non infested soils have outyielded non grafted plants. Apoatã has been also recommend as a normal robusta cultivar in State of São Paulo and Rondônia where several experiments were carried out. Being resistant to all rust races, several nematodes and producing a heavy crop of large, flat beans (90%) it is a very promising cultivar.

EMCAPA 8111, 8121 and 8131 - Released in 1993 the three cultivars are constituted by a mixture of 10, 15 and 14 clones, respectively, selected in commercial farms of Conilon in the State of Espírito Santo. With similar bean size, they differ in maturity being 8111 the earliest.

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TWENTY SEVEN YEARS OF COFFEE BREEDING IN KENYA : PROSPECTS FOR THE RELEASE OF NEW VARIETIES

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SUMMARY

The Coffee Breeding Programme in Kenya was initiated in 1972 with the objective of developing varieties which combine high yields and good quality with resistance to Coffee Berry Disease (CBD), *Colletotrichum kahawae* and coffee leaf rust. Tremendous progress have been made in the areas of CBD resistance, yield and quality improvement

Table 1. Progress in selection for yield and quality traits in Arabica coffee during three cycles of backcross breeding.

Generation of selection	Mean yield	Bean grades						Liquor quality			
		TT	PB	AA	AB	C	T	Acidity	Body	Flavour	Standard
1st cycle	3.45	21.42	24.72	17.84	29.22	4.75	2.05	1.65	1.67	3.30	3.25
2nd cycle	2.44	25.60	12.49	16.88	39.03	4.22	0.94	1.50	1.45	3.27	3.52
3rd cycle	2.99	24.79	9.90	15.26	40.51	5.58	0.87	1.80	1.85	3.39	3.69
Hybrids	3.69	18.53	16.40	22.37	29.19	8.56	1.74	2.07	2.10	3.77	3.78
SL28	2.90	23.15	12.09	25.11	30.28	5.18	1.08	1.63	1.88	3.21	3.58

As a result, a hybrid variety, Ruiru 11 was development and release to the farmers in 1985. The variety combine resistance to CBD and leaf rust with yield and quality similar to SL 28

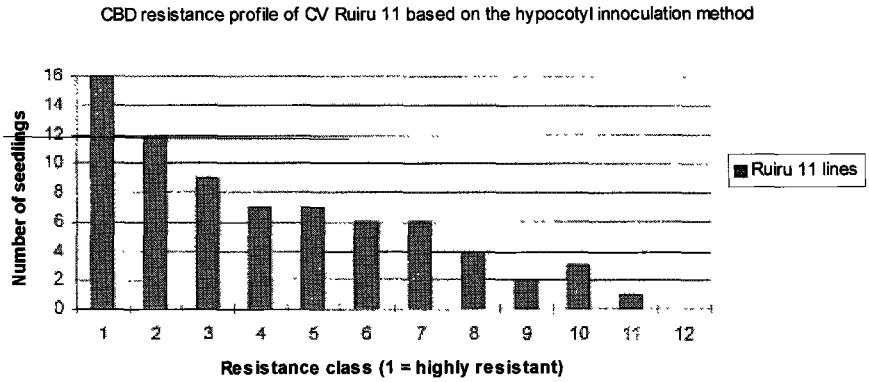


Table 2. Observations on yield and quality of Cultivar Ruiru 11 during two cycles of production

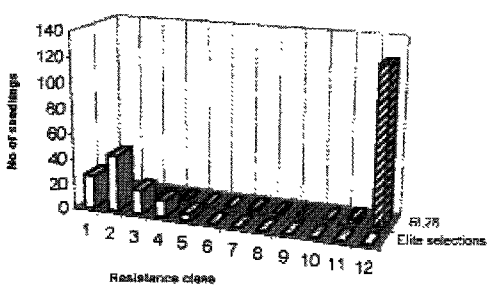
	Mean yield	Bean grades						Liquor quality			
		TT	PB	AA	AB	C	T	Acidity	Body	Flavour	Standard
Progeny mean	3.34	18.53	16.40	22.37	29.19	8.56	1.74	2.07	2.10	3.77	3.78
SL28	2.94	23.15	12.09	25.11	30.28	5.18	1.09	1.63	1.88	3.21	3.58

Further backcross have resulted into the development of breeding lines which have high level of resistance to CBD, good level of field resistance to leaf rust, high yield and good quality

Table 3. Yield and quality performance of advanced selections of Arabica coffee resistant to CBD and rust

Generation of selection	Mean yield	Bean grades						Liquor quality			
		TT	PB	AA	AB	C	T	Acidity	Body	Flavour	Standard
3rd cycle	2.99	24.79	9.90	15.26	40.51	5.58	0.87	1.80	1.85	3.39	3.69
SL28	2.90	23.15	12.09	25.11	30.28	5.18	1.08	1.63	1.88	3.21	3.58

FIG 1, Resistance profiles for elite selection



CONCLUSION

The lines have comparable performance to the traditional Kenyan coffee varieties in all aspects of production and quality and are thus of great potential for use as commercial varieties in Kenya

THE INFLUENCE OF AVAILABLE WATER ON CROP DEVELOPMENT AND YIELD OF COFFEE *(Coffea arabica* L*)* AT AIYURA, PAPUA NEW GUINEA

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INTRODUCTION

In Papua New Guinea (PNG) where most of the coffee is produced the high rainfall is generally experienced between September and March followed by drier period between May and August. However in 1997 between April and October the El Niño weather pattern led to exceptionally little rain through-out PNG including the Highlands where most of the PNG coffee is produced. The coffee industry feared then that the 1998 crop would be significantly influenced downwards compared to the average situation as this extreme weather pattern had not been experienced before. The fear was that due to the little amounts of water supplied to coffee trees, a reduced crop would have led to severe financial problems throughout the coffee sector from producers to exporters. Unfortunately, only little approved scientific literature was available to either verify or falsify the threat. As a result, a discussion within the scientific community and the industry evolved expressing controversial estimates about the quantity of the 1998 crop.

The relationship between the 1997 El Niño and the resulting crop yield in 1998 was examined using the Papua New Guinea (PNG) Highlands as an example. Weather and coffee yield data from the Coffee Research Institute (CRI), Aiyura, Eastern Highlands Province was utilised for the analysis. In a soil water balance model (von Enden 1998), rainfall data, soil characteristics and plant water requirements are brought together to produce charts exhibiting the changing water availability.

OBJECTIVE

It is hoped that the findings will contribute to a better understanding of the behaviour of coffee growth and crop development under varying moisture availability.

PREVIOUS WORK

Research of Coffee (*Coffea arabica* L.) has been carried out in PNG to investigate the growth cycle, crop development and nutrient demands. Research projects were aimed to optimise time and quantity of fertiliser application. In a study of leaf nutrient fluctuations Harding (1994) identified considerable varying nutrient demands of the coffee tree during crop development. A fluctuating nutrient demand implies that varying amounts of water are needed by the plant for optimal growth.

Hombunaka (1998) refined Harding's findings and established different crop development cycles for the Aiyura-Kainantu area compared to the rest of the highlands. Based on this, detailed, geographic related recommendations for fertiliser application were identified. It was shown that even slight climate differences lead to modified crop development cycles.

Both authors emphasised the climate of the PNG Highlands as an important factor influencing crop development cycle. Their findings showed:

- Contrary to most other coffee producing countries the PNG Highlands do not have a clear dry season, although a less wet season does occur
- due to the relatively equable climate, coffee ripens during most months of the year
- if a clear rainfall stimulus can be identified, yields are seen to peak 8-9 months later

Wrigley (1988) refers to research work in Kenya which examined the influence of water deficiency on coffee growth. It was found that a lack of plant available water can directly lead to water stress or indirectly to nutrient deficiencies limiting crop development. As a result, coffee development is reported to be limited when about half of the available soil moisture is depleted. This statement must be examined more closely: a period of water deficiency in the soil – meaning water stress to the plant – followed by strong rainfalls favour the onset of flowering. On the other side, at times when high amounts of water and nutrients are needed (e.g. during the phase of cherry weight gain) water deficiencies strongly hinder crop development.

von Enden (1998) sketched the 1997/98 crop development using a semi-quantitative approach. Sources of information were a quantitative assessment of the 1997 water balance assisted by a qualitative approach putting farmers' perceptions into the centre of attention. The assessment gave insight into the influence of drought and frost on crop development and resulting management strategies in the relatively unexplored environment of smallholders coffee production.

THE AVERAGE WEATHER AND CROPPING PATTERN

Under average conditions, the climate of the PNG Highlands can be divided into two seasons: a wet season between September and March and a slightly drier season between May and August. Aiyura receives an average amount of 2074 mm rainfall/year (Trangmar *et al.* 1995). The soil moisture regime acts according to the rainfall pattern.

The soil moisture model on which the analysis is based assumes a maximum water holding capacity of the soil of 85 mm reflecting average soil characteristics in the Aiyura area. Under this assumption, soil moisture is slightly depleted between June and September (see Figure 1). During this period evapotranspiration (Et) exceeds rainfall so that soil moisture resources are used. This situation, however, does not put coffee growth in danger. It rather provides a situation in which a rainfall event acts as a stimulus for the flowering onset. Figure 1 illustrates central variables of the average weather pattern and soil water regime.

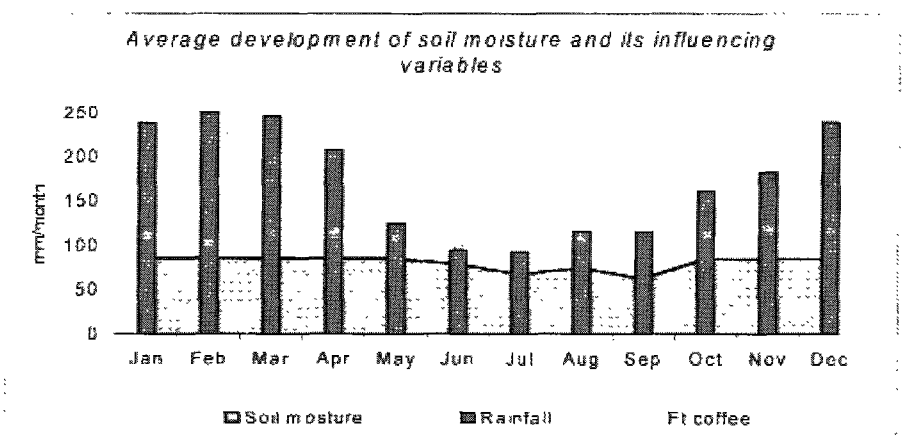


Figure 1: 1997 Monthly Rainfall and Effects on Soil Moisture and Evapotranspiration

The variables acting in the water balance are:

- Rainfall which provides the water input into the system
- Evapotranspiration (Et) which withdraws water from the system
- Soil Moisture which represents the ability of the system to store moisture

The 1997/98 weather and cropping pattern

The 1997/8 weather data are looked at on a 10 day basis which allows a detailed analysis of the coupled development of climate and crop.

Generally, the weather in 1997 was characterised by exceptionally little rainfall which resulted in low soil moisture levels for extended periods. In total, Evapotranspiration (Et) exceeded precipitation for five months in 1997 and led to fully depleted soil water resources at times. Water demands could not be supplied by rainfall inputs, consequently water deficits, which are reported to be rare in the highlands (McAlpine 1970), were experienced. In addition, low temperatures were experienced during dry periods, especially during clear nights: clouds were missing so that long wave radiation was lost to the atmosphere.

The development of the 1997/8 water availability and its influencing variables are shown in Figure 2 (data from Aiyura weather station). Four segments are identified; each segment is characterised by a typical down and up movement in available soil moisture: in the beginning, soil moisture is available before levels are declining; after a period of comparatively low moisture levels, soil moisture levels are increasing again pinpointing the end of a segment and the beginning of the next. The sudden incline of soil moisture favours the onset of flowering of the coffee tree and start a new crop cycle. Each of those stimuli is indicated by an arrow in Figure 2.

1st segment: March to May :
Early in 1997, precipitation and soil moisture levels decreased unusually early: between March and April little precipitation lead to a reduction in available soil moisture. In May 1997, precipitation increased and the soil body was again entirely filled with moisture. However, this state of maximum soil moisture supply did not last long.

2nd segment: May to July :
Soil moisture declined in the end of May leading to more severe drought conditions in the beginning of June. During this period, Et exceeded precipitation diminishing the soil's moisture reserves. For the first time in 1997 water deficits were experienced in the end of June. The segment ends in July when precipitation increased again and water deficits were overcome for a short time.

3rd segment: July to end of September :
The most severe water deficits were experienced during the third segment. Deficits commenced in August lasting until the end of September; hardly any rain fell and Et totally withdrew soil moisture during this period. Consequently, coffee trees suffered fierce water stress. Drought conditions ended when rainfall set in at the end of September; soil moisture levels could recover but there was still too little to meet the requirements of the coffee trees.
In addition to dry conditions unusual low temperatures were experienced during the third segment (per com. Hombunaka). Temperatures below 10 degrees C paired with dry conditions were likely to have acted as a stimulus for flowering. This incident is visualised in Figure 3 within the third segment.

Figure 2: Development of soil moisture and its influencing variables between January 1997 and May 1998

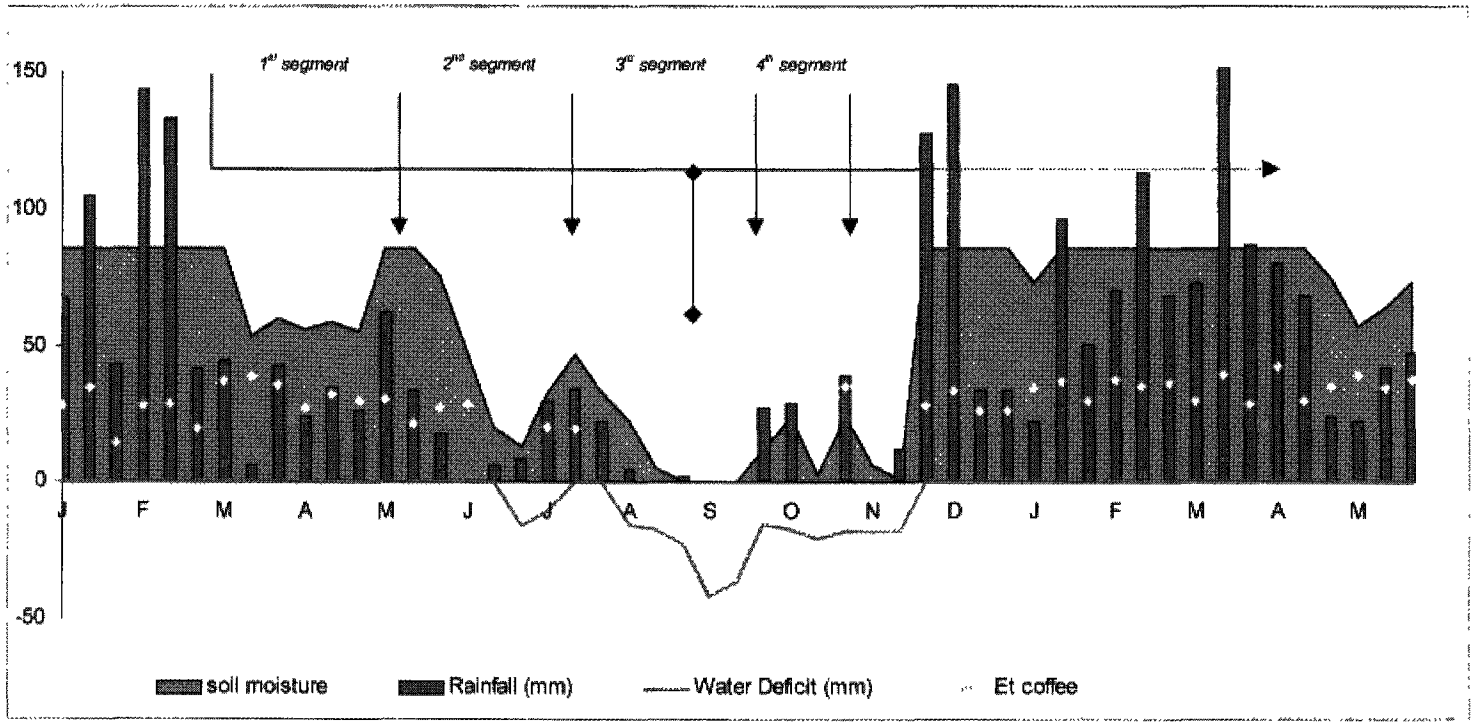
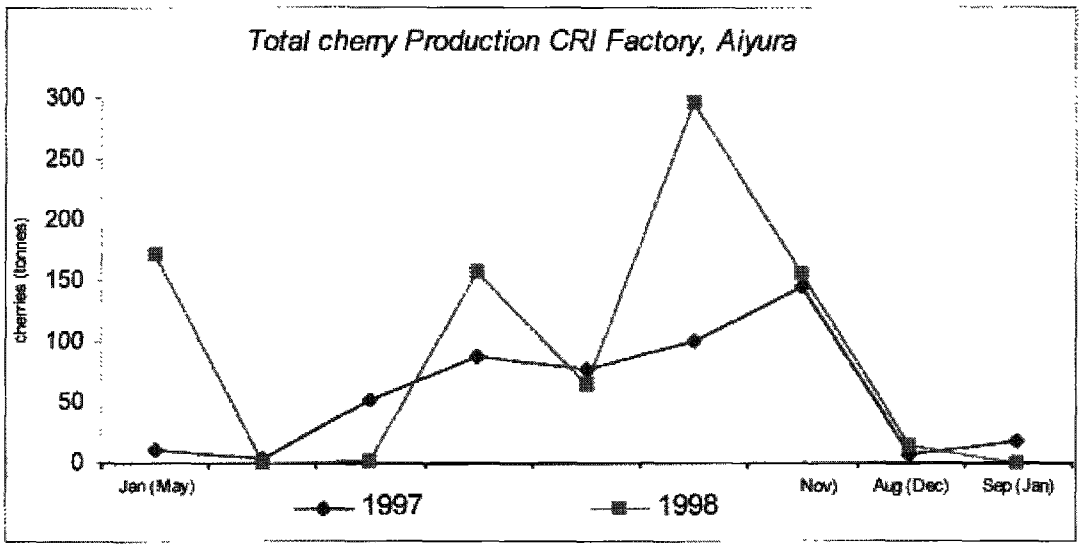


Figure 3: Cherry Production, 1997-98



NOTE: the months in brackets indicate the month of stimulus 8 months earlier

4th segment: end of September to end of November :
This segment starts with relatively low levels of soil moisture and water deficits. Due to a lack of rainfall a further decline in soil moisture can be observed in mid October. Soil moisture slightly recovers in the end of October and can be identified as a stimulus. Drought conditions are finally overcome in the end of November when soil moisture was at maximum storage capacity again. A stimulus can be identified for mid October, and the end of November. From January 1998 until May only slight depleted soil moisture was observed.

An approach which quantifies the unusual up and downs of soil moisture is given in Table 1. In order to identify a strong incline in soil moisture a coefficient was calculated. Whenever the increase of soil moisture succeeded 20 per cent of the total water storage capacity of the soil a situation was given which is likely to have caused the onset of flowering. Table 1 visualises this by using "* * *" in the "Stimulus" line. Four situations were given which are likely to have induced

The 1998 cropping pattern can be expected to act according to the 1997 soil moisture regime; production peaks can be predicted for the time around eight months after each stimulus. Three clear yield peaks (May, June and July 1998) are identified (Figure 3). The up and down movement of 1998 yield quantities can be explained by the repeatedly changing water availability from dry to wet during 1997. Each increase of rainfall and soil moisture was the starting point of a new development cycle.

January 1998 yield
The cherry production in Aiyura in January 1998 can be related to the first water availability segment. Dry weather and reduced soil moisture levels in 1997 are very unusual for the months March to April – under average conditions excess water is constantly available during this period. In 1997, a clear rainfall stimulus in May after a dry period started a crop development cycle. The effect could be seen in a production peak eight months later, i.e. in January 1998. However, it was not the case as most of the flowerings were aborted due to sub-optimal conditions of water supply during cherry development, i.e. between July and October thus lower yield recorded

Table 1 : Quantitative evaluation of 1997/8 soil moisture regime and stimuli

Year 1997	**Jan**			**Feb**			**Mar**			**Apr**			**May**			**Jun**		
Decade	*1*	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*
Soil moisture	85,80	85,80	85,80	85,80	85,80	85,80	85,80	52,99	59,74	55,80	58,43	55,12	85,80	85,80	75,51	46,47	19,87	12,92
Soil moisture coefficient *		0,00	0,00	0,00	0,00	0,00	0,00	-38,24	7,86	-4,60	3,07	-3,86	35,76	0,00	-11,99	-33,85	-30,99	-8,10
Stimulus**													* * *					
Will yield in month	*September*			*October*			*November*			*December*			*January*			*February*		
Year 1997	**Jul**			**Aug**			**Sep**			**Oct**			**Nov**			**Dec**		
Decade	*1*	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*
Soil moisture	32,19	46,51	33,21	22,28	5,05	0,00	0,00	0,00	11,92	23,25	2,30	23,87	6,10	0,52	85,80	85,80	85,80	85,80
soil moisture coefficient*	22,46	16,68	-15,49	-12,74	-20,08	-5,89	0,00	0,00	13,89	13,21	-24,42	25,14	-20,72	-6,50	99,39	0,00	0,00	0,00
Stimulus**	* * *											* * *			* * *			
will yield in month	*March*			*April*			*May*			*June*			*July*			*August*		
Year 1998	**Jan**			**Feb**			**Mar**			**Apr**			**May**					
Decade	*1*	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*			
soil moisture	72,98	85,80	85,80	85,80	85,80	85,80	85,80	85,80	85,80	85,80	85,80	74,16	56,75	63,93	73,39			
soil moisture coefficient*	-14,94	14,94	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	-13,57	-20,29	8,37	11,03			
Stimulus**																		
will yield in month	*September*			*October*			*November*			*December*			*January*					

*Note: coefficient gives the change of soil moisture as a percentage of maximum soil moisture storage capacity

**Note: when a coefficient >20% is calculated a stimulus is assumed

February, March, April 1998 yield
Dry conditions and the lack of a rainfall stimulus in the beginning of the second water availability segment (June) suppressed flowering. Also lack of moisture after flowering may have led to higher percentage of cherry abortion. The result is a poor yield in February, March and April 1998 (ca. -92% compared to 1997).

May 1998 yield
A reasonable crop was delivered in May although a rainfall stimulus could not be identified eight months earlier. The crop is likely to originate from a flowering in September due to low temperatures coupled with dry conditions. Low temperatures were observed for that period -unfortunately no records are available. The reasonable crop was realised because of adequate moisture in October and thereafter which is critical as this is the period of rapid expansion, endosperm formation and weight gain leading to ripening of the cherries. If there was inadequate moisture in late October and onwards most of the cherries would have been aborted.

June 1998 yield
Strongest water deficits during 1997 occurred during the third segment. As soon as a clear rain stimulus was given in October, flowering started leading to a strong production increase in June 1998 (+197% compared to 1997). The peak has been fuelled by the extreme dry spell beforehand. The constellation of severe water stress followed by a period of excellent water supply emphasised the flowering process. The good availability of water from end of November 1997 onwards can be seen as important for optimal cherry development.

Wrigley (1988) stated that the coffee yield is negatively influenced when the coffee tree experiences water deficits between the 8th and 17th week after flowering, during the time of net weight gain of the cherry development. Transferring this statement to the 1997 situation, crop development cycles which started around April/May and July/August were negatively influenced by insufficient water availability during cherry development.

In conclusion, the changing water availability in 1997 led to multiple flowering resulting in three (May, June and July) yield peaks of the 1998 crop. From January to September 1998, a total increase in yield can be seen. The El Niño weather pattern appears to have favoured the coffee yield rather than put the crop in danger - coffee trees as part of its physiology adapted well to the unusual weather situation. Increased yields in peak months more than compensated for the reductions caused by the drought. The 1998 calendar year delivered a large crop with a total 12% increase of received cherries for the time span January to September 1998 (Table 2).

Table 2: Cherry received at CRI factory: monthly figures 1997/1998

	Jan	***Feb***	***Mar***	***Apr***	***May***	***Jun***	***Jul***	***Aug***	***Sep***	***SUM***
1997 (t/month)	10.2	4.2	52.3	88.3	77.3	100.0	144.4	7.1	18.7	**502.5**
1998 (t/month)	17.2	0.1	1.1	15.6	63.9	297.0	155.3	13.8	0.8	**564.8**
Deviation (t/month) '97 to '98	7.0	*-4.0*	*-51.1*	*-72.7*	*-13.4*	197.1	10.9	6.7	*-17.9*	**62.3**
Coefficient of variation (%)	*69*	*-97*	*-98*	*-82*	*-17*	197	8	95	*-96*	**12**

Conclusion

It must be emphasised that assumptions in this paper are based on the conditions of a small area within the PNG Highlands. Dry conditions affected areas differently according to the varying micro climate of the Highlands. Even on village level varying spatial and timely differences in drought impacts on

coffee growth were identified. In addition, appropriate management (e.g. shaded cultivation, weeded gardens, pruning) was found to effectively increase the trees' resilience towards drought (von Enden 1998). However, the findings of this paper remain relevant because they can be translated into any environment when necessary data is provided.

The most important finding is that one of the worst droughts of the century (Bourke 1998) did *not* lead to a crop failure in 1998 particularly for *C. arabica* but not *C. canephora* (Stapleton *et al.* 1998) but even pushed production. The 1997/98 actual production was a record 1.2 million plus, 60 kg green bean bags. This was the highest production figure recorded since records began in 1964 (Stapleton *et al.* 1998). This fact underlines the extreme drought tolerance of the coffee (*C. arabica*) tree and its ability to quickly react to and recover from extreme moisture regimes. Also as most arabica coffee trees are grown under shade trees this has cushioned against the extreme drought effects.

A timely distribution of water availability and temperature was identified to play a central role in the coffee crop development cycle. This was demonstrated by the average distribution of monthly yield quantities in relation to rainfall and soil water regimes. Examining the 1997/8 situation, this relation was even more accentuated. Characteristic up and downs of the 1998 crop were explained by changes from dry to wet conditions in 1997 described above as segments. Rainfall after spells of dry weather was made out to be the impulse for the start of a new crop development cycle. After periods of total soil water depletion, an intensified reaction was observed – represented by a strongly increased yield eight months later.

PNG was fortunate that dry spells were interrupted by several rainfall events. Although relatively small, they acted as stimuli, induced crop development and safeguarded the ongoing process of cherry development. Dry periods which regularly followed a wet period, appeared not to have significantly influenced crop yields.

ACKNOWLEDGEMENT
The CRI Farm and Processing Departments for the yield figures and Breeding Department for the rainfall data. Mrs. Karolyn Gillina for her secretarial services in accessing and sending this document by e-mail, which ensured faster publication of this report

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SMALLHOLDER COFFEE IRRIGATION RESEARCH IN KENYA

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ABSTRACT

A field study was conducted to investigate the feasibility of developing irrigation methods for smallholder coffee farms using basins and readily available packaging materials such as polythène bags, bottles and plastic containers, to supply 25 water per tree per month. All treatments, except the polythène bag resulted in significant yield increases. The best overall treatment (0.5x0.5x0.2 m basin supplying water to four trees) resulted in 4672 kg clean coffee per ha equîvalent to 6.3 kg cherry per tree. The unirrigated control resulted in 1003 kg clean coffee per ha (2.4 kg cherry per tree). The results are discussed. It is concluded that the smallholder yields can be substantially increased by using materials that are readily available in their homes.

INTRODUCTION

Research on irrigation in coffee in Kenya has involved water placement methods such as overhead sprinkler and drip irrigation. The capital required for these systems is enormous. Thus the systems are exclusively used by the large scale coffee planters. However, the smallholder sector which commands about 60% of Kenya's coffee production (Karanja, in press), produces its coffee entirely under rain fed regimes. The smallholder level of coffee production is about 2.3 kg of fresh fruit per tree or 570 kg clean coffee per ha, whereas the large scale sector produces more than twice this level (Roe and Nyoro, 1986). The higher yield per tree in the large scale than in the smallholder is attributed to irrigation (Anon 1986).

This field study was conducted with the aim of developing an appropriate irrigation technology amenable to the smallholder coffee sector in Kenya. The objective was to find out the effect of the irrigation methods on coffee yield.

MATERIALS AND METHODS

One year old *Coffea arabica* L. cultivar Ruiru 11 seedlings were transplanted in the field at the Coffee Research Station (CRS) Ruiru, Kenya. With the exception of the cova planting, all seedlings were spaced at 2 x 2 m resulting in a density of 2500 trees per ha. The CRS is situated at 1.05° S and 36.45° E, at an altitude of 1608 m above sea level. Soils at the CRS are humic nitosols (Shitakha, 1983).

The methods used to apply water to the coffee trees were: one 1.0 x 0.5 x 0.2 m basin dug between two adjacent coffee trees, one 1.0 x 0.5 x 0.02 m basin dug between four trees, one 0.5 x 0.5 x 0.2 m basin between four trees spaced at 1.5 x 1.5 m in a cluster i.e. cova planting, (spacing between covas was 3m, resulting in a density of 4444 trees per ha); 2 litre plastic containers (used in cooking oil packaging), 2 litre bottles (used in fruit juice packaging and polythène bags (used as meat wrappers). Using a 2 mm diameter nail, holes were punched at the bottom of the plastic containers and polythène bags, through which water dripped into the soil. These and the bottles were placed on the ground 50 cm from coffee stem base.

Timing of irrigation was 2 months after blooming. This timing is critical because it is the beginning of the rapid expansion phase of fruit development. Fruit size is positively correlated Ito moisture available during this phase (Wormer, 1966). Water obtained from a water tap located about a meter outside the

trial site was placed in the basins, plastic containers, polythène bags and bottles. The bottles were pushed with the open end into the soil to allow water to drip into the soil. Using these methods 25 1 of water were supplied to each tree per month, which is about V4 of 25 mm depth of overhead irrigation. The treatments were replicated tree times in randomized complete block design. Experimental plots comprised four trees separated by one guard row. Cherry (ripe fresh fruit) yield per tree was recorded. The weight of 100 cherries per treatment was recorded at each harvest , from which the mean weight per fruit was calculated. Yield of cherry per plot was converted into clean coffee (endosperm + embryo) by a ratio of 6 kg fresh fruit weight to one kilogram clean coffee.

RESULTS

All irrigation treatments except the 2 litre polythène bag resulted in significant ($P<0.05$) yield increases above the unirrigated coffee planted at 2x2 m spacing which resulted in 1003 kg clean coffee per ha (Table 1). However, yield from the basin irrigated cova treatment (4672 kg clean coffee per ha) was not significantly higher ($P>0.05$) than the yield from the unirrigated cova (3580 kg/ha). Moreover the unirrigated cova treatment resulted in significantly higher yield than the unirrigated coffee planted at 2x2 m (Table 1). On the basis of cherry yield per tree, all irrigation treatments resulted in signif icant increases above the unirrigated treatments. The cherry yield per tree from the unirrigated cova (4.8 kg) and from the unirrigated 2 x 2 m planting (2.4 kg) were not significantly different ($P>0.05$) (Table 1). There were no significant differences in the mean weight per fruit.

Table 1. Effect of different irrigation methods using locally available materials on clean cof f ee (kg/ha) and cherry yield per tree (kg/tree) of cultivar Ruiru 11.

Irrigation treatment	Clean coffee yie1d (kg/ha)	Cherry yield (kg/tree)
Basin:		
▪ 1.0 x 0.5 x 0.2 (1 for 2 trees)	3158	7.6
▪ 1.0 x 0.5 x 0.02 (1 for 4 trees)	2858	6.9
▪ 0.5 x 0.5 x 0.2 (1 for trees in cova)	4672	6.3
2 litre plastic container	2933	7.0
2 litre bottle	3796	9.1
2 ltre polythene bag	2692	6.5
Unirrigated (2 x 2 spacing)	1003	2.4
Unirrigated (cova)	3580	4.8
LSD (P < 0.05)	**1734**	**3.5**

DISCUSSION

The data indicated that development of an appropriate irrigation technology for the smallholder coffee sector in Kenya is feasible. The yield from the basin irrigated cova planting (4672 kg) was 8.2 times higher than the national mean of 567 kg clean coffee per ha from rainfed smallholder coffee. This 1evel of yield is 4.0 times higher than the mean of 1174 kg per ha from irrigated large scale plantations reported by Roe and Nyoro (1986) . The high yield per ha from the unirrigated cova planting implied the possibility of tripling yields without recourse to irrigation by planting coffee trees close to each other in a cova. The résultant mutual sheltering effect of close-spaced coffee trees is reported to improve their internal water status (Fisher and Browning, 1978) and to result in increased relative humidity within the coffee tree canopy (Gathaara and Kiara, 1984). These may explain the good performance of the cova planted coffee.

The irrigated cova treatment which resulted in the highest yield per ha was not the one with the highest cherry yield per tree. This was due to the higher tree density in the cova planting which resulted in

increased yield per ha but in a decreased yield per tree. Thus description of coffee yield on the basis of cherry weight per tree is apparently imprecise.
The materials used in the placement of water in this study are available in rural homes where coffee is grown. Moreover digging of basins may be done with family labour. The results from this study lead one to conclude that smallholder coffee yields can be increased substantially by using technology and materials that are readily available in their homes.

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RESPONSE OF CLONAL ROBUSTA COFFEE TO ORGANIC AND MINERAL FERTILISER APPLICATION IN LAKE VICTORIA CRESCENT ZONE

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Abstract

A long-term study was conducted at KARI on performance of six (6) high yielding recommended Uganda's indigenous robusta coffee (Coffea canephora Pierre) clones under four major soil fertility management involving application of coffee husks and crop residues as mulch, cow-dug and N.P.K. 25:5:5 fertiliser were compared against a non-soil amendment.

Soil amendments with coffee husks and cow-dug performed significantly at (P=0.05) better that the rest. The overall (1995-98) mean yields increased from 2,799.5 to 3,063.3 kg/ha, clean coffee for the treatments of coffee husks and cow-dug respectively. Clonal differences in biological yields and bean quality were significant at (P=0.05) with erect type varieties showing superiority over the nganda types. Significant yield reduction to 446.7 kg/ha^{-1} c.c was observed in control during 5-th cropping cycle due to etiolated plants and shortened productive primaries.

Mulching clonal coffee with husks and cow-dung improved soil-water conservation regime, and up-take of nutrients significantly. The study confirmed the earlier reports indicating the superior performance of erecta coffee clones over nganda varieties. The advantages of application of coffee husks and cow-dung in soil amendment revealed their potential substitutes for mineral fertilisers.

Key words: Clones, organic and mineral fertilisers, yields and coffee bean quality.

INTRODUCTION

High yielding Robusta clonal coffee (Coffea Canephora Pierre) varities have been adopted by farmers for increased production in Uganda. Significant yield increases have been acheived in use of nitrogenous fertilisers on robusta coffee (Anon., 1962) and determined to be cost effective. Studies on use of nitrogenous mineral fertilisers on recently selected clonal coffee varieties showed substantial profit from 225-305% when applied in the range of 52-125 g elemental nitrogen per tree. Nitrogen application rates of 115.5, 231.1 and 346.6 kg ha^{-1} per year resulted in profit increments of Ug. Shs 788,522; 1,354,686 and 1,039,121 respectively (Kibirige et al. 1993, 1996).

Currently newly introduced coffee varieties require higher up take of nutrient for large berry yields, rational biological vegetative growth and good coffee bean quality. The rate at which the clones have been adopted by large scale farmers is relatively significant. Continues cultivation of small land

holdings under coffee and other food crops due to increased population growth has caused soil exhaustion and degradation (Zake, 1992). The use of mineral fertilisers is limited, expensive and fertilisers are not accessible by farmers.

Consequently, the alternative feasible application of available agricultural crop residues to improve soil fertility and environment is one of the alternatives. It can increase coffee yields and improve upon marketable good quality coffee beans.

Improvement of soil fertility in Uganda showed that mulching robusta coffee was superior to clean weeding (Anon., 1942, 1962). Recent studies in E. Africa have demonstrated that mulching leads to substantial yield increase and improvement in bean quality size (Kabaara, A.M., 1976, 1972, Zake, 1996; Kucel et al., 1996). Clarke and Mac Rae, (1988), Jameson, (1970) attributed the beneficial effects of mulching to suppress weeds, improvement of soil moisture regime and efficient release of plant nutrients during decomposition.

Therefore, a long-term study was conducted to evaluate values of organic materials in form of coffee husks, cow-dung and a variety of plant refuse which commonly available in households to substitute for the expensive mineral fertilisers.

MATERIALS AND METHODS:

The field trials were conducted between 1991 and 1998 at Kawanda Agricultural Research Institute (KARI). KARI lies at an altitude of 1220 m.a.s.l., 32°32'E, 0°25'N) and 13 km north of Kampala. The mean monthly minimum temperature is 15°C and maximum is 26°C. The mean annual rainfall for seven years amounted to 1211.8 mm and maximum annual rainfall registered 1444.9 mm in 1994. Less than 1110 mm was received in 1993, which substantially reduced the crop yields.

The trial was laid out during I and II rains in 1991. Uniform, same age and well developed root system robusta cuttings of Erecta ($1^{s}/_{2}$, $1^{s}/_{3}$, $1^{s}/_{3}$) and Nganda type ($236/_{26}$, $257^{s}/_{53}$ and $258^{s}/_{24(o)}$) were selected and grown at the clonal nursery. Clones were transplanted at 3m x 3m, giving 1111 plant ha^{1} into 1.87 ha plot. The six recommended clones were planted in randomised split-plots with 8 trees per clone per soil amendment. Two of the replicates were planted in April 1991, and the third in October 1991 when more planting materials became available.

The five soil amendments including a control constitute the main plots. The entire experiment received uniform management practices including blanket applied N-fertiliser at 100g per tree (0.52 N) for 2 rain seasons. Then, treatments consisting of 15 ton ha^{1} coffee husks; 10 ton ha^{1} cow-dung; 12 tonnes ha^{1} crop residues (mixtures of maize straws, beans and soya bean refuse); N:P:K 25:5:5 fertiliser and un treated control. These were arranged in RCBD, replicated three times. N:P:K 25:5:5 was applied at the rate of 240 kg ha^{1} in two splits in a year. The rates were adopted from Kabaara and Kimeu, (1972). The organic manures were applied at least after every eighteen months during cropping years.

Wet, washed and dried samples of coffee beans were weighed between 0.25-0.5 kg for all laboratory cup tasting quality analysis. Analyses were conducted to determine the quality characteristics of roast, body, and flavour according to the methods of sample preparation. The interpretation of scores, remarks and results were carried out by using the standard procedures established by Kyagalenyi Coffee Ltd and Uganda Coffee Development Authority (UCDA).

Data were collected on cherry yields, growth parameters and bean quality elements which were eventually analysed by ANOVA. The means were separated using Duncans Multiple Range Test.

RESULTS AND DISCUSSION:

The present study confirms that long-term effects of organic manures on clonal and can increase yields by three folds compared to farmers national yield level. Stable production of coffee cherries was observed after 16-18 months after planting. All treatment replicates performed significantly different at (P=0.05) although the III replicate was planted a season after replicates I and II.

Variable Clonal coffee yields due to Soil Amendments

Yield increases of six clonal robusta coffee due to soil amendments are presented in Table 1. Analysis of variance indicate variations in yield performance among soil treatments being significant (P=0.05). Coffee husks and cow-dung treatments performed better than treatments consisting of N.P.K 25:5:5 fertiliser and crop residues. It was observed that all organic manure and fertiliser treatments were significantly better than control.

Table 1 : Effect of different soil amendments on clonal robusta coffee yields

Mean Yield: Clean coffee (kg ha^{-1})

Soil Amendments	Year 1994/95	Year 1995/96	Year 1996/97	Year 1997/98	Mean for 4-years
Coffee husks	3,648.0	4,154 a	2,837 a	1,108 a	2,699.7
Cow-dung	3,574.0	4,048 ab	2,260 a	843 ab	2,383.7
Crop residues	2,882.0	3,146 bc	1,154 b	469 bc	1,589.7
NPK 25:5:5	2,997.0	2,383 cd	888 b	559 bc	1,276.6
Control	2,663.0	1,986 d	572 b	446 c	1,001.3
Mean	3,152.8	3,143.4	1,542.2	685.2	1,790.2
Lsd 0.05		940.6	286.2	384.7	
C.V, %		44.7	26.9	42.5	

Mean in a column not sharing a common letter are significantly different (P=0.05)

The II and III year of cropping were the best, meanwhile 1995/6 yields ranged from 1986 kg clean coffee per hectare where no – soil amendment to 4,154 kg of clean coffee per hectare where coffee husks were applied. In the firth year of cropping (1997/8), mean yields reduced from 1,108 to 446 kg of clean coffee per hectare. The decline is due to aging unproductive coffee stems and shortened primaries. During this period available soil nutrient in control was at lowest level.

The superior performance of the coffee husks and cow-dung is attributed to in part, better soil moisture retention, better weed suppression, lower damage by scales and mealy bugs (Kucel et al. 1996). Mulch increases the rate of infiltration of rainwater while at the same time conserves moisture during dry spells (Anon, 1987). Therefore, all mulching materials improved the water regime under rooted clonal cutting varieties.

Apparently the control and N.P.K. treatments had greater volumes of water seepage down the profile culminating in higher leaching rate of nitrogen, poor up-take of mineralised nutrients and reduced coffee yields.

Higher supply of potassium which is a key nutrient element of coffee plants in reproductive period of fruit development could have contributed greatly to this superiority. Higher level of potassium in soil should have induced magnesium deficiency following prolonged application (Wilson, 1995).

However, no evidence of magnesium deficiency in soil and leaf plant analysis was established. Adequate supply of potassium by these mulching materials also improves metabolism of nitrogen supplied by them since positive interactions occur between the two treatments (Kabaara, 1976). It is evident that soil amendments are necessary in coffee production.

Relative Yield performance of Robusta Clones.

Tables 2 and 3 indicate the performance of two types of clonal robusta coffee. Clonal robusta differences for yield performance was significant (P=0.05) with erecta type clones $1^{s}/_{2}$, $1^{s}/_{3}$, $1^{s}/_{6}$ performing better than that of the nganda types $236/_{26}$, $257^{s}/_{53}$ and $258^{s}/_{24(o)}$. Genetical cultivar responses indicated in these results are in conformity with previous observations by Kibirige et al. 1993,1996), where erecta clonal coffee persistently performed better than nganda traditional varieties. Clone $1^{s}/_{6}$ had the highest mean yield of 3,758-kg clean coffee ha^{-1} during 1995/6 cropping year. In certain cases clone $1^{s}/_{2}$ yielded between 3,306-3,649 kg clean coffee per hectare in all soil amendments during 1994/5-1995/6 cropping seasons. The increase of the 4-years mean yield of the erecta clones due to the soil amendments are significantly high and registered 2,378.2 kg clean coffee per hectare compared to 1,864.2 kg clean coffee per hectare for nganda types.

Table 2 : Response of Clonal Robusta coffee varieties to soil amendments.

Mean Yield kg clean coffee ha^{-1}

Clone Variety	Year 1994/95	Year 1995/96	Year 1996/97	Year 1997/98	Mean for 4-years
Erecta					
$1^{s}/_{2}$	3,649.3 a	3,306 ab	1,049 c	1156 a	2,290.0
$1^{s}/_{3}$	3,370.0 ab	3,101 ab	2,436 a	918 ab	2,456.3
$1^{s}/_{6}$	2,881.6 b	3,758 a	2,003 ab	927 ab	2,392.4
Nganda					
$236/_{26}$	3,294.4 ab	2,811 ab	956 c	298 c	1,839.9
$257^{s}/_{53}$	2,748.2 b	3,636 a	1,444 bc	383 c	2,052.8
$258^{s}/_{24(o)}$	2,539.4 c	2,248 b	1,366 bc	647 bc	1,700
Mean	3,080.5	3,143.3	1,545.0	721.3	2,129.9
Lsd 0.05	-	1,030.0	642.1	382.0	

Means with same letters are not significantly different at (P=0.05)

Table 3 : Mean Yields of erecta and nganda type of Clonal Robusta

Mean Yield: kg clean coffee per hectare

Coffee type	Year 1994/95	Year 1995/96	Year 1996/97	Year 1997/98	Mean for 4-years
1. Erecta	3,300.3	3,383.3	1,829.3	999.8	2,378.2
2. Nganda	2,860.7	2,898.3	1,255.0	442.6	1,864.2
Mean	3,080.5	3,143.3	1,542.2	721.2	2,121.2

Better performance of erectas may, apart from the significant yield potential, arise from their superior large canopy parameters and compact upright morphological growth. Due to large surface area, the erecta type of clone provides greater leaf area index, which can intercept solar radiation for photosynthesis.

Higher percentage of productive primaries and internode lengths and narrower angles of primaries against the stem, which are basic components of yield in arabica coffee (Van der Vossen, 1985), may have also contributed to an increase in coffee yield.

Nganda type of clones are highly susceptible to leaf rust (Hemileia Vastatrix), and this was observed on $236/_{26}$ which performed poorly and reduced significant mean yield.

In certain cases, all clones performed better and yielded over 3,500 kg clean coffee per hectare and highest yield registered over 4,500 kg clean coffee per hectare for $1^{s}/_{2}$ (fig. 1). At the end of cropping year (1997/8), the yields reduced significantly, lowest mean yield registered 200-kg clean coffee for $236/_{26}$ (fig. 2).

Cow-dung and coffee husks treatments provided better mineralised nutrients in soil compared to the crop residues, NPK-25:5:5 and control. The highest yield is observed in cow-dung soil amendment where clone $1^{s}/_{3}$ gave 4.000 kg clean coffee per hectare (fig. 3).

In figure 4, a similar pattern of performance of coffee husks, cow-dung, crop residues, NPK and control is observed. Organic manures applied in soil improved and sustained the clonal robusta yields significantly. The average mean yield of six clones in coffee-husks, cow-dung and crop residues remained above 2.500 kg clean coffee per hectare in a year. The overall 1st year and 5th year cropping cycle in all soil amendments experienced reduction in coffee mean yields (fig. 5). During 6-18 months, the clones have smaller leaf surface area and canopies, short primaries, less vigorous growth, poor uptake of nutrient elements. However, during 2nd and 4th year cropping cycle, the clones were generally well established with good growth parameters and higher yields were attained under normal field management. The 5th year cropping cycle in all treatments experienced reduced coffee yields due to etiolated plants and shortened being primaries. Onzima et al. (1996) observed that it is uneconomical to practice a cropping cycle more than three years with application of adequate nitrogen mineral fertiliser before stumping old robusta trees.

Table 4 : Clonal robusta coffee bean quality in all soil amendments

Clone	100 Seed weight, (g)	Moisture Content, (%)	Screen 19 %	Screen 18 %	Screen 17 %
Erecta					
$1^{s}/_{2}$	18.93 ab	12.40 a	15.75 abc	19.94 a	28.22 a
$1^{s}/_{3}$	18.60 ab	12.53 a	15.22 abc	20.15 a	29.63 a
$1^{s}/_{6}$	21.59 a	12.47 a	23.65 ab	14.45 a	16.63 ab
Mean	19.71	12.45	18.21	18.18	24.83
Nganda					
$236/_{26}$	22.57 a	13.10 a	24.62 a	22.57 a	13.43 ab
$257^{s}/_{53}$	11.76 b	8.67 a	9.52 c	8.70 a	12.10 ab
$258^{s}/_{24(o)}$	11.78 b	8.60 a	11.26 bc	14.19 a	11.99 b
Mean	15.37	10.02	15.13	15.15	13.84
Overall mean	17.54	11.25	16.67	16.67	16.67
C.V.	32%	32.9%	35.3%	74.3%	79.7%
Lsd 0.05	8.71	6.03	13.06	17.85	27.46

Means with same letters are not significantly different at (P=0.05)

All 6 clones x 5 soil amendments were:

Screen 16 at P=0.05 ns Screen 15 at P=0.05 ns
Screen 14 at P=0.05 ns Screen 13 at P=0.05 ns
Screen 12 at P=0.05 ns

The quality elements of coffee during 5th year cropping cycle are displayed on Table 4. The effect of mean soil amendments on good quality beans of clonal robusta coffee has not been consistent for the varieties. However, it was noted that treatments, which enhanced higher clean coffee yields also, indicated better proportions of good quality beans. The highest 100 seed weight registered 22.57 g for clone $236/_{26}$. It had clear large and bold roasted beans. The 100 seed weight and screen size from 17 – 19 for Erecta type varieties are significant and better than Nganda type coffee. The poorest quality aggregates were observed on clone $257^{s}/_{53}$ although the mean clean coffee yield was medium.

Table 5 : Effect of organic manures on clonal robusta cup tasting qualities (1998)
KYAGALANYI COFFEE LTD, BUGOLOBI

Soil Amendments	Roast	Body	Flavour	Interpretation of scores	
				Roast - type	
1. Coffee Husks	3.766	3.033	2.833	Brillant:	0
				Solid:	1
				Bright:	2
				Ordinary:	3
2. Cow-dungs (animal manures)	3.733	3.167	2.933	Dull:	4
				Irregular:	5
				Body - type	
3. Crops Residues	3.866	3.267	3.167	Full:	0
				Good:	1
				F.Good:	2
				Fair:	3
4. NPK-25:5:5	4.400	3.567	2.967	Light:	4
				Poor:	5
				Flavour - type	
5. Control (no soil amendment)	3.700	3.300	3.300	Fine:	0
				Good:	1
				F.Good:	2
				Fair:	3
Mean	3.893	3.267	3.040	Poor:	4
				V.Poor:	5

Table 5 illustrates the effects of organic manures and NPK-25:5:5 on clonal robusta coffee samples in respect of cup tasting qualities. The visual characteristics of roasted coffee beans ranged from 3.700 – 4.400 (ordinary to dull) in all soil amendments. A slight improvement in roast quality parameters was recorded in all organic manure treatments and no-soil amendment. NPK-25:5:5, (25% N) has a direct effect on roasting and implies dullness. There is no significant difference observed in body characteristics for all treatments. Coffee husks and cow-dung provided fairly 3.033-3.167 body quality characteristics. At the same time, coffee beans harvested from NPK-25:5:5 plots showed a degree of fair body. Fairly good flavour (2.833-2.967) is observed in treatments of coffee husks, cow-dung and NPK.

The results of cup tasting on clonal Robusta varieties are shown in Table 6. No significant difference is observed for all the clones at P=0.05 in roast, body and flavour quality characteristics. $1^{s}/_{2}$ and $1^{s}/_{3}$

Table 6 : Results of cup tasting of clonal robusta coffee (1998)
KYAGALANYI COFFEE LTD, BUGOLOBI

CLONES	ROAST	BODY	FLAVOUR	REMARKS
ERECTA TYPE				
$1^{S}/_{2}$	4.000 a	3.333 a	2.867 a	$\underline{1^{S}/_{2}}$: good cup - coffee husks; clean cup-NPK; (1/2) half cup-cow-dung; herbal taste - control; insecticide taste - crop residues.
$1^{S}/_{3}$	4.200a	3.400a	3.133a	$\underline{1^{S}/_{3}}$: 1/2 good cup-coffee husks; fair cup-control
$1^{S}/_{6}$	3.933a	3.267a	3.000a	$\underline{1^{S}/_{6}}$: hash cup-cow-dung; good cup-coffee husks.
NGANDA TYPE				
$236/_{26}$	3.733a	3.267a	2.933a	$\underline{236/_{26}}$: general cup-cow-dung; woody cup-control; clean cup-cow-dung; good-coffee husks.
$257^{S}/_{53}$	3.733a	3.267a	2.067a	$\underline{257^{S}/_{53}}$: No remarks
$258^{S}/_{24(o)}$	3.800a	3.333a	3.267a	$\underline{258^{S}/_{24(o)}}$: good cup-cow-dung; fair taste-coffee husks.
MEAN	3.899	3.311	3.044	
C.V.	14,90%	17,50%	17,90%	
Lsd	0,954	0,946	0,895	

SCORES:
Roast: Brilliant = 0; Solid = 1; Bright = 2; Ordinary = 3; Dull = 4; Irregular = 5.
Body: Full = 0; Good = 1; F.Good = 2; Fair = 3; Poor = 4; V. Poor = 5.
Flavour: Fine = 0; Good = 1; F.Good = 2; Fair = 3; Poor = 4; V.Poor = 5.

erecta type clones have indicated dullness, 4.000 – 4.200 according to visual characteristics of roasted beans. Slightly ordinary roast is achieved in samples of $236/_{26}$, $257^{S}/_{53}$ and $258^{S}/_{24(o)}$ all nganda type of clones. The six (6) varieties basically indicate inconsistence in flavour according to the quality parameters determined. However, clones $1^{S}/_{2}$, $236/_{26}$ and $257^{S}/_{53}$ have fairly good flavour. The remarks of insecticide cup taste, herbal and tainted cup tastes are due to pesticide application to control mealy bags in crop residues and secondly due to poorly wet processing procedures.

CONCLUSION

It is unquestionable that use of organic manures as an input improves yield performance of clonal robusta provided field practices are optimal. Superiority of coffee husks in soil amendments is significant, but requires more investigations as it can be easily infected with Fusarium wilt (Tracheomycosis) which is prevalent in robusta coffee growing areas. Coffee husks, cow-dung and crop residues provide good potential substitutes for mineral fertilisers.
A cropping cycle of more than six years for clonal robusta cannot be maintained as most of the varieties become etiolated, unproductive, consequently significant reduction in yields. Cost-effective application of organic manures and numeral fertilisers in cropping systems of clonal robusta varieties needs to be included in the terminal report of this experimental project. The results of valid, statistical data analysis on plant and soil samples are incomplete. Robusta coffee varieties grown in organic manure plots have demonstrated a better cup tasting quality characteristics than control and NPK-25:5:5. Major physical defects and poor aggregates of liquor (bitterness, astringency, acidity) were not recorded, except the herbal, insecticidal and woody cup tastes were caused by chemical appreciation to control mealy bags (*Planococcus ureineus* De lotto) and secondly due to poor wet processing procedures.

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THE EFFECT OF GREEN MANURE APPLICATION TO COFFEE PLANTS GROWTH, YIELD AND QUALITY IN KENYA

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ABSTRACT

Green manures from leguminous plants have been used extensively as source of plant nutrients mainly in annual food crops. The current study focused on whether they can be used successfully in coffee in order to reduce the heavy costs associated with fertiliser application.Green manures from différent leguminous plants were applied to young coffee plants at establishment phase at Coffee Research Station, Ruiru, Kenya between 1992 and 1997. The coffee plants were planted at a plant density of 2500 trees/ha while the green manure plants were either interplanted with the coffee or grown outside the coffee plots. Foliage from the plants were cut at three month intervals and the foliage applied around the coffee trees as mulch.

The applied green manures except that from desmodium did not significantly affect the coffee plant height, stem diameter, number of priinaries or nodes per primary. Application of the green manures did not significantly affect clean coffee yields, bean size, coffee liquor qualities during the first production cycle. They can therefore be recommended for use in coffee production. Further work on their management, décomposition and nitrogen mineralization and utilisation is suggested.

INTRODUCTION

Coffee is an important crop in Kenya's economy. To ensure high coffee yields and quality, tiinely application of agroinputs is recommended (Anon, 1989). Due to the high cost of inputs, farmers have adopted sub-optimal input regimes. This have had adverse effects on coffee yields and quality (Karanja 1992). To ensure sustainability of coffee production in Kenya, alternative sources of nutrition are required. One such alternative is the use of green manures. Green manures have been used successfully as sources of plant nutrients in crops such as rice, maize, coconut(Bottenberg, 1981; Kang *et al.,* 1981, Vioayakumar *et al.,* 1986). They could be used to substituts to some extent the inorgarnic fertiliser requirements for coffee, especially in nitrogen deficient coffee soils (Michori, 1981).

The objective of this study was to investigate the effect of green manures on growth, yield and quality of newly established coffee plants.

MATERIALS AND METHODS

The trial was carried out at Coffee Research Station, Ruiru from May 1992 to December 1997. The station is located at l° 06' S, 36° 45' E, and 1620 m above sea level. The rainfall is bimodally distributed with the main season being April to July (long rains) and a shorter season from October to December (short rains). The soils are humic nitosols with a deep profile and reddish brown to dusky brown clays (Jaetzold and Schimdt, 1983). They are moderate in bases, low in phosphorus and slightly

acidic with a pH range of 4.0 - 5.4 (CaCI2) (Siderius and Muchena, 1977). Arabica coffee hybrid Ruiru 11 was used as the test crop. The coffee plants were planted at a plant density of 2500 plants/ ha. The coffee plants were raised on single uncapped stem with minimum pruning (Mwangi, 1983).
The green manure tested were obtained from leucaena *(Leucaena leucocephala),* sesbania *(Sesbania sesban),* and calliandra *(Calliandra calothyrsus),* desmodium *(Desmodium intortum), lucerne (Medicago sativum)* and pigeonpea *(Cajanus cajan).* These plants were interplanted with coffee and cut in March, June, September and December in each year and the foliage were applied as mulch to the coffee plants. Leucaena, sesbania and calliandra were also grown outside the coffee plots and their cut foliage also applied to separate coffee plots. The effect of the green manures on coffee was compared to that of application of cattle manure, napier grass or inorganic fertilizers. These are the common practices in coffee production in Kenya.(Mwangi, 1983)

Cattle manure was applied twice a year in March and September at the rate of 10 tonnes per hectare per annum (13 kg/tree/year). Napier grass mulch was applied along the coffee trees during the first year after establishment and thereafter on alternate coffee inter rows at the rate of 10 tonnes per hectare per year (Mwangi, 1983). Nitrogen fertiliser was applied to the coffee plants at the rate of 50 kg N/ha/year during the first year and 160 Kg N/ha/year in subsequent years split three times in a year in April, May and November (Mwangi, 1983). In April and May the fertiliser was applied in form of calcium ammonium nitrate (26% N) while in November it was applied in the form of 20: 10: 10 NPK fertiliser Non fertilized coffee trees were also maintained as a control.
The thirteen treatments were laid in a completely randomised block design, with three replications. The plot size was 48 ml and consisted of 20 coffee trees. Plant growth was recorded during the first two years after establishment.The six central coffee plants in each plot were marked and plant height from plant base to the tip of the growing point, stem diameter at 15 cm from stem base, extension growth of the middle canopy primary branches, total number of bearing primaries per tree and the total number of bearing nodes per primary branch recorded at three month intervals. Ripe coffee cherries were picked from the six central plants from 1995 and a 10% sample per picking taken for assessment of yield of clean coffee and the percent grade A beans. Clean coffee are those whose parchment skin has been removed by hulling reducing their weight by 20%, while grade A beans are those retained by a 6.75 mm screen. Coffee liquor quality was determined organoleptically as described by Devonshire (1956).

RESULTS

Application of green manures from desmodium reduced significantly the coffee plant height, stem diameter, primary branch extension and number of primary branches per tree. Application of the other green manures did not have any significant effect on the coffee plant growth (Table 1).
Interplanting the green manure plants with coffee or bringing in their foliage had no significant effect on the coffee growth parameters. Similarly the type of green manure plant (shrub or annual) did not affect the coffee plant growth parameters.

Application of green manures did not significantly affect the yields of clean coffee which averaged 600kg/ha . The use of green manures did not also affect the coffee quality in terms of grade 'A' sized beans which averaged 68.3 per cent.

DISCUSSION

Most of the green manures applied to young coffee plants did not affect coffee plant growth. However. interplanting desmodium and lucerne plants with coffee adversely affected the increase in coffee plant height, stem diameter and primary length.
Desmodium being a creeping plant had a choking effect on the young coffee plants, thus affecting the coffee plant growth. Sirnilar effects have been observed when young coffee plants are intercropped with sweet potatoes in Kenya (Njoroge and Kimemia, 1995).

Table 1 : Effects of green manure application on coffee plant growth (1992 1993)

Sources of green manure	Height	diameter	Primary branch extension	Number of primary brances
Leucaena intercrop	39.7 ab	1.25 bc	39.3 abc	44 a
Leucaena purestand	34.9 ab	1.19 abc	40.8 abc	42 a
Sesbania intercrop	34.8 ab	1. 19 abc	29.3 cd	38 ab
Sesbania purestand	36.8 ab	1.39 ab	42.8 a	52 a
Calliandra intercrop	52.4 a	1.04 abc	35.7 a-d	44 a
Calliandra purestand	40.3 ab	1.39 ab	45.2 a	46 a
Desmodium intercrop	29.7 b	0.75 c	25.5 d	26 b
Lucerne intercrop	30.8 b	0.83 c	29.7 bcd	42 a
Pigeonpea intercrop	33.2 b	0.95 abc	34.7 a-d	40 a
Napier grass mulch	36.5 ab	1.15 abc	37.7 a-d	54 a
Cattle manure	33.6 ab	1.31 abc	44.3 a	48 a
Inorganic nitrogen	44.4 a	1.53 a	46.7 a	46 a
Unfertilized control	44.0 a	1.48 a	44.9 a	46 a
Mean	37.8	1.18	38.2	44
SED	6.6	0.2	5.8	3.5

Means followed by the same letter down the column were not significantly different according to Duncan's Multiple Range test, 5 % level.

To avoid the adverse effects on coffee growth, harvesting at shorter intervals is suggested.
The use of green manures has been reported to increase yields of annual crops like maize and rice (Prussner, 1983).
Kang *et al.,* (1981) reported a 146% increase in maize grain yields following the addition of 10 tons of leucaena prunings (Vioyakumar, 1986). In the current study, application of green manures did not affect either the clean coffee yields and the coffee quality in terms of grade 'A' beans. The non effect could be attributed to the availability of nitrogen - released during decomposition of the organic matter from litter fall and also nitrogen fixation. The retum of nitrogen and organic matter from the green manure crops hale been reported to lead to better tree growth and increised palm oil nut yields in West Africa (Peoples and Craswell, 1992). Venikateswarlu et *al* (1990) reported that nitrogen fixation *in situ* and leaf fall are the two important processes that nitrogen fixing trees contribute to soil fertility.
Application of green manures did not affect the raw, roast and liquor quality. The overall assessment indicated that the beans were of Fair Average Quility (FAQ), light, light medium and FAQ. These observations concurred with those of Gathaara and Kiara (1990) who observed that fertilizers do not appear to influence the organoleptic characteristics of coffee beans. It was therefore concluded that green manures could be used as sources of nutrients to coffee plants. This would be a good basis for producing organic coffee, in Kenya particularly with the disease resistant arabica hybrid variety Ruiru11

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MISE EN EVIDENCE DU TRANSFERT D'AZOTE DES LEGUMINEUSES AUX CAFEIERS PAR L'UTILISATION DES METHODES ISOTOPIQUES.

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Résumé

L'objectif de l'étude est d'évaluer l'économie en azote obtenue lorsqu'on associe des plantes légumineuses aux caféiers. Des échantillons de feuilles et de branches ont été prélevés dans des plantations de caféiers cultivés soit sous ombrage de légumineuses (*Calliandra, Erythrina* et *Leucaena*), soit en cultures intercalaires (*Flemingia, Leucaena*). La méthode des abondances isotopiques naturelles d'azote (A.I.N.A.) a été utilisée pour évaluer les quantités d'azote atmosphérique fixées par les légumineuses et effectivement transférées aux caféiers.

On a pu démontrer que la disponibilité en azote pour le caféier est soumise à certaines conditions culturales. Lorsque ces conditions permettent à la légumineuse de réaliser au minimum 20 à 25% de ses besoins en azote grâce à la fixation biologique de l'azote atmosphérique, le transfert est effectif dès la première année.

Connaissant les quantités d'azote dans les légumineuses et les proportions obtenues à partir de la fixation biologique ou prélevées dans le sol par chacune des plantes, on a pu calculer les quantités d'azote apportées au caféier par les légumineuses dans les divers types d'associations culturales étudiées.

La plantation intercalaire de légumineuses présente l'avantage de constituer un système écologique durable qui permet au planteur de préserver son capital caféier en période de non entretien et de l'optimiser en période de bon entretien et de faible utilisation d'intrant.

Summary

The objective of this study is to evaluate the amount of nitrogen saved when legume crops are associated to coffee trees. Leaf and branch samples were taken in coffee plantations with legume crops planted either as shade trees (*Calliandra, Erythrina*, and *Leucaena*) or as cover crops (*Flemingia, Leucaena*). The Nitrogen Natural Isotopic Abundance method was used to measure the amounts of atmospheric nitrogen fixed by the legume crops and actually transferred to coffee trees.

We could show that the N available for coffee is subject to some cultivation conditions. When the legume crop can fix more than 20 to 25% of its needs from biological N fixation, the N transfer is effective from the first year.

Knowing the amounts of N in the legume crops and the ratio taken from biological N fixation and from the soil, we could compute the amounts brought to the coffee trees in the various associations.

Legume intercropping has the advantage of making up a durable and ecological system that allows preserving the coffee capital when the farmer does not maintain its plantation and optimise it in conditions of good management and low input.

INTRODUCTION

Les caféiers préfèrent les sols riches en matière organique et en azote. Les plantes fixatrices d'azote en général et les légumineuses en particulier peuvent participer à cette alimentation. C'est pourquoi, de nombreux essais d'associations caféiers - légumineuses ont été mis en place dans les pays producteurs

de café et ont démontré l'intérêt de ce type d'association (CARVAJAL, 1984; BOUHARMONT, 1978 et 1979).
Dans le cas de cultures associées, l'azote atmosphérique fixé par la légumineuse peut être partiellement transmis à la plante non fixatrice associée. Ce transfert a été mis en évidence dans le cas particulier des associations à base de caféiers de façon indirecte. Au Kivu, SNOECK J. (1961) a observé une augmentation de 22 % d'azote total dans les caféiers associés au *Leucaena leucocephala*. Au Costa Rica, une augmentation du pourcentage d'azote total dans des caféiers associés avec *Erythrina* par rapport à des caféiers cultivés seuls a aussi été observée (ALPIZAR *et al.*, 1985 ; GLOVER *et al.*, 1986). Une meilleure minéralisation de l'azote associée à une meilleure nitrification a été observée dans les sols de plantations de caféiers associés à des *Erythrina* (15 $g \cdot m^{-2} \cdot an^{-1}$ de N) par rapport aux plantations de caféiers seuls (11 $g \cdot m^{-2} \cdot an^{-1}$ de N) par BABBAR et ZAK (1994). Au Venezuela, ARANGUREN *et al.* (1982) ont observé que l'*Erythrina* ou l'*Inga* cultivés comme arbre d'ombrage dans la plantation de caféiers permettent de compenser les quantités d'azote exporté par la récolte du café, les pulpes étant restituées au champ.
La méthode des abondances isotopiques naturelles d'azote (A.I.N.A.) a été utilisée pour étudier les mécanismes de transferts d'azote entre deux cultures pérennes dont l'une fixe l'azote (KURDALI *et al.*, 1990). Ces auteurs ont montré que cette technique permet d'estimer les quantités d'azote atmosphérique (N_2) fixé et transféré aux plantes associées.
Nous avons voulu exploiter cette méthode pour confirmer l'existence d'un transfert d'azote aux caféiers et quantifier la part de N_2 fixée par les légumineuses et effectivement apportée au système cultural associatif de caféiers. Pour ceci, nous avons employé diverses espèces de légumineuses dans différentes conditions de cultures.

MATERIELS ET METHODES

Description des sites

Les observations ont été réalisées au Burundi dans diverses plantations de caféiers utilisant les légumineuses soit comme arbres d'ombrage, soit comme haies vives, situées dans deux des centres de recherche café.
Le centre de recherche café de ***Kayanza*** est situé à 2,7° au sud de l'équateur et 29,6° Est. L'altitude du centre est de 1.800 m. La région est caractérisée par un climat équatorial d'altitude avec une seule saison des pluies d'octobre à mai. La pluviométrie annuelle est de 1.300 mm. La température moyenne est d'environ 19°C tout au long de l'année avec des amplitudes thermiques de 10°C. Les sols sont modérément acides (pH = 5,5) et assez bien pourvus en éléments minéraux. Les essais ont été plantés en décembre 1990 ; les prélèvements d'échantillons de feuilles ont été effectués en avril 1994.
Le centre de recherche de ***Rukoba*** est situé à 3,1° au sud de l'équateur et 30,2° Est. La région est caractérisée par une altitude plus faible (1.400 m) et une pluviométrie annuelle de 1.240 mm ; la température moyenne est légèrement plus élevée qu'à Kayanza (23°C). Les sols y sont fortement acides (pH = 4,7 à 5,2) et désaturés. Les essais ont été plantés en décembre 1991 ; les prélèvements d'échantillons de feuilles ont été effectués en avril 1994.

Dispositif expérimental

Les caféiers sont cultivés en tiges multiples à des écartements de 2,5 m x 1,5 m (2 666 $plants \cdot ha^{-1}$). L'espèce utilisée est le *Coffea arabica* cultivar Jackson 2 qui est un caféier de type Bourbon à port haut (2,5 à 3,0 m).

Dans certains essais, les légumineuses sont utilisées comme plantes de couverture (*Flemingia congesta*, *Desmodium intortum*, *Leucaena leucocephala*). Ces légumineuses sont alors plantées en doubles haies séparées de 30 cm. Les plants sont plantés à 30 cm d'écartement en quinconce (26 666 $plants \cdot ha^{-1}$). Chaque essai compare ces trois légumineuses en association avec des caféiers à un témoin composé de caféiers en culture pure. La parcelle élémentaire est composée de 10 caféiers séparés par une ligne de bordure. Les traitements sont répétés dans six blocs randomisés.

Dans d'autres essais, les légumineuses ont été utilisées comme arbres d'ombrage (*Calliandra calothyrsus, Erythrina abyssinica, Leucaena diversifolia*). Dans ce cas, les arbres ont été plantés à 10 m d'écartement (100 plants·ha^{-1}). La parcelle élémentaire est alors constituée d'un bloc de 24 caféiers (4 x 6) avec un arbre d'ombrage en son centre. Les traitements sont répétés dans six blocs randomisés.

Observations et mesures

Les parcelles de caféiers ont été choisies de manière a avoir à la fois :

- des caféiers adjacents aux légumineuses et suffisamment proches de celles-ci pour bénéficier de l'azote atmosphérique fixé à la fois par la litière et par les racines ;
- des caféiers suffisamment éloignés de la zone d'influence de la légumineuse mais qui restent dans le même type de sol. Dans ce cas, la litière de légumineuse a été remplacée par une quantité équivalente de paillis afin de conserver le même environnement chimique et organique pour ne pas modifier les niveaux d'abondance isotopique naturelle d'azote.

Des échantillons de feuilles ont été prélevés sur les légumineuses, les caféiers adjacents à ces légumineuses et sur des caféiers plus éloignés dans des parcelles de caféiers âgés de quatre ans à Kayanza et de trois ans à Rukoba. Ce qui correspond à des prélèvements effectués après respectivement 2 ans et 1 an de fixation de N_2 et de transfert.

Les quantités de biomasses produites et les pourcentages d'azote total ont été mesurés pour les différents traitements. Des échantillons de sols ont été prélevés sous les mêmes plantes.

Méthode des A.I.N.A. pour l'analyse des pourcentages d'azote fixé

A partir de la mesure des abondances isotopiques naturelles d'azote (A.I.N.A.) d'échantillons de feuilles d'une plante fixatrice d'azote et de feuilles d'une plante non fixatrice, il est possible de calculer les pourcentages d'azote atmosphérique fixés par la plante fixatrice. Cette méthode est basée sur les observations suivantes (AMARGER *et al.*, 1977 ; BARDIN *et al.*, 1977) :

- il existe naturellement deux isotopes stables de l'azote : ^{14}N et ^{15}N.
- le taux d'isotopes ^{15}N de l'air est constant et égal à 0,3663 %, tandis qu'il varie dans le sol entre 0,3630 % et 0,3730 % en fonction des conditions de sols et de cultures (SHEARER *et al.*, 1974 ; LEDGARD, 1989).
- une légumineuse qui puise une partie de ses besoins en azote de l'atmosphère (Ndda : N dérivé de l'atmosphère) et le reste dans le sol (Ndds : N dérivé du sol) aura une composition isotopique (% $^{15}N_{lég.}$) intermédiaire entre la valeur isotopique de l'atmosphère (% $^{15}N_{fixation}$) et celle du sol (% $^{15}N_{sol}$) du fait de la fixation biologique de l'azote atmosphérique. Ce calcul est possible dès lors qu'il existe une différence entre les rapports isotopiques de l'air et du sol.

Du fait de la très petite différence entre les abondances naturelles d'isotopes ^{15}N dans le sol, dans la plante et dans l'air ambiant, les données sont généralement exprimées en milliers. On utilise alors l'unité suivante :

$$\delta^{15}N = [(\%^{15}N_{échantillon} - \%^{15}N_{air}) / (\%^{15}N_{air}) \times 1000]$$

où le % d'isotopes ^{15}N de l'air sert de référence. Par définition, on a :

$$\delta^{15}N_{air} = 0 \text{ ‰}.$$

Le pourcentage d'azote introduit dans le système cultural par la plante fixatrice d'azote peut dès lors être calculé en posant le système de deux équations à deux inconnues décrit par BARDIN *et al.* (1977):

$$\begin{cases} Ndda\% + Ndds\% = 100\%_{dans\ la\ lég.} \\ Ndda\% \times \delta^{15}N_{fixation} + Ndds\% \times \delta^{15}N_{sol} = 100\% \times \delta^{15}N_{lég.} \end{cases}$$

La résolution de ce système d'équations donne la formule :

$$Ndda\ \% = \frac{(\delta^{15}N_{sol} - \delta^{15}N_{lég.})}{(\delta^{15}N_{sol} - \delta^{15}N_{fixation})} \times 100.$$

La valeur de fixation pure ($\delta^{15}N_{fixation}$) est obtenue en cultivant les plantes fixatrices dans un milieu complètement dépourvu d'azote pour être sûr que l'azote mesuré dans la légumineuse ne puisse provenir que de la fixation. Cette valeur varie d'une espèce de légumineuse à l'autre (MARIOTTI *et al.*, 1980 ; SHEARER et KOHL, 1986) et aussi chez une même légumineuse lorsque différentes espèces de rhizobium sont impliquées dans la symbiose (YONEYAMA *et al.*, 1986 et 1989). Ces valeurs ont été calculées pour les trois légumineuses utilisées dans nos essais à partir de plants inoculés avec les bactéries isolées au Burundi et cultivés en serre dans des pots contenant un milieu de croissance dépourvus d'azote. Les résultats suivants ont été obtenus pour les $\delta^{15}N_{fixation}$ analysés dans les feuilles de : *Leucaena* = -1,90 ; *Desmodium* = -1,00 ; *Flemingia* = -1,20 ; *Calliandra* = -1,00 ; *Erythrina* = -1,00 ; (SNOECK *et al.*, 1994b).

Mise en évidence du transfert global d'azote de la légumineuse au caféier

Un caféier associé à une légumineuse bénéficie du N_2 fixé par cette dernière par l'intermédiaire de sa litière déposée aux pieds des caféiers et par les échanges dans le sol entre les systèmes racinaires (via les exsudats racinaires, rhizobium en décomposition, etc.). De ce fait, l'abondance isotopique naturelle mesurée dans le caféier va évoluer au cours du temps pour se rapprocher de celle de la légumineuse. Tandis que l'abondance isotopique naturelle d'un caféier cultivé seul reste proche de celle du sol.
KURDALI *et al.* (1990) ont montré qu'une plante fixatrice apporte à l'association culturale de l'azote qu'elle puise à partir de deux sources : X% qui viennent de la fixation biologique du N_2 et Y% qui viennent de l'azote du sol.
On peut donc établir le système de deux équations à deux inconnues suivant :

$$\begin{cases} \mathbf{X\% \times \delta^{15}N_{fixation} + Y\% \times \delta^{15}N_{sol} = 100\% \times \delta^{15}N_{association}} \\ \mathbf{X\% + Y\% = 100\%} \end{cases}$$

Où, dans notre cas particulier, le $\delta^{15}N_{fixation}$ a été obtenu comme décrit précédemment, le $\delta^{15}N_{sol}$ correspond à la mesure de l'excès isotopique mesuré dans le caféier éloigné et le $\delta^{15}N_{association}$ correspond à l'excès isotopique mesuré dans le caféier bénéficiant de l'association. La résolution de ce système d'équations donne le pourcentage (X%) d'azote apporté à l'association caféier – légumineuse par la fixation de N_2 :

$$\mathbf{X\ \% = \frac{(\delta^{15}N_{sol} - \delta^{15}N_{association})}{(\delta^{15}N_{sol} - \delta^{15}N_{fixation})} \times 100.}$$

RESULTATS

Le tableau 1 résume les valeurs moyennes de 5 répétitions (± écart–type) des mesures de $\delta^{15}N$ effectuées dans les différentes parcelles d'essais d'associations caféiers – légumineuses installées à Rukoba et à Kayanza ainsi que les résultats des calculs de pourcentages d'azote atmosphérique fixés par les diverses légumineuses et apportés à l'association culturale.

Dans le cas des légumineuses utilisées comme cultures intercalaires, quand les capacités fixatrices de N_2 sont suffisantes (plus de 20%), les caféiers associés présentent des valeurs isotopiques intermédiaires entre les valeurs mesurées dans les légumineuses et les valeurs du sol (caféiers éloignés). Ces différences se retrouvent dans les pourcentages d'azote fixé (Ndda%) et transféré (X%). Les résultats des calculs des deux dernières colonnes montrent que le bénéfice de l'association augmente proportionnellement au pourcentage d'azote fixé.

Les caféiers associés aux légumineuses utilisées comme arbres d'ombrage (*Calliandra, Erythrina*) bénéficient de façon moins efficace de l'association. Une raison essentielle à cette différence peut être trouvée dans la différence entre le nombre de plants utilisé dans chacune des deux associations ; 100 plants·ha^{-1} dans le cas des arbres d'ombrage contre 26.666 plants·ha^{-1} dans le cas de cultures intercalaires. Cette explication a été vérifiée dans d'autres essais (SNOECK *et al.*, 1994a).

Tableau 1 : Pourcentage de N_2 total et fixé calculé à partir des valeurs isotopiques d'échatillons foliaires

Caféiers associés à		$\delta^{15}N$ (‰)			**Ndda** (%) N dérivé de l'atmosphère	X (%) N de la fixation dans le caféier
		Légumineuses	Caféier associé	Caféier éloigné		
Rukoba						
Leucaena leucocephala	(i)	4.14 ±2.0	7.63 ±0.6	8.12 ±0.4	39 ± 19	5 ± 4
Flemingia macrophylla	(i)	3.85 ±0.6	7.55 ±0.5	8.12 ±0.4	42 ± 5	6 ± 3
Kayanza						
Flemingia macrophylla	(i)	2.86 ±0.5	3.38 ±0.2	3.85 ±0.3	20 ± 9	6 ± 4
Desmodium intortum	(i)	1.30 ±0.5	3.60 ±0.3	4.50 ±0.3	50 ± 5	16 ± 2
Leucaena leucocephala	(i)	-0.4 ±0.3	1.20 ±0.2	2.10 ±0.2	52 ± 4	22 ± 1
Leucaena leucocephala	(i)	2.65 ±0.5	2.94 ±0.2	2.83 ±0.3	0	0
Leucaena diversifolia	(o)	1.46 ±0.5	4.55 ±0.2	5.48 ±0.3	48 ± 5	15 ± 4
Calliandra calothyrsus	(o)	3.82 ±0.2	5.13 ±0.1	4.57 ±0.6	20 ± 13	0
Erythrina abyssinica	(o)	3.17 ±0.3	4.32 ±0.4	3.38 ±0.3	21 ± 4	0

(i) légumineuse utilisée comme culture intercalaire ; (o) légumineuse utilisée comme arbre d'ombrage.

Des analyses de sol ont été faites aux pieds des caféiers et des légumineuses dans toutes les parcelles. Les résultats (qui ne sont pas présentés ici) montrent des différences significatives de fertilité des sols (acidité et saturation en bases échangeables) entre les essais, mais on n'a pas observé de différences significatives entre les traitements d'une même parcelle.

DISCUSSION

L'absence de différences significatives entre les analyses de sol à l'intérieur d'une même parcelle permet de supposer que le sol n'est pas une source de variation et donc que les différences entre les valeurs de $\delta^{15}N$ observées sont essentiellement dues à la fixation du N_2 et au transfert dans la plante voisine. Les différences entre les diverses parcelles et entre les deux stations expliquent en partie les différences entre les abondances isotopiques des différents essais.

La fixation de N_2 n'est possible que dans des sols qui sont suffisamment saturés en bases échangeables (K, Ca, Mg) et ne sont pas trop acide (pH > 5,5). Ces deux facteurs sont indispensables à l'activité microbienne dans le sol. Sinon, il n'y a pas de fixation de N_2 et dans ce cas, les deux cultures rentrent en concurrence et la culture intercalaire de légumineuse peut même avoir un effet dépressif sur les rendements des caféiers (SNOECK *et al.*, 1994b).

Si la fixation d'azote existe mais est faible (de l'ordre de 20% dans les exemples du tableau 1), ce qui est le cas dans les sols assez acides (pH entre 5,0 et 5,5), il n'y a pas ou très peu de transfert. On ne peut pas présumer de la concurrence entre les deux cultures, mais dans ce genre de cas, les planteurs ne voient pas l'intérêt d'une culture intercalaire.

Le caféier bénéficie de l'azote introduit dans le système cultural via la fixation de N_2 quand celle-ci est suffisamment importante (plus de 20 %). On a vu qu'alors, plus la légumineuse fixe d'azote, plus son impact sur la quantité d'azote introduit dans le système cultural est important. Il y a un coefficient de proportionnalité (X% / Ndda%) assez constant entre les différents essais d'une même station de recherche de 15 % à Rukoba et de 33 % à Kayanza. Cette différence entre les stations peut être imputée à plusieurs facteurs, dont probablement la différence d'âge des essais (1 an de fixation et transfert à Rukoba et 2 ans à Kayanza) et aussi la différence de fertilité entre les deux stations (sols plus fertiles à Kayanza qu'à Rukoba).

La densité de plantation de légumineuses influence la quantité de N_2 fixé et introduit dans le système par deux types d'actions complémentaires : une partie importante (75% du N_2 fixé) est transférée par la litière et le reste (25%) est obtenu par la cohabitation des systèmes racinaires via les exsudats racinaires, nodosités de rhizobium en décomposition, etc. (SNOECK *et al.*, 1994b). Ces modes de transfert expliquent que des légumineuses cultivées comme arbres d'ombrage qui produisent moins de matière organique que des cultures intercalaires régulièrement fauchées et dont les systèmes racinaires sont moins en contact avec ceux des caféiers transfèrent aussi moins d'azote aux caféiers.

Le bénéfice de la légumineuse pour l'association culturale peut être chiffré en équivalent d'engrais azoté en multipliant la quantité d'azote fixé par le pourcentage d'azote transféré au système.

De nombreux auteurs ont étudié les quantités d'azote fixées par diverses légumineuses et certains se sont plus particulièrement intéressés aux associations avec les caféiers (ARANGUREN *et al.*, 1982 ; ALPIZAR *et al.*, 1985 ; GLOVER et BEER, 1986 ; SNOECK *et al.*, 1994a). Sur base des informations fournies par les divers auteurs, on peut estimer que, dans les conditions normales d'association avec des caféiers, les légumineuses utilisées comme arbres d'ombrage (à densité de 100 à 150 arbres·ha^{-1}) peuvent apporter à l'association entre 50 et 100 kg d'engrais N par ha et par an ; tandis que les légumineuses utilisées en cultures intercalaires (plus de 20.000 plants·ha^{-1}) peuvent apporter à l'association entre 150 et 250 kg d'engrais N par hectare et par an.

Si l'on admet que, annuellement et dans de bonnes conditions, 20 à 25% de l'azote fixé va aux caféiers (meilleurs cas ci-dessus), la légumineuse apporte une dose équivalente de 12 à 62 kg d'engrais N·ha^{-1}·an^{-1} selon le type d'association. Cet apport représente environ 10% des besoins des caféiers puisqu'on estime qu'il faut de 100 à 500 kg N·ha^{-1}·an^{-1} pour compenser les exportations par les récoltes (35 kg N·ha^{-1}·an^{-1} par tonne récoltée), la croissance, l'entretien et les tailles des arbres (CARVAJAL, 1984).

Malgré tout, cet apport n'est pas du tout négligeable. En effet, lorsqu'on observe une courbe de réponse des rendements des caféiers aux doses croissantes d'azote, on constate une réponse rapide (plus que proportionnelle) pour les faibles doses et qui tend vers une réponse moindre pour les plus fortes doses comme le montre le graphique ci-dessous.

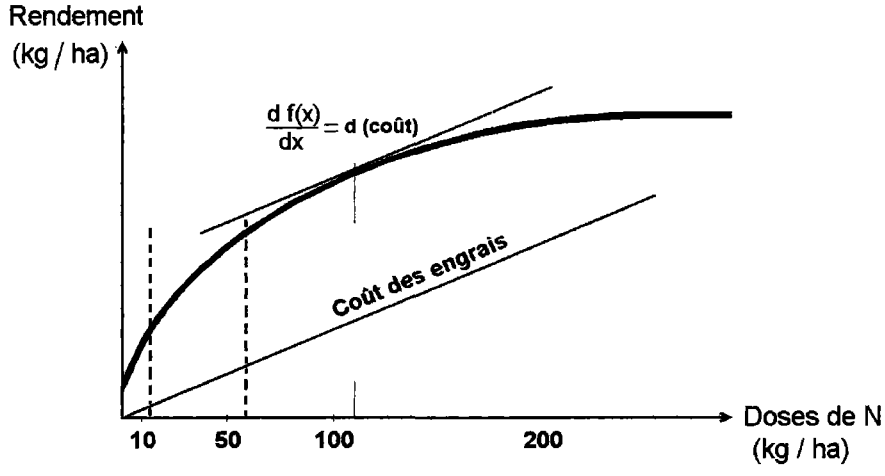

Figure 1 : Courbe type de réponse des caféiers aux engrais montrant la zone de plus grande efficacité

La culture intercalaire de légumineuses permet de mieux rentabiliser les engrais. Ce qui a pour conséquence un moindre coût en intrants pour le planteur et lui assure aussi une culture plus durable grâce à des plants plus vigoureux et donc plus résistants aux aléas. Cela lui permet aussi de valoriser son capital caféier, voire même de mieux le conserver en période de non culture (cas fréquent en Afrique lorsque les prix d'achat du café sont trop faibles pour inciter les planteurs à entretenir leurs plantations) en conservant une ambiance favorable à une culture qui ne reçoit pas d'intrants et qu'un minimum d'entretien, grâce à l'apport naturel d'éléments minéraux et de matière organique.

Le choix de la plante utilisée en association sera fonction des conditions écologiques et topographiques locales et des possibilités de fauchage et d'entretien. Il n'est pas possible de recommander une plante qui soit universellement adaptée à toutes les conditions de culture des caféiers.

L'effet bénéfique des légumineuses associées n'est pas limité au seul apport d'azote. Elle apporte aussi les mêmes avantages que le paillage : réduction de l'envahissement par les mauvaises herbes, protection du sol, amélioration de l'activité microbienne dans le sol et apport d'autres éléments nutritifs (K, Ca, Mg, ...). Si les racines sont profondes (*Leucaena*, *Flemingia*), ils peuvent même récupérer des éléments nutritifs en profondeur.

Grâce à cette combinaison d'effets favorables, la culture intercalaire de légumineuse peut avoir un effet sur les rendements supérieur au seul effet de l'apport d'azote. Au Cameroun, BOUHARMONT (1978) a observé qu'il est possible d'obtenir les mêmes rendements en café avec une culture intercalaire de *Flemingia* qu'avec un apport de 83 kg $N \cdot ha^{-1} \cdot an^{-1}$ apporté sous forme de sulfate d'ammoniaque. D'une manière générale, il estime que la culture de légumineuses intercalaires (*Flemingia* ou *Mimosa*) entraîne une augmentation des rendements de 200 à 500 kg de café marchand par hectare dans la toute la région de culture du Robusta de ce pays.

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EVALUATION OF SOME LEGUMINOUS SPECIES FOR THE ESTABLISHMENT OF ROBUSTA COFFEE IN GHANA

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SUMMARY

Some tree/shrub species like *S. siamea*, *F. macrophylla*, *M. griffonis* and *G. sepium* can tolerate repeated pollarding and can be used in hedgerows and pruned for mulching coffee. However, *S. siamea* and *F. macrophhylla* can over-shade the coffee and possibly take too much nutrients from the soil to the disadvantage of the development of the coffee. *G. sepium*, *M. griffonis* and *C. cajan* enhanced the early development of a sturdy plant whilst *S. siamea* and *F. macrophylla* are likely to encourage tall but weak coffee plants. Species which are not too aggressive in terms of above and below ground competition can enable the farmer to obtain returns from the plantain used to nurse the coffee but low biomass output may not provide sufficient prunings for mulching the coffee. Further evaluation of this trial into the bearing stage of the coffee continues.

POSTER

INTRODUCTION

Coffee has traditionally been cultivated under the temporary shade of *Glyricidia sepium* and plantains in Ghana (Ampofo and Osei-Bonsu, 1988). This system qualifies as an agroforestry package (Young, 1991) but has not been accorded the importance it deserves. However, the inclusion of a leguminous species like *G. sepium* is likely to improve soil nitrogen fertility through nitrogen fixation and mineralization of litter (Budelman, 1989) and enhance the soil physical characteristics through the addition of organic matter (Yamoah *et. al.* 1986, Bahiru Duguma *et. al.*, 1988). Wood and Burley (1991) advocated the need to incorporate and evaluate other woody perennial species into the cultivation of cash crops for similar ecological benefits. *Senna siamea* is known to grow on poor soils and produces a lot of litter but its soil improving potential is not well known (Young, 1991). In Ghana, *Milletia griffonis*, an indigenous leguminous species and *Flemingia macrophylla*, an exotic species which produce a lot of biomass have not been evaluated in tree crop agroforestry. An investigation was therefore carried out at the Cocoa Research Institute of Ghana to assess the possibilities for incorporating some of these species as temporary shade in the cultivation of robusta coffee.

MATERIALS AND METHODS

The experiment was set up at the Cocoa Research Institute sub-station at Afosu in 1996 to evaluate five leguminous tree/shrub species, namely; *Milletia griffonis*, *Flemingia macrophylla*, *Cajanus cajan*, *Glyricidia sepium* and *Senna siamea* planted in hedgerows, during the establishment of robusta coffee. The area used for the trial was cleared from a secondary bush with all the trees felled, chopped and cleared off the field. The plot size used was 12m x 18m and improved coffee clones raised from cuttings were planted at 3m x 3m. The coffee rows were also interplanted with plantain as temporary shade at 3m x 3m in all the plots including the control. The hedgerows for the shade trees were established from nursed seedlings of *F. macrophylla*, nursed rooted cuttings and seedlings of *M. griffonis* and from 50cm cuttings of *G. sepium* planted at stake whilst *C. cajan* and *S. siamea* were planted at stake with seeds. A hedge of each species was established at 0.5m on either side of the coffee line and spaced 0.5m within the row. The treatments were replicated five times in a randomised complete block design. Coffee growth was monitored by measuring stem girth at 15cm from the ground and plant height. Biomass production of the tree species was assessed at 6 to 8 weeks intervals

by pruning the hedges at 1m from the ground, weighing the prunings and sub-sampling for dry matter determination in a ventilated oven at 105°C for 48 hours. The prunings were subsequently spread in the rows as mulch for the coffee. Litter dropping on $1m^2$ polythene sheet placed under the hedgerows at two points within a plot was collected at the time of pruning, bulked per plot and dried to constant weight as for the fresh prunings. Weed cover was estimated by scoring periodically on a scale of 1 to 10 where complete weed cover was reckoned as 10. The performance of the plantain was also assessed at 9 months after planting.

RESULTS AND DICUSSION

Establishment was poor in *M. griffonis* (61%) whilst the other species scored over 80 percent survival (Table 1). Poor rooting from *M. griffonis* cuttings together with its fragile seedlings accounted for this result thus indicating that some woody species may not be easily incorporated into an *agroforestry* system.

Table 1: Evaluation of the performance of the hedgerow species

Species	% Establishment success at 12 months after planting	Year 1			Year 2		Total	
		No of prunings	Biomass **kg ha-^{1}**	Litter fall **gm^{-1}**	No. of Prunings	Biomass kg ha-^{1}	Biomass kg ha^{-1}	Wood tonne ha^{-1}
Milletia griffonis	61.3	4	7 042	16,2	5	12 006	19 048	10,1
Senna siamea	80.0	5	14 574	58,1	6	21 091	35 665	13,4
Flemingia macrophylla	87.7	5	12 236	56,8	6	13 485	25 721	7,1
Cajanua cajan	81.9	5	9 616	180,4	2	4 372	13 988	0
Glyricidia sepium	86.0	5	10 879	6,8	5	6 620	17 499	6,7

C. cajan and *S. simea* were easy to establish by planting at stake with seeds but the other species required nursery care for the fragile seedlings. *G. sepium* could be easily established from cuttings but care had to be taken to select hardened cuttings and to make holes prior to planting. In the first year, 4 to 5 prunings could be carried out on the hedges but in the second year 2 to 6 prunings were taken. The highest biomass was produced by *S. siamea* (35,665 kg ha^{-1}) whilst the least was produced by *C. cajan* (13,988 kg ha^{-1}) which could not withstand the repeated pollarding and died early in the second year. At the time of grubbing the hedgerows owing to competition with the coffee by some species, considerable wood production was also recorded in the *S. siamea* (13.4 tonnes ha^{-1}). Litter fall was heaviest from *C. cajan* especially during the dry season and this helped to mulch the coffee.

Girth of the coffee plants was significantly enhanced with the use of *M. griffonis*, *C. cajan* and *G. sepium* hedgerows six months after planting (Table 2). The least girth attainment was observed in coffee grown under *S. siamea* and *F. macrophylla* hedgerows probably owing to severe competition from the fast growing hedgerows. The hedgerows did not influence girth increments after six months. Taller plants were produced in coffee growing under *S. siamea*, *F. macrophylla* and *G. sepium* hedgerows at 21 months after planting. This was due to the competition of the coffee plants for light under these fast growing species. Tall and spindly growth is not a desirable trait in coffee where a strongly developed main frame is required to

ensure sustained yield (Ampofo and Osei-Bonsu, 1987). Plantain yield was very poor where *C. cajan* was planted (Table 3) whilst yield of plantain from *M. griffonis*, and *G. sepium* was similar to the control.

Weed cover was consistently as high within *M. griffonis* and *C. cajan* hedgerows as in the control for two years because of the poor ground shade produced by these species. It was obvious that the profuse canopy development from the *S. siamea* and *F. macrophylla* kept weed regrowth in check as a weed control strategy.

Table 2: Performance of coffee under hedgerow shade
Growth increments (cm) in coffee plants

Species	6 months		9 months		21 months		Total	
	Girth*	Height	Girth	Height	Girth	Height*	Girth	Height
M. griffonis	2.8[a]	21.8	5.8	38.5	11.6	64.8[b]	20.2	125.1
S. siamea	1.9[c]	20.0	3.9	37.8	9.9	80.1[a]	15.7	137.9
F. macrophylla	1.8[c]	18.4	4.3	36.7	11.6	89.6[a]	17.7	144.7
C. cajan	2.4[ab]	19.1	4.6	34.9	10.1	59.1[b]	17.1	113.1
G. sepuim	2.8[a]	21.3	4.4	32.5	10.7	78.1[a]	17.9	131.9
Control	**21.1[b]**	**19.1**	**5.7**	**38.3**	**12.3**	**60.4[b]**	**20.1**	**117.8**
S.e.d	0.23	NS	NS	NS	NS	6.72	NS	NS

* Figures followed by different alphabets in the column are significant at $P < 0.05$.

Table 3: Effect of the hedgerow species
on companion crop and weed regrowth

Species	% Fruiting Plantains at 9 months after planting	Year 1		Year 2	
		% Weed cover	Weeds dw gm^{-1}	% Weed cover	Weeds dw gm^{-1}
M. griffonis	31	72	97.6	55.3	418.0
S. siamea	20	44	58.8	35.7	380.6
F. macrophylla	24	52	58.8	39.3	243.0
C. cajan	14	54	103.0	53.3	494.0
G. sepuim	34	68	80.0	48.7	440.6
Control	32	110	64.7	64.7	741.4
Mean	25.8	59	84.7	49.5	453.0

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PRELIMINARY INVESTIGATIONS INTO THE USE OF INTERCROPPING FOR WEED MANAGEMENT IN YOUNG COFFEE IN GHANA

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SUMMARY

The possibility of using intercropping in combination with manual weed control to manage weed problems in young coffee was investigated for the first two and half years of establishment using canavalia (*Canavalia ensiformis*), cowpea (*Vigna unguiculata*), maize (*Zea mays*), cassava (*Manihot utilisima*) and plantain (*Musa paradisiaca*) and compared with the conventional methods of manual weeding and herbicide application using *Glyphosate* in sole crop coffee. The presence of the food crops did not significantly affect the growth and early yield of the coffee plants. Intercropping however slightly increased the frequency of manual weeding and also the time taken to complete the operation. Intercropping generally increased the net returns of the various packages. However, the negative effect of cassava on the second year yield of coffee suggests that prolonged intercropping with this crop should be discouraged.

POSTER

INTRODUCTION

Weed control is a major management problem during the establishment of coffee farms particularly where no shade is used. In Ghana, work on weed control in coffee has concentrated on the efficacies and economics of chemical and manual methods of control with reported frequencies of 3-4 times per annum for the recommended chemicals, *Glyphosate* and *Paraquat*, and 5-6 times for manual weed control (Ampofo *et al.*, 1988; Oppong *et al.*, 1995). Such high frequencies of application result in high cost of using these methods. With emerging evidence indicating that weeds are evolving resistance to herbicides (Lebaron and Gressel, 1982) and repeated slashing resulting in the emergence of grass species and sedges (Mitchell, 1988) which pose very severe competition to coffee (Wrigley, 1988), the need to develop an integrated approach to weed control in coffee with the view to reducing cost of application cannot be over-emphasized. This paper reports on the preliminary findings of a study aimed at exploring the possibility of using intercropping for weed management during the establishment of a coffee farm.

MATERIALS AND METHODS

Six month old coffee (*Coffea robusta*) clones were transplanted into the field in June, 1996 at a spacing of 3m x 3m after an initial weed control with the herbicide *Glyphosate* at the rate of 1.0 l in 100ml of water per hectare (low volume application) . After transplanting, the following treatments were imposed in a randomised complete block design with four replicates: sole crop of coffee with manual weed control (T1), sole crop of coffee with chemical weed control (T2), coffee + canavalia (T3), coffee + cowpea (T4), coffee + maize (T5), coffee + cassava (T6) and coffee + plantain (T7). Weeds in the intercropped plots were managed manually. The canavalia, cowpea, maize and cassava were spaced at 0.5, 0.25, 1.0 and 1.5m respectively, away from the coffee plants. The within and between row spacings for these crops were 0.25m x 0.25m for cowpea, 0.5m x 0.5m for canavalia and maize and 1.5m x 1.5m for cassava. The plantain which was planted at the beginning of the trial in

June 1996 was spaced at 3.0m x 3.0m in the centre of four coffee plants. Planting of the cassava was carried out once a year in the month of June after harvesting the previous crop. Double cropping of the canavalia, cowpea and maize was attempted in each year. Data collected during the first two and half years of establishment included the growth of the coffee plants at 3-monthly intervals, the first two years' yields of coffee berries, yields of the component crops and frequency and cost of weed control. The monetary value of the yields of the food crops and coffee was calculated based on prevailing market prices during the experimental period.

RESULTS AND DISCUSSION

Growth and early yields of coffee and intercrops

Contrary to findings reported elsewhere (Mitchell, 1965), the presence of the intercrops did not exert any significantly adverse effects on the growth of coffee plants (Table 1). Although intercropping did not significantly affect the early yields of coffee berries, there was an indication of an adverse effect of cassava and plantain on coffee yield in the second year confirming the findings of Mitchell (*loc. cit.*) and Njoran and Snoeck (1987). The coffee plants were shaded by cassava during the latter part of the trial and, therefore, might have suffered from competition for light and probably nutrients. This indicates that intercropping with cassava could be conveniently carried out only in the first year of transplanting since the first yield of the coffee was not adversely affected.

Table 1: The effect of treatments on the growth at 2 years after planting and early yields of coffee plants and food crops.

Treatment*	Growth increments		Yield				
	Girth (mm)	Height (cm)	Fresh coffee berries (kg/ha)		Food crops (kg/ha)		
			1997	1998	1996	1997	1998
T1	21.7	109.9	20.1	1812.5			
T2	23.4	119.2	46.2	2006.9			
T3	19.9	99.2	5.9	2000.0		750	140
T4	20.7	98.1	121.5	1520.8	683**	297	30#
T5	23.0	117.1	28.5	2126.4	1426**	1332#	537**
T6	18.9	119.5	29.5	650.0	1426	16437	9233
T7	18.4	107.8	55.6	1180.6		3288	5236
S.E.D. (18df)	ns	Ns	33.3	ns			
CV %			107.3	58.0			

*Treatment descriptions can be found in *Materials and Methods*; ** only minor crop yield; # only main crop yield.

The establishment of canavalia, cowpea and maize encountered some problems as the trial progressed which adversely affected their yields. The minor season maize of 1997 failed to establish as a result of damage caused by birds and rodents. The same factor, in addition to erratic rainfall in the early part of the season, resulted in the poor establishment of these crops in 1998. The major season cowpea of the same year was completely destroyed by the fungus *Collectotrichum* sp. The cowpea yields recorded in this trial compare favourably with 91kg/ha achieved in similar studies in Bolivia (Janicki, 1983). It is interesting to note that the yield of the cassava decreased in the second year plantings, which is an indication of gradual reduction in the availability of soil nutrients.

Frequency of weed control and labour requirement

Mitchell (1988) stated that weeds in coffee farms could be suppressed by the lateral shade provided by intercrops. This implies that intercropping could reduce the frequency of weed control. On average, intercropping in this trial slightly increased both the frequency of weed control and the amount of

labour needed to undertake the operation (Table 2). This was mainly due to the need to prepare the land for replanting of the food crops, their respective weed control requirements during cultivation and the extra care required to avoid damage to the crops during weeding. The overall effect of these factors was an increase in the cost of weed control in the intercropped plots.

Table 2 : Frequency of weed control and total labour used for weed control during the year.

Treatment	Frequency of weed control			Labour needed for weed control (mandays/ha/year)		
	1996	1997	1997	1996	1997	1998
T1	2.0	4.0	5.3	10.6	32.4	45.6
T2	1.0	2.0	2.0	1.6	6.6	6.4
T3	2.0	5.0	6.0	11.4	49.0	63.0
T4	2.0	5.0	5.0	10.8	40.5	65.0
T5	2.0	6.0	6.0	9.0	54	91.8
T6	2.0	4.5	5.8	11.6	37.4	68.4
T7	2.0	5.5	5.0	13.8	52.8	52.5

Economic benefit of the packages

One of the criteria for selecting the best establishment strategy is the economics of production. Table 3 shows that intercropping coffee with the food crops during the first two and half years of planting was economically beneficial than sole coffee cropping (T1 and T2), except in the case of cowpea (T3) which was not better than the sole crop of coffee with chemical weed control (T2). Although Table 3 indicates that the best net returns from the intercropping package could be derived from the inclusion of cassava and plantain, this result ought to be taken cautiously since these treatments gave the lowest coffee yields in the second year. It is evident that even though the cost of weed control using intercropping may be high , this could be offset by the additional income provided by the intercrops.

Table 3 : Economics of the treatments for the 1996 - 1998 period

	Cost of production per ha*(x1000 ¢)			Revenue per ha (x1000¢)					Total net returns per ha(¢)
				Food crops			Coffee		
	1996	1997	1998	1996	1997	1998	1997	1998	
T1	31,8	129,6	205,0	-	-	-	12	1019,4	665000
T2	25,2	124,83	142,63	-	-	-	25,8	1128,6	861740
T3	52,2	228,8	319,5	677,47	949,5	168,3	3	1125,0	2322758
T4	53,4	218,0	313,2	144,47	334,65	30,6	68,4	855,6	848577
T5	65,4	363,2	449,1	468,31	1111,55	246,8	16,2	1195,8	2161005
T6	48,7	280,0	377,55	-		2203,24	16,8	365,4	5930908
T7	86,4	227,2	263,25	-		3260,46	31,2	664,2	5845336

* Production cost is made up of weed control, planting and harvesting of food crops except for canavalia and cowpea whose cost of harvesting could not be estimated because of the small quantities of pods harvested at a time which made it difficult to record the time taken to perform the operation. Conversion rate US$ = ¢2500.

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COFFEE PRUNING AND SPACING - MANAGEMENT OF TALL ARABICA COFFEE (*Coffea arabica* L.) IN PAPUA NEW GUINEA

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POSTER

INTRODUCTION

Pruning and spacing are two important aspects of husbandry practices in coffee management. Pruning influences the tree growth and yield responses to other cultural practices, while inducing favourable conditions for pests and disease control (Kiara, 1987). While tree density or number of trees per unit land area is an important yield component (Cannell, 1985), it influences the pruning methods to be employed, weed control, impact of rainfall on soil erosion, utilisation of light, efficiency in the applications of fungicides, pesticides and fertilisers. Economical optimum tree densities are required for different varieties and under different management systems. The optimum tree density gives an individual coffee tree opportunity to utilise resources and least competition with adjacent trees for growth and yield. Most of current husbandry practice used in Papua New Guinea (PNG) have been adapted from other coffee growing countries. Agronomic trials have been set in PNG to determine best practices for PNG under PNG conditions.

MATERIALS AND METHODS

A trial was established on tall arabica coffee (*Coffea arabica* L.), variety Mundo Novo at the Coffee Research Station, Aiyura, PNG. The objective of the trial was to determine the economical tree density optimum and the best pruning (handling and desuckering) methods on the yield of tall arabica coffee. Three tree densities (2500, 3460 and 4444 trees per hectare) and three pruning methods were organised in a 3 x 3 factorial design, replicated five times. The three pruning methods involved handling and desuckering, were (1) secondary growth and suckers were removed after two crops (2) secondary growth and suckers within 20 cm of the main stem and primary branches below 60 cm were removed and (3) desuckering was carried out where it was necessary. Table 1 gives the number of bearing uprights per hectare per density. The trees were brought up on three bearing uprights by bending the seedlings in the field.

Table 1 - Tree densities and their respective number of bearing uprights per hectare

Tree Density (trees/ha)	Number of Uprights/ha
2500	7500
3460	10380
4444	13332

Field costs during the first two years of coffee establishment covered the costs of seedlings, labour for drainage, planting and pruning, inputs like fertilisers and pesticides. Production costs during the third to the fifth year covered field maintenance, cherry harvesting and processing at K0.39 and K0.38 per kilogram of green beans (Table 2 & 3).

Table 2 - Annual Production Costs in Kina per hectare

Trees/ha	1992/93	1993/94	1994/95	1995/96
2500	1384.30	1221.20	3316.90	4719.90
3460	1763.40	1149.60	3738.20	5486.30
4444	2155.00	1081.40	3991.00	5454.60

Table 3 - Production Costs in Kina per hectare broken down into field, harvesting and processing

Trees/ ha	1994/95			1995/96		
	Field	Harvesting @ K 0.39/kg	Processing @ K 0.38/kg	Field	Harvesting @ K 0.39/kg	Processing @ K 0.38/kg
2500	1463.20	940.91	916.79	1985.10	1385.16	1349.64
3460	1491.10	1138.14	1109.00	1614.09	1614.09	1572.71
4444	1525.30	1248.86	1216.84	2468.70	1512.34	1473.56

Annual returns were calculated from annual green bean yields multiplied by an average price of K 2.87 per kilogram of green bean (Table 4). The net present value (NPV) formula was applied to the net returns using the relevant rate of interest. Summaries of the Net Present Value are present in Figure 1.

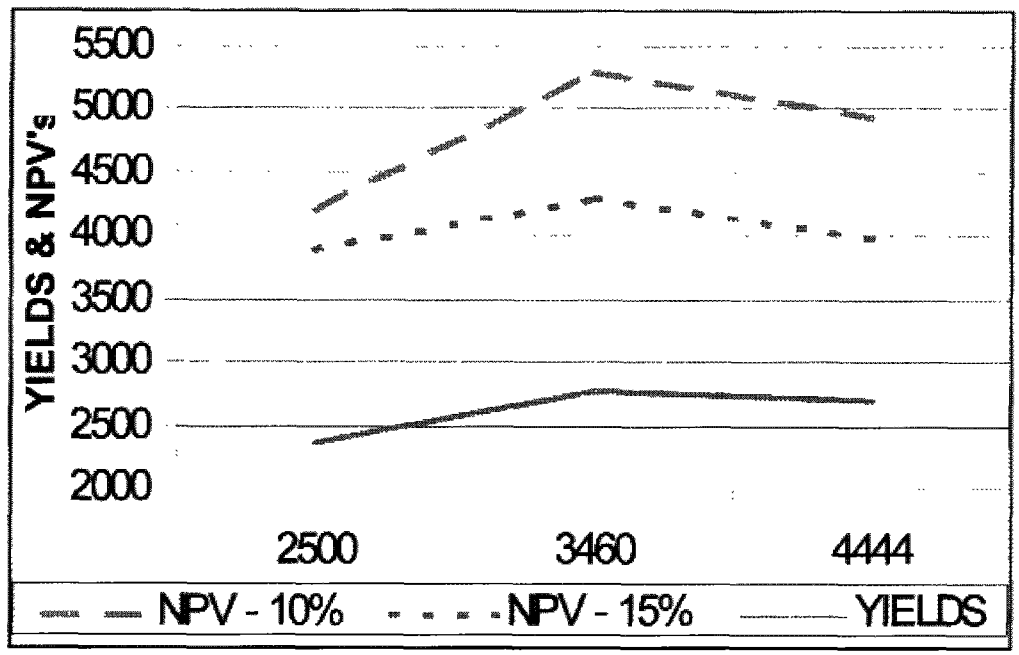
Table 4: Annual Returns in Kina per hectare K 2.87 per kilogramErreur! Signet non défini.

Trees/ha	1994/95	1995/96
2500	6909.30	10,193.00
3460	8375.40	11,878.00
4444	9190.30	11,129.00

Net Present Value (NPV) is the aggregate discounted cash flows over the period of the project, i.e. four years in this case. NPV represents the extra capital gains generated by the investment after paying back the initial outlay and earning an interest on the money invested. In this respect, an investment that yields positive NPV at the opportunity cost of capital is considered viable.

Green bean (clean coffee) yields were recorded for three coffee seasons and the Net Present Values (NPV) were determined for the period.

Figure 1: MUNDO NOVO PRUNING AND SPACING TRIAL
Green Bean Yields and Net Present Values at 10% and 15% Rate of Return



RESULTS AND DISCUSSIONS

The results showed that, the differences in the mean green bean yields obtained due to the tree densities used were significant ($p<0.05$) between 2500 and 3460 trees ha^{-1}. The differences in the yield between these two densities and 4444 trees ha^{-1} were not significant. The mean green bean yield figures for the three crops were higher at 3460 trees ha^{-1} as compared to the lowest (2500 trees per hectare) and the highest (4444 trees per hectare) tree densities used in the study. The respective yield figures from lowest to the highest densities were 2362, 2784 and 2689 kg green bean per hectare per year (Table 5).

Table 5: *Effect of Spacing on Coffee yields given in Green Bean (clean coffee) yields per hectare*

Tree Density trees/ha	Coffee Yields kg/ha			
	1995	1996	1997	Mean
2500	2401	3656	1059	2362
3460	2918	4221	1213	2784
4444	3133	3950	982	2689
Mean	2817.5	3932.3	1084.9	2611.6
LSD (p=0.05)	501.6	478.9	182.8	333.46

Economic analysis results showed that the highest Net Present Values were obtained at 3460 trees per hectare. Despite the fact that the mean yields for the two high tree densities were close, 2784 kg green bean per hectare per year at 3460 trees per hectare and 2689 kg green bean per hectare per year at 4444 trees per hectare (a difference of 95 kilograms of green bean), the annual returns in PNG Kina per hectare were much lower at 4444 trees per hectare. Therefore, 3460 trees per hectare was the economical optimum for tree density, which also gave the highest biological yields.

CONCLUSION AND MANAGEMENT IMPLICATIONS

The existing recommendations for tall rust susceptible arabica coffee in PNG is 2500 to 3000 trees/ha (PNG Coffee Handbook pp 5-31, 1994). Comparing these recommendations with the present results, the optimum biological and economical tree density of 3460 trees per ha carries an additional 460 trees per ha over and above the maximum tree density currently recommended for tall varieties. The existing recommendations were based on information and practices used elsewhere in the coffee growing world and suitable under their conditions. These tree densities were also sparsely adequate to combat coffee leaf rust and ease its management. The impact of leaf rust, although need further verification, for now is not a major deterrent for yield as it was thought otherwise when the problem first appeared in 1986. The present results now leave the farmers with room to go for higher tree densities which are economically manageable.

The implications for the management of tall arabica coffee, is that, it is now locally proofed the existing spacings for unshaded tall arabica coffee, and even more, unshaded coffee can be planted at slightly higher tree densities than original thought under PNG conditions. The practical tree spacings for around 3460 trees/ha would be 1.70 m x 1.70 m square planting, and for hedgerow (rectangular) plantings at 2.00 m x 1.40 m, which will give a tree density of around 3571 trees per hectare; the latter arrangement will allow adequate working space between coffee rows for field operations.

The green bean yields due to the method of pruning in terms of handling and desuckering applied during the three years did not significantly influence the yield differences. This results show that farmers can carry out desuckering and handling only as necessary using their intelligence - to encourage growth of potential crop bearing wood by removing unnecessary growth of suckers, shoots and twigs.

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USE OF HUMIC ACID IN PROMOTING GROWTH OF YOUNG COFFEE ROBUSTA SEEDLINGS IN NIGERIA

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ABSTRACT

Coffee seedlings grows very slowly on the field, and this has been found to be responsible for over 60% of the cases of fail establishment on the field, since the plants are too feeble to withstand the first dry season following transplantation to the field. In Nigeria, coffee is usually transplanted in June, and the dry season set in around October the same year. Establishment rates are been put at 40% without irrigation with irrigation.

Humic acid is known to have growth promoting properties, being an organic substance is less harzadous to handle the mineral fertilizer. Besides Humic acid are used in very small dosages [plant per million (PPM)] and comes less expensive than mineral fertilizers in Nigeria.

Five levels of humic acid 8000ppm, 4000ppm,2000ppm,1000ppm and 500ppm were compared with the control 0ppm in a green house experiment. The coffee seedlings were one year old as at the time of transplantation and the soil is an Alfisol belonging to the Olorunda series.

Results show significantly better growth of seedlings treated with humic acid over the control. It was also observed, that concentration of humic acid higher than 4000ppm did not cause any significant plant treated with humic acid were able to withstand the dry season better than the control when no water was supplied.

The result encourages use of humic acid treatment for good establishment of coffee.

INTRODUCTION

Coffee seedlings are usually small in size at the time they are transplanted to the field after one year in the nursery. Due to this size, quite a number of time (about 60%) grow very slowly with feeble roots and consequently fail establishment result especially where field maintenance is inadequate. Also as a result of slow growth, the plants do not survive the three month dry season following the transplantation very well. This is therefore always the need to irrigate and mulch to curtail the lost of seedling within 40-60%(Obatolu et al 1998).

Humic acid is known to the rich in organic nitrogen in forms which are readily released into soil solution for intercorporation into microbial biomass and for plant uptake. In Nigeria, at the Cocoa Research Institute the beneficial effect of humic acid as growth hormone for rooting coffee cuttings has been validated and exploited (Omolaja and Obatolu, 1998).

This study examine the use of humic acid as a growth promoting substance for coffee canephora (robusta coffee) seedlings in order to boost the growth rate and drought resistance of the seedlings.

MATERIAL AND METHODS

Humic acid was extracted from the soil using IN NaOH solution and allowed to dry at room temperature 1.6g of the Humic acid was taken and dissolved in about 5ml ethanol (96%). The solution

was heated slowly to quicken dissolution. 200ml of water was added and therefrom 100ml, 8000pp, 4000ppm, 2000ppm, 1000ppm, 500ppm solutions were made accordingly.

One year old coffee robusta seedlings were transplanted into a 15 litre pot containing 10kg of soil. The soil was an Alfisol-Olorunda series of dedium fertility status and already certified good soil for coffee robusta (Obatolu, 1978). The coffee seedlings were allowed to grow for humic acid.
After 30 days, 20ml of humic acid were administered at different concentrations of 8000ppm, 4000ppm, 2000ppm, 1000ppm, 500ppm and the control 0ppm received 20ml of distilled water.

Growth records were taken every four weeks taking note of the height, girth and number of leaves. After about five months the plants were watered only once a week with 50ml of water to stimulate dry season conditions. Percentage survival of plants under each treatment was noted.

There were five replicates of each treatment and L50 was used to compare the means of the treatments.

RESULTS AND DISCUSSIONS

In general, plants treated with humic acid showed better growth performance than the control.

Table 1: Effect of Humic Acid Growth of Coffee Seedlings

Humic Acid Level (ppm)	Average Growth per four Weeks		
	Height (cm)	Girth (mm)	Number of leaves
0	0.84	0.06	0.68
500	1.39	0.09	1.97
1000	2.39	0.12	4.72
2000	2.42	0.12	4.03
4000	2.50	0.13	4.74
8000	2.46	0.11	4.13
LSD (5%)	0.14	0.09	0.19

Growth in height increased every month by between 65.5% and 198% over the control as a result of humic acid treatment. Similarly, growth in Girth increased by between 50% and 116% every four weeks when plants were heated with humic acid as compared to the control. Significantly more leaves were produced by plants that received humic acid treatment (between 190% and 600% more leaves were produced).

Between the humic acid treatments, 400ppm appeared to be optimum as treated levels above or below did not perform better.

Table 2: Percentage survival of coffee seedlings during stimulated dry season

Humic Acid level (ppm)	Percentage survival
0	45.0
500	68.0
1000	100.0
2000	100.0
4000	100.0
8000	100.0

As shown in table 2, plants that received 1000ppm and above survived the stimulated dry season and remained green, whereas those that received 500ppm had 69% survival and the control only 45%.

The efficacy of humic acid as a growth hormone has been evident from various trials.

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PREDICTION OF YIELD STABILITY IN ARABICA COFFEE BASED ON THE STABILITY OF MORPHOLOGICAL COMPONENTS

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INTRODUCTION

The significance of the environment in determining performance in Arabica coffee has been demonstrated in a number of studies (Srinivasan *et al,* 1979; Walyaro, 1983; Reddy *et al,* 1986; Srinivasan & Vishveshwara, 1978; Walyaro, 1983; Montes *et al,* 1987; Agwanda & Owuor, 1989; Bittenbender *et al, 1991).* In this study, the use of morphological traits in predicting stability of yield and bean qualities was examined.

RESULTS

Strong family and family by locality interactions were observed for all the traits studied. Family ecovalences for height of the main stem, internode length of the main stem and the internode length on primary branches were all significantly correlated with family ecovalences for mean yield over four years.

Table 1. Family means and mean squares for growth, yield and bean quality traits during the second year of production

		Mean Squares (1)						
Traits	Means	Locations	P	Families	P	Interaction	P	Error
df		4		22		88		220
GMS	10.87	195.8	****	3.4	****	2.3	****	0.9
HMS	99.82	2800.5	****	667.0	****	133.3	****	51.2
ILMS	3.70	4.0	****	1.3	****	0.2	***	0.1
PBP	48.07	18412.0	****	308.8	****	129.5	****	62.0
CR	64.46	5338.8	****	81.2	****	73.9	****	22.1
ILPB	2.97	3.2	****	0.1	****	0.1	****	0.0
NL	5.89	127.6	****	5.4	****	3.9	****	2.0
BR/N	6.45	125.2	****	4.1	**	3.7	***	2.3
PB	8.25	930.3	****	31.5	****	14.8	****	8.9
AA	14.52	3912.0	****	295.1	****	134.3	****	35.7
AB	45.01	199.4	****	550.9	****	226.1	****	100.8
C	8.14	3237.0	****	28.5	****	22.4	****	9.8
T	1.17	34.1	****	2.1	**	1.3		1.3
AA + AB	59.75	4104.1	****	248.0	****	202.0	****	72.8
Yield	2.98	119.5	****	1.0		1.6	****	0.8

(1) P≤ 0.1 : * - P ≤ 0.05 : ** - P ≤ 0.01 : *** - P ≤ 0.001 : ****

Table 2. Correlations[4] between family ecovalences over years for growth and yield traits during five years of production

Trait	Yieldl	Yield2	Yield3	Yield4	Yield5	Mean years	Mean locs
GMS	0.02	0.32	-0.12	-0.14	***-0.05***	0.11	*-0.05*
HMS	0.27	-0.04	0.44	0.47	0.20	*0.15*	*0.51*
ILMS	0.44	0.05	0.47	0.59	0.18	0.22	0.73
PBP	0.43	-0.03	0.34	0.32	0.28	0.10	0.44
CR	0.33	0.46	0.33	0.09	0.00	0.23	0.42
ILPB	0.31	0.03	0.36	0.48	0.22	0.21	0.52
NL	-0.01	0.18	0.39	0.01	0.47	0.45	0.00
BR/N	0.19		0.03	0.13	0.36	0.36	0.07
Yield 1		0.02	0.58	0.75	0.35	0.18	0.70
Yield2			0.17	0.09	0.08	0.51	0.13
Yield3				0.75	0.77	0.34	0.73
Yield4					0.76	0.41	0.81
Yield5						0.60	0.33
Mean years							0.41
Mean locs							

[4] $P_{(0.05)}$ for $| r | \geq 0.38$, $P_{(0.01)}$ for $| r | \geq 0.49$

CONCLUSIONS

Interactivities for height, internode length on the main stem and internode length on primary branches could be used as indicated of yield interactivities in Arabica coffee.

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TOWARDS EFFICIENT COFFEE MARKETING IN KENYA

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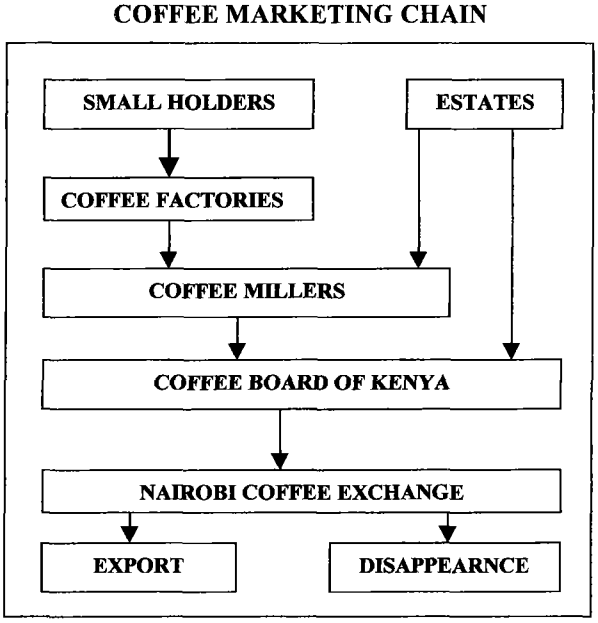
INTRODUCTION

Coffee is important in the Kenyan economy because it is a major foreign exchange eamer and takes into considération food security issues, employment and income. The country's production is distributed among the smallholders and the estates sector with a 54 % and 46 % respectively.
Presently, the industry has faced a decline in performance. The production dechned from 128, 926 metric tonnes in 1987/88 to 55,042 metric tonnes in 1997/98. The decline has been partly attributed to inefficiencies of the overall marketing chain and other issues that could have unfavourable implication on the ability of some of the traditional coffee organisations to render effective services.

Figure 1: a schematic représentation of the coffee marketing chain in Kenya.

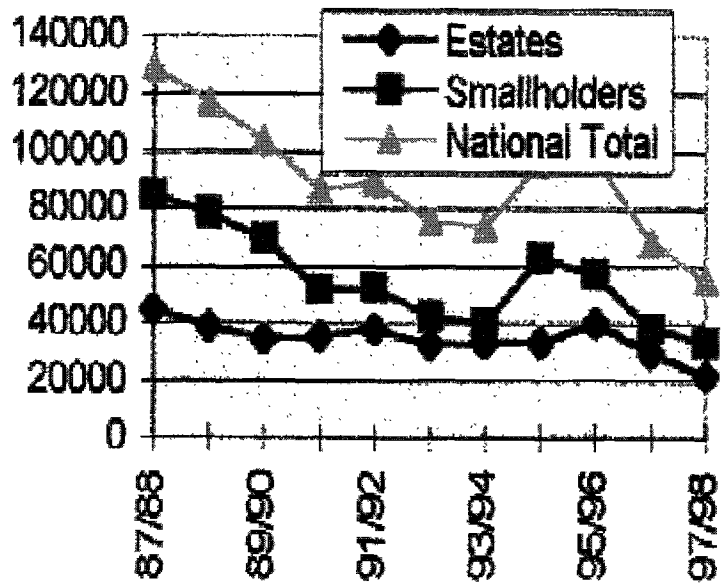


KENYA COFFEE PERFORMANCE

a) Kenya coffee production

Kenya coffee has production has been declining for the last several years. Several reasons have been floated. Possible reasons for declined production

- Escalating cost of coffee production
- High processing cost
- Lack of cheap credit
- Inefficient marketing chain
- Adversarial relationship



b) Product characteristie

Coffee quality

Although coffee production has declined coffee quality has been maintained over the years with the smallholder sector having better quality coffee.

Coffee quality performance (percentages)

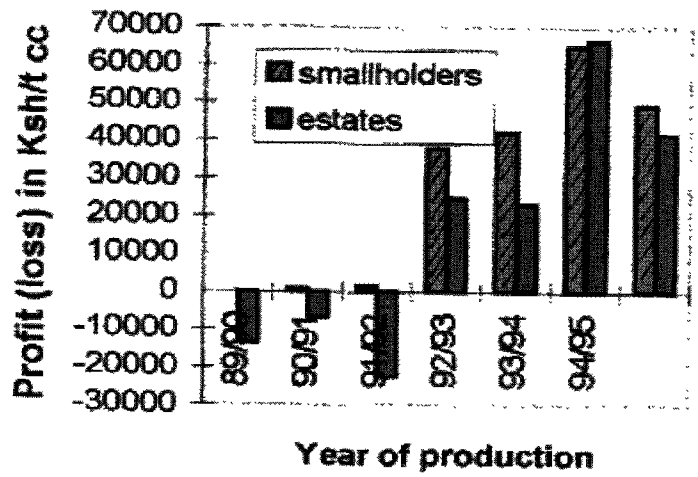
sector	class	87/88	89/90	91/92	94/95	96/97	97/98
Small scale	1 - 3	14.12	22.07	15.60	17.76	25.32	24.14
	4 - 6	33.95	50.56	53.76	56.33	48.20	51.24
	7 - 10	13.01	4.83	12.37	10.74	7.92	8.85
	Buni	18.9	22.54	18.27	14.07	18.30	15.72
Estates	1 - 3	4.70	4.45	4.29	1.42	5.15	9.69
	4 - 6	80.29	79.18	74.49	77.67	75.49	74.83
	7 - 10	8.76	7.52	4.47	12.92	9.46	8.71
	Buni	6.22	8.84	6.67	7.99	9.83	6.73
National total	1 - 3	10.77	16.13	10.77	10.14	16.50	18.17
	4 - 6	62.19	60.04	62.19	67.01	60.14	60.98
	7 - 10	13.63	5.88	13.63	11.83	8.59	8.80
	Buni	13.41	17.98	13.41	11.83	14.65	12.00

Kenya coffee is known for its distinctive cup qualit whichis key to her marketing

c)The level of proritability

Coffee profitability is usually a factor of several issues. Of pertinent concem are factors like :

- Coffee prices
- Cost of coffee production
- Cost of marketing
- Smooth operation of the marketing system



RECOMMENDATIONS AND CONCLUSIONS

- Co-operative reinforcement especially at the primary processing stage in order to enhance production and quality
- Allocative accuracy to match consumer and farmer goals
- Technical and operational efficiency
- Re-design and repositioning of supply chain
- Responsive consumer driven system
- Market diversification i. e reliable domestic market
- Alternative marketing options white maintaining the traditional buyers
  - ➢ The fair trade initiative
  - ➢ Gourmet and speciality
  - ➢ Organic coffee

ACKNOWLEDGEMENT

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HOW TO AVOID MOULD TROUBLES IN GREEN COFFEE PREPARATION

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Summary

Coffee infection by moulds has been sometimes reported: a highly regrettable phenomenon both from a helth and a sensory perspective. Tainted coffee is, of course, of no appeal to the consumer, but some moulds can even produce the harmful mycotoxin Ochtratoxin A (OTA).
In this article are pointed out several remarks on farming routines, as derived from the author's personal experience matured in decades of contact with coffee field activities. Some rules of good agricultural practice may be infered from these remarks.
During post-harvest operations, all prolonged situations of high humidity during the drying process should be avoided. The same apllies for storage and transportation. Also all re-wetting of cherries, parchment and green beans during the steps of drying, storage and transport can spoil quality, including OTA formation, and must therefore be hindered.

INTRODUCTION

It is generally agreed that a modern coffee plantation needs both good productivity and fine quality of the final product, healthiness being of course the most important issue.

Coffee infection by moulds has been sometimes reported, and this is a highly regrettable phenomenon both from a health and a sensory perspective.
A tainted coffee is of course of no appeal to the consumer, but some moulds can even produce a harmful mycotoxin known as Ochtratoxin A (OTA). (PETRACCO,1998)

It may be interesting to point out in this article several remarks on farming routines, as derived from the author's personal experience matured in decades of contact with coffee field activities.
Some rules of good agricultural practice may be inferred from these remarks. Sadly, the application of such rules, which should be taken for granted thanks to their down-to-earth feasibility, is not universal: the author can bear witness of countless cases of disinformation and negligence.

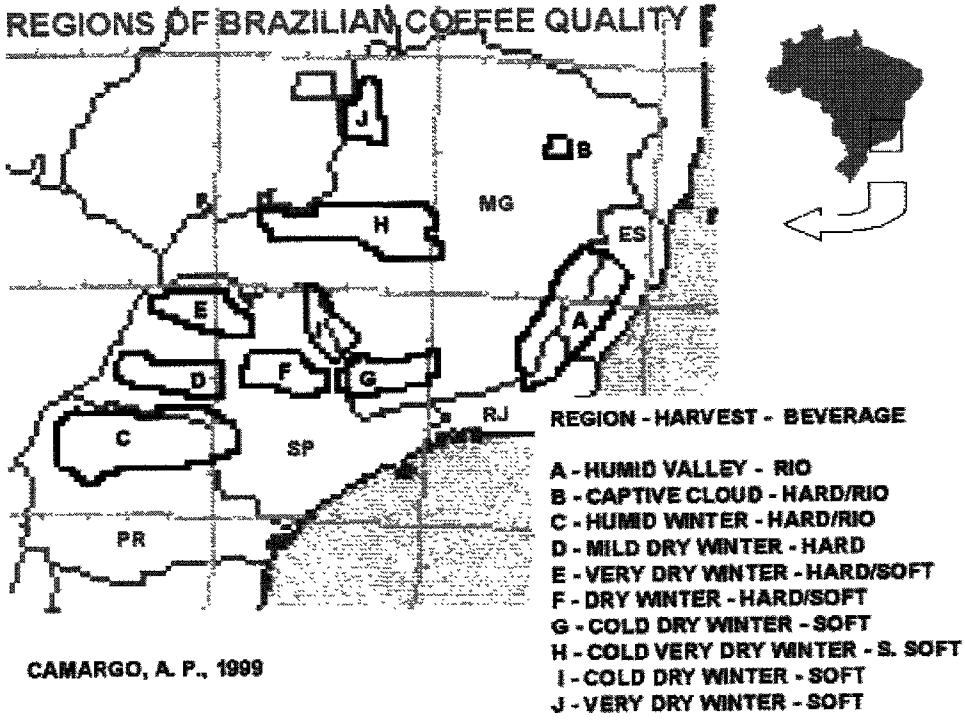
When one wants to conduct a farm to produce high quality problem-free coffee, some steps are very fundamental and will be described in detail.

THE CHOICE OF PLANTATION AREA AND OF PREPARING AND STORAGE FACILITIES

Before anything else, one has to know the ecological conditions of the region to be selected for the plantation and the facilities for post-harvest and storage operations. Orography, hydrography, vegetation, and climate: every parameter must be carefully studied.

Of these, climate is the most important element when referring to quality. (TEIXEIRA et al.,1968, TEIXEIRA et al., 1969). From planting to coffee storage, the climatic conditions have to be satisfactory. However, it is during harvesting and drying time that climate has its major influence on quality. During this phase it is very important to know:

- temperature
- relative humidity of the air
- rainfall distribution
- formation of fogs
- proximity of rivers and dams
- insolation.



The facilities for post-harvest processing must not be built in humid valleys, where an accumulation of cold air can often build up. A good practice recommends that these facilities be situated on the upper third of the hillsides.

When the climate is hot and humid during harvest time, there is a higher probability of an alteration of the beverage quality, often described to the developing of some kinds of moulds. On the other hand, in regions where the climate is colder and dryer usually the natural coffees are of higher quality.(CAMARGO et al.,1992)

When, however, the plot and the facilities are already installed, one should exert more care mainly during the drying stage, to avoid that humidity and temperature favour the proliferation of unpleasant micro-organism. In this case the problem can be solved, or at least reduced, by an alternative choice of more adequate handling, like artificial drying in mechanical dryers and storage in other areas less prone to extreme climatic variations, like the warehouses of the Cooperatives.

SELECTION OF THE VARIETIES

The selection of botanical varieties, or cultivars, is very important not only in relationship to productivity, but also to the characters related to quality.(FAZUOLI et al.,1977).

Nowadays several Research Institutes are actively breeding for more productive varieties, along with selection activities of lineages with a potential for higher beverage quality.

HARVESTING

The harvest can be done either by picking the fruits one by one or by stripping all of them onto the ground, or onto a sheet.
In Brazil, the harvest is done usually by stripping onto a sheet, either by hand or by automatic harvesters. All the fruits of the tree are harvested at the same time.(TEIXEIRA, 1970).

Depending on the numbers of blossoming periods, the fruits that will be harvested could be at different stages of ripeness. On studying the phenomenon of fructification in a coffee plantation, one can see that a period of rest is needed, in order that the ripe flower buds can remain in dormant status of hydric stress, to bloom after the first rain.
For instance, in the arid region of Minas Gerais the rains start usually in October, and there is high probability of having just only one blossoming period: a favourable circumstance for good coffee quality. In the state of Parana, on the contrary, there are many blossoming periods because it rains all year round.
In the latter case, depending on the year and on the region, one will find fruits at different stages of ripeness like unripe cherries (60 to 65% of moisture content), ripe cherries (55 to 60%), "raisin" cherries (40 to 50%) and dry cherries (30 to 35%).
With such a heterogeneous material, it is fundamental to have a careful preparation procedure to obtain a good sundried coffee.

PREPARATION

Soon after the harvest, the coffee crop should be taken as soon as possible to the drying patio, preventing it to remain waiting accumulated in the plot, on the trucks or in the reception tanks. It is important that the drying starts immediately, evenly spread in thin layers. (TEIXEIRA, 1977).

When there is a portion of ripe cherries, the initial drying layers should not be thicker than 3 cm. Moreover, coffee should be stirred all day long, with breaks not longer than 30 minutes. During the first days of drying, and always at the end of the day, the drying cherries should be aligned in thin rows in the same direction of the declivity of the patio.
By doing this, one will minimise any undesirable fermentation of the cherries, and will be imparting the correct and uniform drying of fruits in different stages of ripeness. Heaps should be allowed only after reaching the half-dry status.

In this phase coffee must not be wetted. Should this happen, some OTA-producing micro-organisms could appear and contaminate the coffee, inasmuch as they need little moisture to develop and they no longer find competition by other more hydrophilic micro-organism. The important rule is: the shorter the drying time, the lower the probability of contamination by unwelcome micro-organisms.

Attention should be paid also to the cleanliness of the drying patios and of the storage facilities, where OTA contamination has sometimes been observed.

Likewise, the harvest of the cherries should be done in the same day of processing of "washed" coffee, which passes through the pulping machine and is put into fermentation tanks. After the complete disappearance of the mucilage, the parchment coffee should be well rinsed and immediately put to dry. Again, the drying step is extremely important and should be fast, by spreading the beans in thin layers and stirring them regularly.

Nowadays an intermediate process, called with some misinterpretation "descascado", has gained some popularity. In that style, immediately after pulp removal the coffee beans are put to dry with all their mucilage. The layers should always be very thin, and under constant stirring until the completion of drying.(MATIELLO, 1977).

It is worth to stress one more time: any inopportune fermentation during the drying phase could cause the loss of all the quality work and favour the development of micro-organisms liable for the contamination of the beans.

STORAGE

The storage of green coffee, when correctly executed, should keep its chemical composition in the original condition. (MELO et al., 1980).

Coffee should only be stored when properly dry, that corresponds to an average moisture content of 11%. If the drying has been well executed, one is able to observe that all beans show the same uniform aspect, with no spot or splash. This can be confirmed by instrumental measurement of moisture content. Ideally, the humidity variation among the beans should never exceed 0.5% (absolute). However, this is a topic that deserves some discussion, because the author has verified a great deal of heterogeneity deriving from the drying process, mainly when natural (sundried cherry) coffee is involved.

For good storage, not only warehouse location is important, but it is also necessary that it be appropriately built to preserve the characteristics of satisfactory dry coffee. Warehouses should have a good impermeable floor, should be well aerated, their temperature should never exceed 22°C and should always be dark. Humid locations, large variation of temperature and humidity inside the warehouses, bad coverings, etc. might lead to water vapour condensation and, along with coffee stowed too close to the walls, to re-wetting of the beans and their deterioration. (TEIXEIRA et al.,1977).

Furthermore, it must be considered that coffee seeds go on living even after the harvest and, like all living organism, they breath (i.e. they consume sugars to produce energy along with carbon dioxide, and water!). A rising of the temperature accelerates breathing two or three times more, where beans that are stored cool and dry, near 11% moisture, keep a moderate breathing process. In the same way an increase of the level of humidity induces an accelerated breathing with a deterioration of the product. (PUZZI, 1973).

It is also important to keep in mind that coffee beans, like all hygroscopic material, are able to adsorb and release water. This implies that they try to remain in equilibrium with the relative humidity of the surrounding air: in contact with the environment, they will release or receive moisture proportionally to the rising or decreasing relative humidity.

The micro-flora of the stored coffee beans consists of a great variety of fungi and bacteria, all of them depending for survival on water activity. The effects of their survival produce several obnoxious effects, among which impairment to the germinative power, spoiling of the organoleptic qualities, and production of mycotoxins.

TRANSPORT

Both the transfers from the production areas to the ports, usually in trucks, and to the importing countries, usually in containers, should not be submitted to excessive fluctuations of temperature and humidity. (PITT, 1999).

Unfortunately, for the sea trip coffee is shipped in containers, normally not sheltered, which undergo large variation of both temperature and humidity during the transport. With these differences, reinforced by weather alterations, an important migration of humidity may certainly occur and, what is worse, this can provoke condensation and consequent wetting of the grains.
To improve transportation conditions and avoid the formation of noxious moulds, the feasibility of a thermal insulation to reduce temperature variations should be investigated.

CONCLUSIONS

A scientifically studied and carefully designed harvesting praxis, carried out in well selected producing regions, is the only way to avoid all undesired fermentation, including the attack of those micro-organisms that can be responsible for OTA formation, with harmful effects on human health.

In post-harvest operations, all prolonged situations of high humidity during the drying process must be avoided. The same applies for storage and transportation. Last but not least, all re-wetting of cherries, parchment and green beans during the steps of drying, storage and transport can be harmful to quality, including a higher possibility of mould attack that can cause the formation of OTA, and therefore must be hindered.

By laying down these recommendations, derived from decades of experience in good agricultural practice, the author offers his modest contribution to the preservation of the initial quality of good coffee crops with the confidence that a healthier product, free from any kind of contamination, can be obtained.

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CELL WALL POLYSACCHARIDES OF COFFEE BEAN MUCILAGE. HISTOLOGICAL CHARACTERIZATION DURING FERMENTATION

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SUMMARY

The mucilage of pulped coffee beans before and after fermentation was examined by light microscopy. The mucilage of unfermented beans is constituted by two to three layers of elongated palisade-like cells with thin and folded walls attached at their base to the sclerenchymatous parchment. Ruthenium red staining specifically demonstrated the presence of pectic substances. After 20 hours of fermentation, the mucilage tissue is still present with apparently intact cell walls, but it is separated from the parchment. The walls are still stained by ruthenium red suggesting that pectic substances are still present. Therefore it is assumed that no pectinolysis occurs during fermentation or if so it must be to a very restricted and non detectable extent. In conjunction with our biochemical data, it is hypothesized that consumption of sugars by bacterial microflora induces an osmotic pressure gradient from outside to inside the mucilage layer, thus provoking a fracture of mucilage cell walls at their basal site of attachment to the sclerenchymatous parchment.

RESUMEN

El mucilago de las cerezas despulpadas de cafe antes y despues de fermentacion fue examinado con microscopia optica. El mucilago de las ceresas frescas se compone de dos o tres capas de largas celulas con delgados paredes. Estas celulas se pegan a sus bases con el esclerenquimatoso pergamino. La coloracion con el rojo de ruthenium permite demostrar la presencia de pectinas. Despues de 20 horas de fermentacion, el tejido mucilaginoso existe todavia con su paredes aparentemente intactos, pero esta separado del pergamino. Sus paredes estan todavia tenidos con el ruthenium rojo sugeriendo que las pectinas estan todavia presentes. Entonces, se supone que no hay pectinolisis durante la fermentation o que sino es a un nivel muy reducido y no detectable. De acuerdo con nuestros resultados bioquimicos, se supone que el consumo de los azucares por la microflora induce una presion osmotica de la parte externa a la parte interna del tejido mucilaginoso, provocando una rotura de los paredes de las celulas del mucilago a su base, sitio de adhesion al esclerenquimatoso pergamino.

COMMUNICATION

INTRODUCTION

Coffee cherry is a drupe consisting of smooth tough outer-skin or exocarp, soft yellowish pulp or outer mesocarp, mucilaginous layer or inner mesocarp, and greyish-green fibrous endocarp (parchment) surrounding seeds (Purseglove, 1974). In the wet processing of coffee, cherries are first pulped and then fermented to remove the mucilage; then wet parchment coffee is washed and dried. The mucilage,

formerly described as an hydrogel exhibiting no cellular organization (Carbonell and Vilanova, 1952 ; Coleman *et al.*, 1955) is a thin hydrated layer essentially made of pectic substances and soluble sugars (Oliveros and Gunasekaran, 1996 ; Avallone *et al.*, 1999a). Due to its viscous texture, pulped beans cannot be washed without preliminary removal of mucilage by natural fermentation or mechanical demucilagination. During fermentation, endogenous coffee and/or microbial enzymes are supposed to degrade pectic substances of the mucilage (Carbonell and Vilanova, 1952 ; Wilbaux, 1956 ; Rolz et al., 1982), changing its texture from an hydrogel to an hydrosol, but no definite proof was ever brought to sustain this assertion. Furthermore, *Erwinia dissolvens*, a major pectolytic microorganism of the microflora from fermenting beans, was shown to produce only an exo-pectate-lyase having a very restricted action on coffee high methoxyl pectins (Castelein and Pilnik, 1976). With regards to these conflictual assumptions, we decided to study by light microscopy the structure of the mucilage layer and its alterations during fermentation.

MATERIAL AND METHODS

Plant material

Coffee variety used in the present study was *Coffea arabica* L. var. *typica* Cramer. It was grown in Coatepec area (Xalapa, Veracruz, Mexico). Coffee cherries were harvested at the mature stage during the 1998 season.

Coffee wet processing

Coffee cherries were immediately pulped with a DV-255C PENAGOS® pulper. Pulped coffee beans were then conveyed in a water stream to the fermenting tank where they were left to ferment for 20 h. Samples of frozen (- 20 °C) coffee beans at times zero and 20 h of fermentation were rapidly air-freighted and delivered to our laboratory. Whole sound mature cherries were also kept for later light microscopy examination.

Light microscopy

Prior to fixation, the fleshy portion of whole mature cherries was obtained by hand-dissection while pulped beans (times zero and 20 h) were carefully husked to separate the mucilage and the parchment from the seeds. Samples were fixed in 2% glutaraldehyde (0.2 M phosphate buffer, pH 7.0 containing 1% acrolein and 1% caffeine) for 15 min under vacuum at room temperature, then for 24 h at 4 °C. Subsequently they were dehydrated in a graded ethanol series then impregnated for 24 h in a medium containing : Technovit 7100 resin (100 ml; Kulzer), Technovit 7100 accelerator (1 g; Kulzer), Technovit polyethylene glycol PEG 400 (1.5 ml; Kulzer) and triethylene glycol dimethacrylate (0.5 ml). They were finally embedded in the impregnation medium (5 ml) added with Technovit 7100 hardener (0.35 ml). Sections (3 µm) were obtained with a Historange LKB microtome and examined with a DMRB Leica microscope.

- ***Revelation of the cell wall polysaccharides (cellulose, hemicelluloses, pectic substances)*** : sections were oxidised for 5 min in 1% periodic acid, washed with distilled water, then stained for 10 min in the dark with Schiff reactant prepared as follows : *p*-rosaniline chloride (1 g) was dispersed in boiling water (200 ml) and the solution is cooled to 50 °C, then filtered ; sodium metabisulfite (2 g) and 1 M HCl (20 ml) were then added and, after 24 h in the dark, activated charcoal (0.5 g) was added and the medium filtered.
  Sections were then washed with water until washing liquor is colorless and stained with Naphtol blue black [NBB (1 g), acetic acid (7 ml), up to 100 ml water] for 5 min at 60 °C. Sections were washed with water, then treated with 7% acetic acid and finally dried for 15 min at 60 °C.
- ***Specific revelation of the cell wall pectic substances*** : sections were stained with 2% ruthenium red in water for 15 min at room temperature, washed with distilled water, then dried.

RESULTS AND DISCUSSION

Microscope examination before fermentation (time zero)

In a preliminary step we examined sections of different zones of whole mature cherries before they were pulped and fermented. The pericarp is constituted of :

- exocarp (= skin) consisting in a layer of small epidermic cells (~ 10 x 30 µm) (Photo1).
- outer mesocarp (= pulp)(thickness ~ 1 mm) built of roughly isodiametric cells of increasing diameter from outside (~ 40 µm), to middle portion (~ 100 µm), to inside (~ 110 µm) the cherry. Elongated cells are also present from (~ 30 x 80 µm) to (~ 50 x 100 µm). Cell walls are intensely stained fuschia red in the upper zone while in the bottom area the tissue seems a bit loosened with walls far less densely stained red. Tannin containing cells are visible in outer mesocarp (Photo 1).
- inner mesocarp (= mucilage)(thickness ~ 300 µm) constituted of two to three layers of elongated cells (length ~ 160 µm; width ~ 15-50 µm). Sectioning teared a bit the surface of the mucilage (Photo 2).
- endocarp (= parchment)(thickness ~ 50-110 µm) made of sclerenchyma with transverse sclereids (~ 25 µm)(Photo 2) and longitudinal sclereids (~ 150 x 25 µm) which are only visible in Photo 5.
- inner epidermis built of small flattened cells (length ~ 40 µm; width ~ 10 µm)(Photo 3).

Mucilage left after industrial pulping is made of elongated cells attached to parchment sclereids (Photo 3). Residues of cytoplasmic material and nucleus stained greenish-blue by naphtol blue black are visible (Photo 3). Cell walls are thin and stained fuschia red confirming presence of polysaccharides. Accordingly, we have previously shown that mucilage alcohol insoluble residue contained cellulose in addition to pectic substances (Avallone *et al.*, 1999a). Thus the mucilage layer has actually a conventional tissue structure with cells surrounded by cell walls in contradiction with previous assertions describing the mucilage as an hydrogel having no cellular structure (Carbonell and Vilanova, 1952 ; Coleman et al., 1955). The loosened appearance of this tissue with thin walls might explain why these authors previously missed the presence of cell walls. However Beille (1947) followed by Rabéchault (1959) had already described in details the tissue structure of the mucilage layer : cells were drawn in a palisade-like arrangement like in our case with walls either dotted (Beille, 1947) or continuous (Rabéchault, 1959). It is worth to mention that Menezes and Maniero (1955) drawn the mucilage as one layer of cells with thick walls resembling palisade cells of seed coat of legumes (Esau, 1977) ; although having a palisade arrangement, cells of coffee mucilage have not secondarized walls.

Cells walls of the mucilage are stained pink by ruthenium red (Photo 4) confirming the presence of pectic substances (Coleman et al., 1955 ; Avallone *et al.*, 1999a) and not stained by the phloroglucinol/HCl reagent (Avallone *et al.*, 1999a) indicating abscence of lignin. These walls can be characterized as primary cell walls, conversely to the sclerenchymatous parchment not stained by ruthenium red. It is worth to mention that a noticeable amount of water-soluble pectic substances must have been lost during the fixation step when the samples were treated with aqueous glutaraldehyde (Avallone *et al.*, 1999b). Thus, pectic substances evidenced with ruthenium red (Photos 4, 6, 8) must be the water-insoluble fraction only. According to our observations, the tissue constitution of coffee cherry pericarp is summed up in Figure 1.

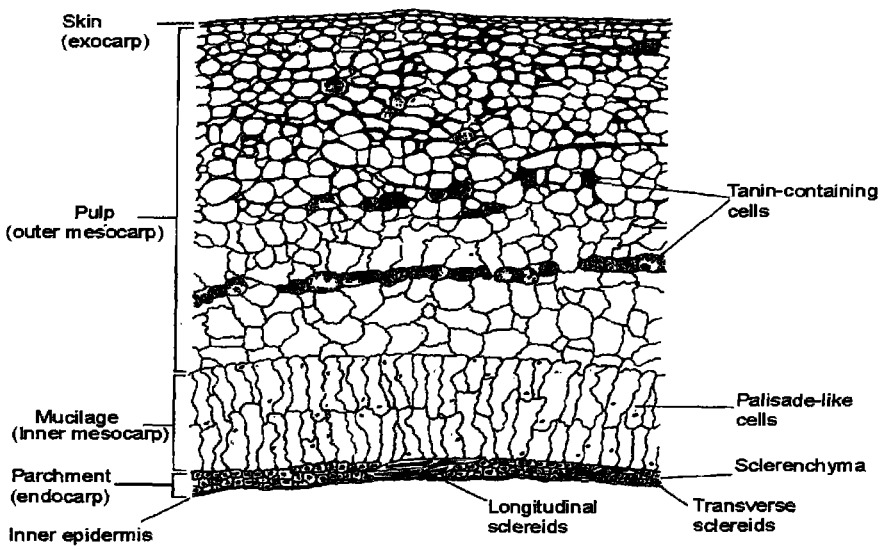


Figure 1. Tissue constitution of *Coffea arabica* L. var. *typica* Cramer pericarp. Microscope examination after fermentation (time 20 h)

First examinations of parchment from fermented beans revealed that cell walls of the mucilage layer were no longer visible except at their basal site of attachment to the sclerenchyma (Photos not shown). This tended to confirm previous assumptions on degradation (pectinolysis) of mucilage pectic substances by endogenous and/or microbial enzymes (pectinases)(Carbonell and Vilanova, 1952 ; Wilbaux, 1956 ; Rolz *et al.*, 1973). However, when sections were examined at a low magnification (Photos 5, 6), clusters of cells of the mucilage layer dissociated from the parchment with stained cell walls were visible. Higher magnification (Photos 7, 8) shows that cell walls of the mucilage layer at time 20 h are apparently intact. They are stained by ruthenium red indicating that pectic substances are still present. Fermenting bacteria are visible in Photo 7. Cells of the mucilage layer are seen in transverse section with ondulated walls while in Photos 2, 3 and 4 they are in longitudinal view.

According to our biochemical (Avallone *et al.*, 1999b). and histological (this study) data and contrary to previous assumptions (Carbonell and Vilanova, 1952 ; Wilbaux, 1956 ; Rolz *et al.*, 1982), mucilage pectic substances are either not splitted during fermentation by endogenous and/or microbial enzymes or, if they are splitted, it must be to a very restricted and non detectable extent Our findings are in agreement with the work of Castelein and Pilnik (1976) who found that *Erwinia dissolvens*, a major coffee fermenting bacteria, produces only an exo-pectate-lyase which is unable to depolymerize coffee mucilage high methoxyl pectins.

At time zero just after pulping, the mucilage layer forms a continuous, although loosened, tissue covering the parchment (Figure 2-A). Mucilage being exposed to air, natural microbial flora starts developping on the surface of the mucilage layer; sugars are progressively metabolised inducing an osmotic pressure gradient from outside to inside. Thus a water flux will compensate leading to turgor of the cells. Although it had not been possible to model this phenomenum, it is possible that such an osmotic pressure gradient would exert a pressure sufficient enough to rupture cell walls at the basal site of attachment of the cells (Figure 2-B). Indeed, this zone of junction between walls of two different tissues, a parenchyma with thin primary walls and a lignified secondarized sclerenchyma, was likely more cohesive than in the rest of the mucilage.

Simultaneously organic acids are produced inducing a pH gradient. It is likely that the pH lowering induces physico-chemical alterations in the cell walls (*e.g.* activation of cell wall enzymes, alteration of the pectic gel)(Carpita and Gibeaut, 1993). There is definitively an unknown alteration of the cell walls since after fermentation they have stiffened and were less hydratable than before fermentation ;

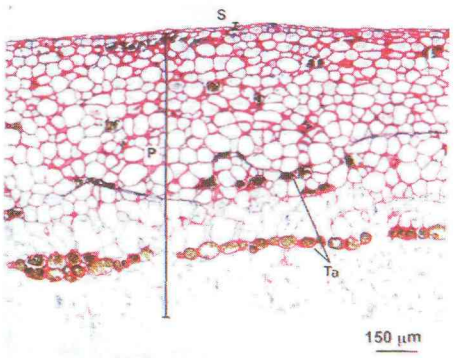


Photo 1. Outer portion of coffee cherry pericarp.
S : skin (exocarp). P : pulp (outer mesocarp). Ta : tannin containing cells.
Periodic acid-Schiff-Naphtol blue black.

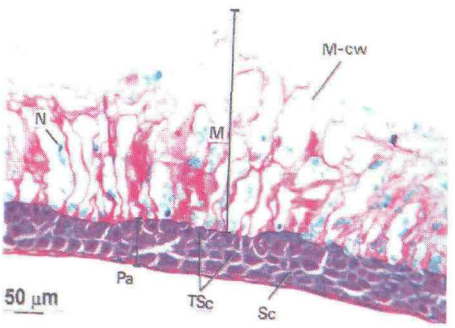


Photo 2. Inner portion of coffee cherry pericarp.
M : mucilage (inner mesocarp). M-cw : mucilage cell walls. Pa : parchment (endocarp). Sc : sclenrenchyma. TSc : transverse sclereids. N : Residual nuclei.
Periodic acid-Schiff-Naphtol blue black.

Photo 3. Mucilage layer and parchment of coffee bean <u>before</u> fermentation. (right)
M-cw, Pa, Sc as in Photo 2. IE : Inner epidermis.
Periodic acid-Schiff-Naphtol blue black.

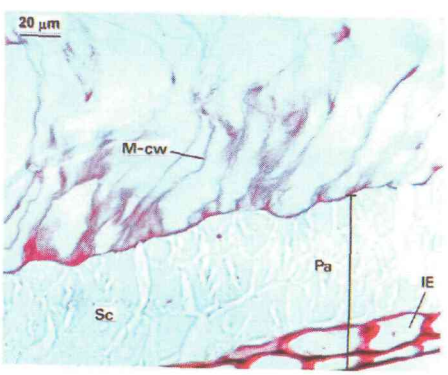
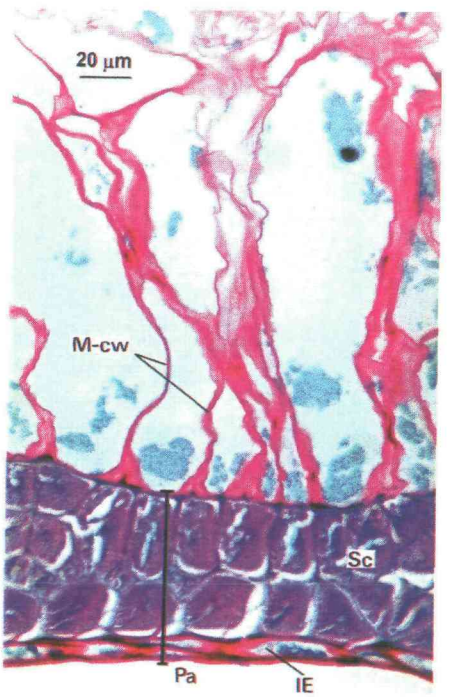


Photo 4. Mucilage layer and parchment of coffee bean <u>before</u> fermentation.
M-cw, Pa, Sc as in Photo 2. IE : Inner epidermis.
Ruthenium red.



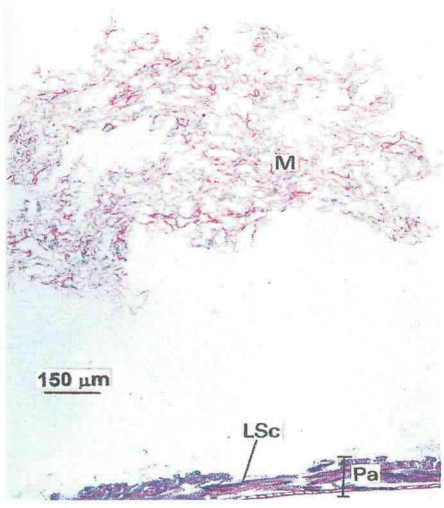


Photo 5. Mucilage layer and parchment of coffee bean <u>after</u> fermentation.
M, Pa as in Photo 2. LSc : Longitudinal sclereids.
Periodic acid-Schiff-Naphtol blue black.

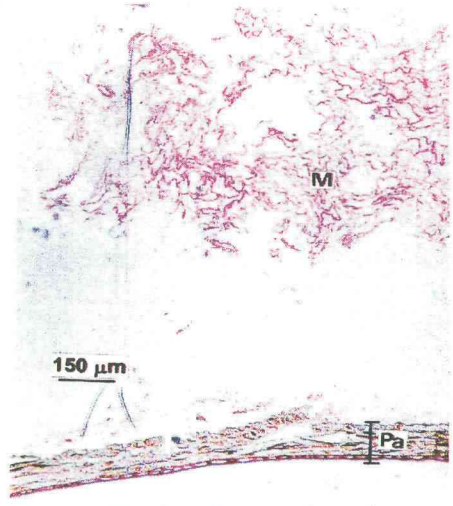


Photo 6. Mucilage layer and parchment of coffee bean <u>after</u> fermentation.
M, Pa as in Photo 2.
Ruthenium red.

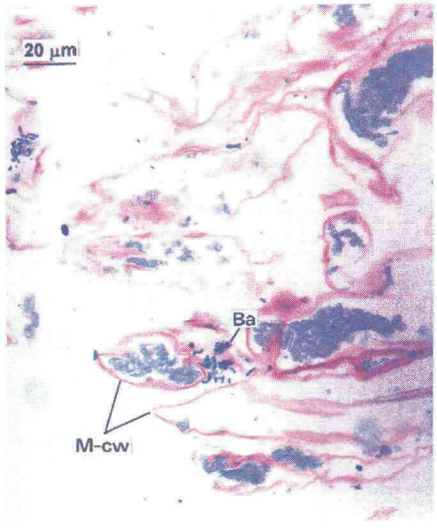


Photo 7. Mucilage layer of coffee bean <u>after</u> fermentation.
Cells are seen in tranverse section. M-cw as in Photo 2. Ba : bacteria.
Periodic acid-Schiff-Naphtol blue black.

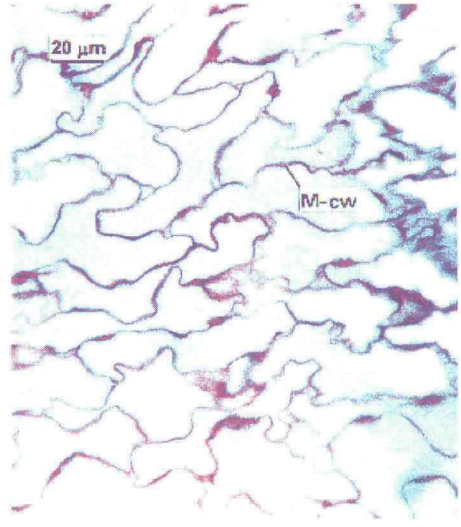
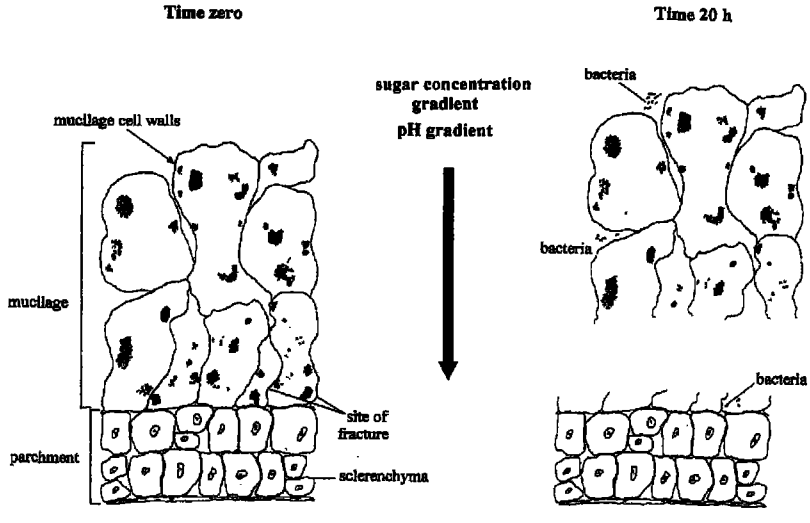


Photo 8. Mucilage layer of coffee bean <u>after</u> fermentation.
Cells are seen in tranverse section. M-cw as in Photo 2.
Ruthenium red.

however with regards to their polysaccharide composition, both types of cell walls exhibit no measurable differences (Avallone *et al.*, 1999b).



2-A 2-B

Figure 2. Schematic view of events occuring during fermentation of coffee bean mucilage. (2-A) before fermentation ; (2-B) after fermentation.

CONCLUSION

After fermentation, the mucilage tissu is still organized with larg cells and its cell walls still contains polysaccharides and pectins. During this step, there is not a total pectic degradation of the tissu and its components as it is said in the literature, but just a separation from the parchment. In pratical terms, this mean that if we want to control the fermentation step with an inoculum addition, it would be better to use a good fermentative strain than a pectinolytic micro-organisms.

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EFFET DE DIFFERENTES CONDITIONS AGRO-ECOLOGIQUES SUR LE DEVELOPPEMENT DE L'ANTHRACNOSE DES BAIES DU CAFEIER ARABICA DANS L'OUEST DU CAMEROUN

Incidence sur le mode de conduite des plantations et l'application des traitements phytosanitaires

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Résumé

The Arabica coffee production zone is located in western Cameroon and extends over a region approximately 150 km in diameter, at an altitude of between 900 and 1,800 m above sea level. The climate is characterized by unimodal rainfall distribution from March to November and a dry season from December to February. Most of the plantings cover 2 to 3 ha or less and are planted with a local variety susceptible to coffee berry disease, under plantain banana or fruit tree shade.
A precise analysis of berries fall in technically limited smallholdings representative of the farming methods in this production region made it possible to distinguish between losses actually attributable to the disease and those due to other phenomena. The disease mostly occurs at medium to high altitude, where around 40% of plantings are located.
By monitoring berry fall on a weekly basis, it was determined that losses due to « physiological fall » could be substantial, reaching up to half of total losses. The infection process appeared to occur earlier and induce greater losses on trees in full sunlight than on shaded trees.
Knowledge of agroecological conditions propitious to disease development is essential for establishing a plantation management strategy and providing information as to the best time to trigger the phytosanitary treatments to be applied depending on the trees' environment.

INTRODUCTION

Le culture du caféier Arabica au Cameroun est située en zone d'altitude entre 900 et 1800 m, dans les provinces de l'Ouest et du Nord Ouest. Les premières plantations ont été installées dans les années 1910 et l'extension de la culture s'est développée de 1940 à 1970. Il s'agit essentiellement de plantations villageoises en culture pure ou associée, d'une superficie de 1 à 4 ha, conduites en règle générale sous ombrage.

Le régime pluviométrique de cette région est caractérisé par une répartition des pluies unimodale de mars à octobre-novembre et une saison sèche de novembre à mars. La pluviométrie moyenne est comprise entre 1500 et 2200 mm en fonction de l'altitude.

Depuis le début des années 80, dans un contexte de diversifications des exploitations qui se traduit par le développement des cultures vivrières intercalaires, on observe d'importantes modifications des comportements des planteurs vis à vis de l'utilisation des intrants agricoles, avec un abandon quasi général de l'apport de fertilisants et des traitements phytosanitaires (Varlet et Berry,1997).

Cette situation préoccupante est renforcée notamment par la présence d'une maladie, l'anthracnose des baies (CBD) due à *Colletotrichum kahawae*, qui est responsable d'importantes chutes de fruits pouvant atteindre des niveaux supérieurs à 50% dans les parcelles potentiellement les plus productives en zone de moyenne et haute altitude, sur des variétés sensibles en l'absence de traitements phytosanitaires (Berry *et al.*, 1991).

Depuis le milieu des années 1980, on enregistre ainsi une chute régulière de la production qui paraît se stabiliser actuellement autour de 8 000 tonnes. Aussi, la recherche nationale s'est donné notamment comme priorité, en plus de la recherche de variétés résistantes (Bouharmont, 1992 et 1995 ; Van der Graaf, 1992 et Van der Vossen *et al.*, 1976), l'identification précise des conditions agro-écologiques favorables au développement de cette maladie afin d'identifier des modes de conduite des plantations susceptibles de réduire les pertes de production et de délimiter les zones d'interventions prioritaires pour rationaliser les traitements phytosanitaires

DISPOSITIF EXPÉRIMENTAL

Les parcelles expérimentales sont installées sur deux sites en plantations paysannes, conduites sous couvert de fruitiers divers et de bananiers plantains, représentatives de la culture dans cette zone. La première est située à Babadjou en zone de moyenne altitude (1550 m) et la deuxième à Santa à 1760 m.

Les deux plantations sont constituées de caféiers appelés Avariété locale@ apparentés à la variété Jamaïque. La densité de plantation est de 1 100 caféiers/ha. Les parcelles ne reçoivent aucun traitement phytosanitaire.

L'incidence de l'exposition à la lumière a été prise en compte en retenant des arbres préférentiellement exposés à la lumière et des arbres exposés préférentiellement à l'ombre.

Le dispositif de suivi de l'évolution de chutes des baies s'est largement inspiré des travaux effectués dans cette zone (Aubin *et al.*, 1991) et des simplifications du dispositif statistique apportées à la suite des travaux de Regazzoni (1996).

Les observations sont réalisées sur un échantillon de 50 arbres à l'ombre et 50 arbres au soleil en randomisation totale. Sur chaque arbre 3 rameaux sont identifiés à 3 hauteurs différentes et dans 3 directions différentes. Sur chaque rameau après la floraison principale le segment portant les noeuds florifères est marqué. Environ 4 à 5 semaines après la floraison la totalité des baies visibles au stade tête d'épingle sont comptées. C'est le nombre total de baies initial. Ensuite, les comptages sont hebdomadaires. A chaque passage, les baies nouvellement malades présentant des symptômes sont repérées par la pose d'une étiquette. Les baies saines sont comptabilisées.
Cette méthodologie de comptage global permet également de distinguer et de dénombrer les éventuelles chutes physiologiques.

Les observations ont été effectuées durant 4 ans de 1995 à 1998 à Santa et de 1996 à 1998 à Babadjou. Les résultats qui sont présentés n'ont pas fait l'objet d'un traitement statistique et sont présentés sous forme de tendances.

RESULTATS

Importance de la maladie

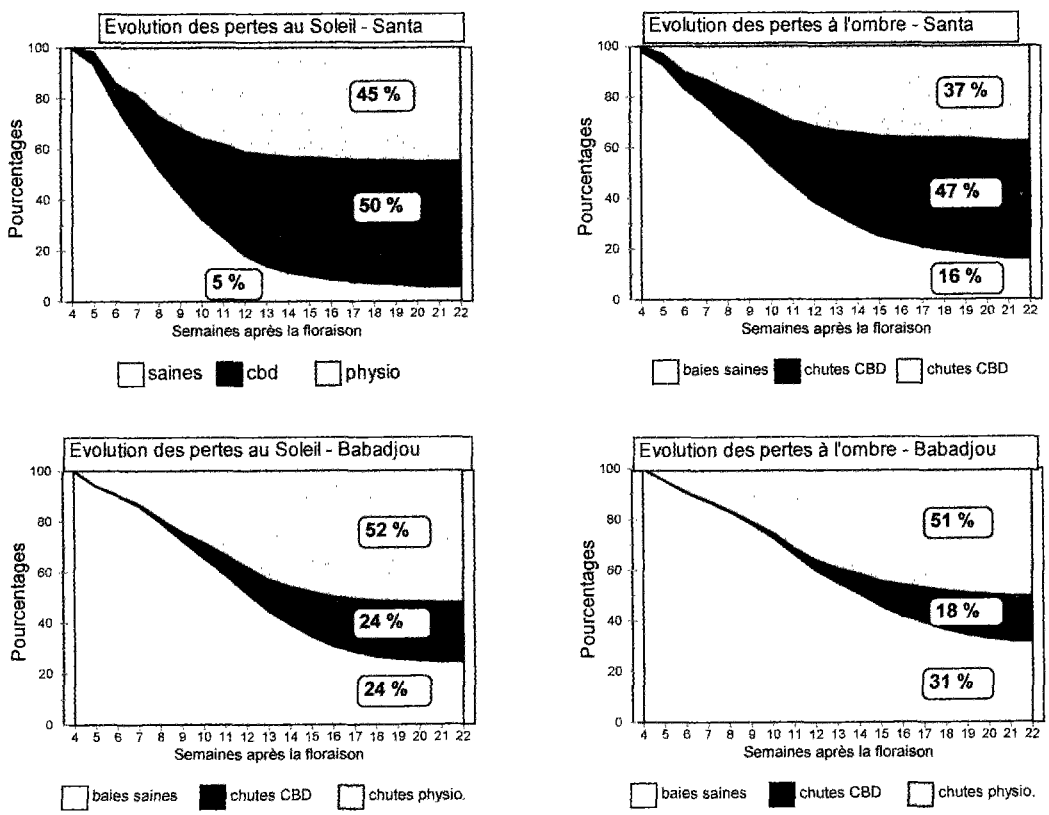
Le taux de perte total obtenu en moyenne (au cours des 4èmes et 3èmes années d'observations) est de 89% à Santa en haute altitude et de 72% à Babadjou en moyenne altitude, la part des pertes dues au CBD étant de 48% à Santa et de 21% à Babadjou. Au sein de ces valeurs générales des situations contrastées sont observées selon les sites et les années (Tableau 1).

En haute altitude, les premiers symptômes de la maladie apparaissent entre la 4ème semaine et la 8ème semaine après la floraison selon les années. Pour une année donnée aucun décalage n'est perceptible entre les arbres situés au soleil et les arbres situés à l'ombre. Au cours des 4 années d'observations les pertes réellement dues au CBD ont variées de 34 à 68% au soleil et la part des «chutes physiologiques» représente de 22 à 63%. A l'ombre, les pertes imputables au CBD représentent de 38 à 55% selon les années et la part de chutes physiologiques est de 21 à 48%. (Figure 1).

Tableau 1 - Pertes de production (en %) enregistrées dans les différents sites et années

Années	SANTA (1 760 m)						BABADJOU (1 550 m)					
	SOLEIL			OMBRE			SOLEIL			OMBRE		
	Chutes physio	Chutes CBD	Baies saines	Chutes physio	Chutes CBD	Baies saines	Chutes physio	Chutes CBD	Baies saines	Chutes physio	Chutes CBD	Baies saines
1995	22	68	10	21	55	24						
1996	34	62	4	36	45	19	60	32	8	47	23	30
1997	63	34	3	48	38	14	58	31	11	51	22	27
1998	60	35	5	43	38	9	39	9	52	53	10	37
Moyennes	45	50	5	37	46.5	16.5	52	24	24	51	18	31

Fig 1 : Evolution des chutes dues au CBD et des chutes physiologiques dans différentes conditions agroécologiques



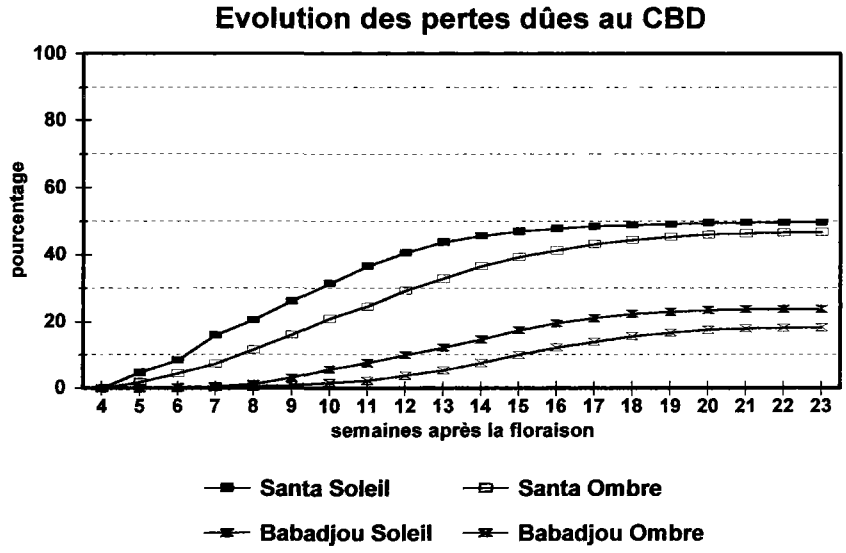
En moyenne altitude, les pertes de production réellement dues au CBD varient de 9 à 32% au soleil et de 10 à 23% à l'ombre. La moyenne sur les 3 années d'observations indiquent que les pertes dûes au CBD au soleil (24%) sont supérieures aux pertes enregistrées à l'ombre (18%). Les chutes physiologiques sont de 52% au soleil et de 51% à l'ombre. Elles sont supérieures aux pertes dues au CBD. Les pertes de production totales sont plus élevées à l'ombre qu'au soleil.

Evolution de la maladie

L'apparition des premiers symptômes entre la 5ème et la 8ème semaine varie en fonction des années et des lieux. Elle est toujours plus précoce en altitude de l'ordre de 1 à 2 semaines (Figure 2).
Indépendamment de l'altitude et du niveau d'ensoleillement, quelle que soit l'importance des dégâts, les courbes d'évolution des pertes cumulées suivent une progression de type logistique, selon un modèle classique :

$$Y = \frac{H}{1 + e^{-a\,(sem-b)}}$$

H : correspond au pourcentage maximum de baies malades
a : représente le taux d'accroissement maximal de la maladie
b : indique la date à laquelle le taux d'accroissement maximal de la maladie est atteint

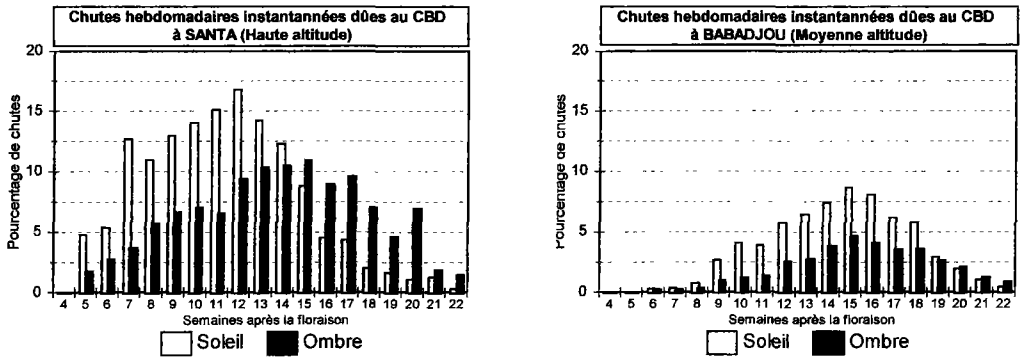


Le point d'inflexion, qui traduit le moment où la vitesse de la maladie est maximale est atteint selon les sites et les années entre la 6ème et la 17ème semaine après la floraison. Le développement de la maladie est plus précoce en altitude de 2 semaines en moyenne quel que soit le lieu et l'année. Pour l'ensemble des sites à l'exception de Babadjou en 1998, le point d'inflexion est atteint de 2 à 4 semaines plus précocement sur les arbres situés au soleil. Exceptionnellement, il est atteint dans certains cas (Santa 95 soleil) dès la 6ème et la 7ème semaine après la floraison.
Il est vraisemblable que le rythme de développement des baies sur les arbres situés à l'ombre et au soleil n'est pas synchrone. Du fait que la sensibilité des baies est fonction de leur stade de développement, il est probable que les baies situées au soleil atteigne le stade de réceptivité plus précocement que les baies situées à l'ombre.

Rythme des chutes

Le rythme hebdomadaire des chutes de baies dues à la maladie fait apparaître dans tous les cas un pic de chute décalé entre les baies exposées à la lumière et les baies exposées à l'ombre, la chute des baies étant plus précoce au soleil. Ce décalage est en moyenne de 2 semaines (Figure 3).

Figure 3 : Cinétique de la chute des fruits dues au CBD



CONCLUSIONS

Les dynamiques du développement de la maladie sont variables selon les années et selon les sites. Malgré la diversité des situations observées une dynamique moyenne de la maladie peut être décrite qui dans leur globalité suivent l'ajustement au modèle logistique et confirment des descriptions antérieures (Muller, 1980, Van der Graaf, 1992, Vermeulen, 1979).

En première conclusion provisoire de ces travaux qui font actuellement l'objet d'analyses statistiques détaillées, plusieurs éléments peuvent être retenus:

- 1 ces observations précises ont permis de mettre au point une méthodologie d'évaluation spécifique des pertes dues au CBD,

- 2 plusieurs conditions agro-écologiques ont une incidence sur ces pertes. On enregistre notamment:

> ☞ des pertes plus élevées en zone d'altitude ou la plus forte pluviométrie et les températures plus basses favorisent les attaques et la progression de la maladie,
> ☞ une apparition plus précoce des chutes et une plus grande intensité du CBD sur les caféiers situés préférentiellement au soleil,
> ☞ les « chutes physiologiques » interviennent pour une part non négligeable dans les pertes de production.

En première recommandation , dans les conditions du Cameroun, l'ombrage pourrait être aménagé pour réduire les pertes en zones de forte pression infectieuse. Cela devrait ainsi permettre de raisonner l'emploi de la lutte chimique qui reste actuellement toujours nécessaire dans les régions les plus atteintes par la maladie.

Enfin l'analyse de l'ensemble des résultats devra permettre à terme de dégager les facteurs principaux rendant possible une identification précise des zones les plus favorables à la maladie et adapter en conséquence les modes de conduite des plantations.

La réalisation de cette étude fait partie d'un projet de recherche financé par l'Union Européenne dont le thème principal est orienté vers l'identification de sources de résistance au CBD et l'étude de la variabilité génétique de *Colletotrichum kahawae,* agent responsable de cette maladie.

Ce projet qui se termine fin 1999 a été mis en place en partenariat avec le réseau RECA (ACRN), conduit

en collaboration avec les institutions de recherche du Kenya (CRF), du Cameroun (IRAD) et du Portugal (CIFC) et coordonné par le CIRAD.

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HISTOCHEMICAL DIFFERENCES DURING INFECTION OF *Coffea arabica* VARIETIES BY *Colletotrichum kahawae* ISOLATES

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SUMMARY

Hypocotyls, attached and detached green coffee berries from *Coffea arabica* varieties varying in resistance to Coffee Berry Disease *(Colletotrichum kahawae)* were inoculated with monoconidial isolates of the fungus. The tissues were observed under a microscope and tested for phenolics, lignin and callose deposition. There were differences in hyphal growth and appearance being more extensive and longer-celled in the susceptible varieties. Concentration of lignin- and phenolic-like compounds was higher in resistant varieties and so was callose deposition around hyphae. Formation of scabs and cork barriers followed the other reactions but not always. Detached coffee berries lacked these induced resistance mechanisms. Penetration of a cork barrier was also observed.

INTRODUCTION

Colletotrichum kahawae infects green coffee berries to cause coffee berry disease which is a major limiting factor in Arabica coffee production in Africa. (1) This study was carried out to increase the knowledge of mechanisms of CBD resistance (2) by studying the involvement of phenolics, lignin and callose which are known to be active in plant disease resistance (3,4).

MATERIAL AND METHODS

Seedling hypocotyls, attached and detached green arabica coffee berries of varieties differing in field resistance to CBD were inoculated with monoconidial isolates of *C. kahawae.* The inoculated areas were sectioned and observed under bright field and UV light fluorescence tests for lignin and callose (5).

RESULTS

Rate of tissue colonisation was most rapid in susceptible varieties (Plate 1) but differed with isolates.

Autofluorescence indicating phenolics and lignin was more intense in resistant varieties (Plate 2). Further resistance reactions were scab formation, callose deposition on cell walls and intracellular hyphae and cork barrier formation (Plate 3). One isolate penetrated a cork barrier.

DISCUSSION

The demonstrated involvement of phenolics, lignin and callose in this host - pathogen system increases the complexity of its resistance mechanisms (2). Thus a variety like Rume Sudan exhibiting most of them is rather stable but pathotypes may occur as penetration of cork barrier indicated. These mechanisms are also active against *Hemileia vastatrix (5)* hence pathogen non-specific.

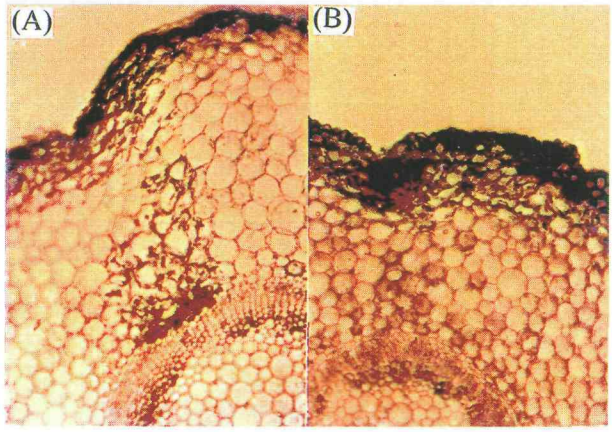

Plate 1: (A) Deep tissue colonisation (dark staining hyphae) in susceptible variety (SL 28). (B) Limited tissue colonisation despite necrosis in medium resistant variety K7.

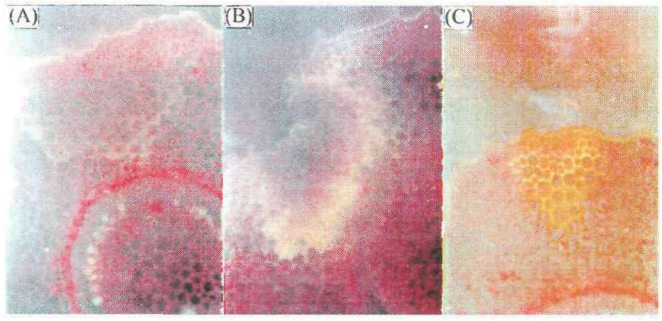

Plate 2: (A) Light autofluorescence in susceptible variety (SL 34)(B) strong autofluorescence in a 'susceptible' hypocotyl of resistant variety Rume Sudan (C) strong autofluorescence in resistant hypocotyl of variety catimor

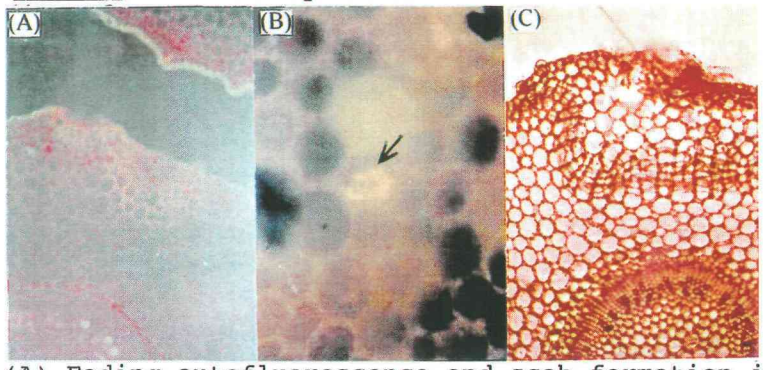

Plate 3: (A) Fading autofluorescence and scab formation in medium resistant variety Pretoria. (B). Callose deposition in intracellular hyphae in resistant variety Rume Sudan. (C). Fully formed cork barrier below necrotic lesion in Pretoria.

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PIÉGEAGE DE MASSE DU SCOLYTE DU CAFÉ *Hypothenemus hampei* Ferr. (Col., Scolytidae) EN CONDITIONS RÉELLES : PREMIERS RÉSULTATS

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Résumé

Le piégeage de masse du scolyte du café est une technique de lutte en cours d'expérimentation en El Salvador, destinée à être utilisée dans le cadre d'une protection intégrée. Appliqué pendant plus de trois mois sur des parcelles infestées, peu de temps après le dernier passage de récolte et à raison de 26 pièges/ha, le piégeage du scolyte se caractérise par une succession de forts et de faibles niveaux de capture. Cette alternance coïncide avec les pics d'émergence et de migration du ravageur, déclenchés par les rares pluies intervenant durant la seconde moitié de la saison sèche. Les populations résiduelles présentes dans les cerises desséchées tombées au sol, sont en grande partie à l'origine des premières migrations. Les vieux fruits encore présents au niveau des rameaux semblent servir de refuge temporaire pour les scolytes en quête de nouveaux hôtes. L'efficacité du piégeage de masse est évaluée en fonction du niveau des infestations de la nouvelle fructification par les femelles colonisatrices ayant échappé à la capture, par rapport à un témoin sans piégeage. Avec le mélange attractif de base (traitement 1) constitué d'éthanol et de méthanol 1:1, la réduction des populations est de 34.8%. Elle atteint 50.7% lorsque les effluves de ce mélange sont enrichies de deux terpènes (traitement 2), l'effet de ces terpènes n'intervenant qu'en début de piégeage. Pour chaque traitement, la quantité totale de scolytes capturés ne représente qu'une infime partie de la population résiduelle, estimée juste avant les premières migrations, dans chaque aire de piégeage. Cette quantité d'insectes semble toutefois participer activement à la diminution des attaques des nouvelles générations de fruits. Un meilleur ajustement de la période de capture et surtout une augmentation de l'attractivité des pièges sont les solutions à envisager pour améliorer les performances de la technique.

Abstract

Mass trapping of coffee berry borer (CBB) is a monitoring technique in process of experimentation in El Salvador, to be used in an integrated pest management approach. Applied just after the last harvest, with 26 traps/ha and for more than 3 months, the mass trapping with hostplant allelochemicals presented series of high and low levels of capture. These abundances of catches, matched with peaks of emergence and migration of the pest, related with scarce rains late in the dry season. The pest residual population into the dry berries remaining on the ground were the major source of first migrations. The old dry fruits still present on the branches, seemed to act as temporal refuge for the CBB, seeking for new hosts. The efficacy of the mass trapping were evaluated according to the infestation level of new berries, caused by untrapped colonizing females and compared with a control field without trapping. The reduction of damage were 34.8% with the basic mixture: ethanol, methanol 1:1 (treatment 1). It reached 50.7% with the alcoholic mixture associated with two terpenes (treatment 2). The effect of these terpenes only occured at the beginning of the trapping period. For both mixtures, the total amount of trapped CBB, represented a small part of the residual population, evaluated just before the first migration. However, this small part seemed to participate actively in reducing the attack on the new berries. A better adjustment of the trapping period and an improvement in both allelochemical blend and trap might be the solutions to increase the performance of the mass trapping technique.

INTRODUCTION

Les récentes études conduites en El Salvador sur l'optimisation du piégeage de masse du scolyte du café (Dufour *et al.*, à paraître), ont montré qu'il était possible de capturer d'importantes quantités de femelles colonisatrices, au cours de la période de migration qui coïncide avec la fin de la saison sèche et le début de la saison des pluies. Ainsi, dans certains sites très infestés et à des moments précis, les niveaux de capture peuvent atteindre plusieurs milliers de scolytes par piège et par jour. Ces études font suite à un ensemble de travaux portant sur l'attraction allélochimique du scolyte (Giordanengo *et al.*, 1993; Mathieu, 1995; Mendoza Mora, 1991), l'identification de substances chimiques volatiles émises par les cerises fraîches de café (Mathieu *et al.*, 1998) et le piégeage au champ (Gutiérrez-Martínez *et al.*, 1995; Mathieu *et al.*, 1997; Mendoza Mora, 1991). Avant d'envisager le piégeage comme une véritable méthode de lutte, il semblait important d'étudier tout d'abord, le déclenchement et l'évolution des migrations, sachant qu'elles ont pour origine l'ensemble des populations présentes dans les fruits non récoltés. Par ailleurs, il était nécessaire de soumettre la méthode à une première étude d'efficacité afin d'en apprécier les potentialités. En effet, il n'est pas certain qu'un bon niveau de capture en période de migration, puisse diminuer de manière sensible les populations résiduelles de scolytes, et plus précisément le nombre de femelles capables de coloniser les nouvelles fructifications. En d'autres termes, on ne sait pas si le piégeage de masse peut contribuer à faire baisser les niveaux d'infestation sur la future récolte.

La première partie de ce travail présente donc une description des aspects bioécologiques observés dans une caféière de moyenne altitude, cultivée sous ombrage, représentative du parc caféier en El Salvador. La deuxième partie est consacrée à l'étude de l'efficacité, mise en oeuvre à l'aide d'un matériel de piégeage encore privé de toute amélioration technique.

MATÉRIELS ET MÉTHODES

Conditions expérimentales

Ce travail a été réalisé en El Salvador, dans une caféière de type "organique" située à une altitude de 900 m et fortement attaquée par le scolyte. Les parcelles expérimentales ont été délimitées sur des surfaces planes, plantées de vieux caféiers de variété "Bourbon". La densité de caféiers productifs n'est pas parfaitement homogène, surtout dans les parcelles témoin. L'ombrage est constitué d'essences forestières et de quelques espèces cultivées, les plus communes appartenant au genre *Inga*.

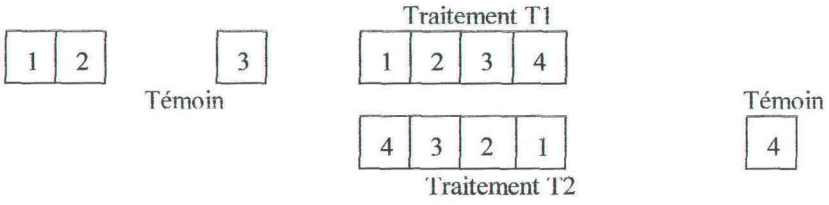
Les activités culturales annuelles sont tout à fait classiques pour ce type de caféière: taille appréciative, réduction de l'ombrage, desherbage manuel, fertilisation organique avec application de chaux. Avant le début de cette étude, la lutte contre le scolyte s'est concrétisée par une récolte partielle des fruits résiduels, véritables réservoirs de scolytes durant l'intersaison. Elle s'est poursuivie par une récolte stricte de la fructification dite "prématurée", laquelle constitue généralement le refuge idéal pour les femelles migrantes.

Les données de température ont été enregistrées à la station météorologique la plus proche (El Boquerón), les précipitations ont été mesurées directement dans la caféière.

2.2. Dispositif expérimental

Le dispositif ne correspond à aucun schéma classique utilisé en agronomie. Etant donné que les principales variables, telles que la distribution et la quantité de fruits résiduels infestés par le scolyte, sont fortement liées au phénomène de récolte et donc relativement indépendantes du milieu, il est possible de rassembler les 4 répétitions d'un même traitement dans les parcelles d'un même bloc. Cet arrangement a l'avantage de donner à chaque traitement une aire d'influence plus grande et de diminuer les interférences avec le milieu extérieur. Le dispositif comporte 2 traitements avec piégeage (T1 et T2) et un témoin sans piégeage (Fig. 1). La surface de chaque parcelle est de 3025 m² (55 m x 55 m).

Fig. 1. Dispositif expérimental



2.3. Piégeage

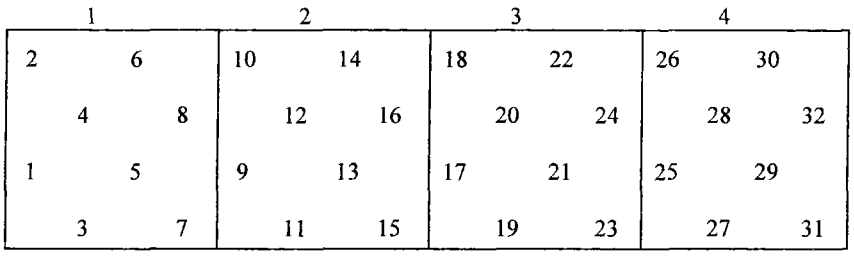
La méthode utilisée a été mise au point à partir des résultats obtenus lors des récentes études sur le piégeage du scolyte (Dufour *et al.*, à paraître). Huit pièges par parcelle avec un même attractif, soit 32 par bloc ou par traitement (Fig. 2) sont suspendus aux caféiers, à une hauteur de 1,2 m et fonctionnent en permanence pendant 3 mois.

Le traitement T1 correspond à la diffusion d'un mélange 1:1 d'éthanol absolu et de méthanol pur, à un taux moyen de 0.21 g/jour. Le traitement T2 correspond à la diffusion séparée du même mélange alcoolique et de 2 terpènes: l'un à 0.023 g/jour et l'autre à 0.014 g/jour.

Le liquide dans lequel viennent se noyer les scolytes capturés, est une solution aqueuse à 0.2% d'un mélange antiseptique à base de glutaraledéhyde et de glyoxal, qui évite la décomposition des insectes entre chaque collecte.

Les captures sont relevées périodiquement puis comptées, principalement lorsqu'interviennent des changements de temps pouvant agir sur l'intensité des migrations.

Fig 3 : Distribution des pièges pour un traitement



2.4. Echantillonnages

En dehors du piégeage, des échantillonnages de fruits attaqués et de populations de scolytes sont réalisés, ainsi qu'un comptage des caféiers productifs.

✏ Les échantillonnages de fruits attaqués sont effectués sur les fruits résiduels, juste avant le début du piégeage (début mars) et sur les nouvelles fructifications en cours de développement, qui vont constituer l'essentiel de la future récolte (début août). Les fruits prématurés présents sur les rameaux, récoltés en cours d'essai ne sont pas pris en compte. Au niveau de chaque parcelle, l'échantillonnage porte sur 24 caféiers productifs, désignés de manière systématique, ainsi que sur la surface au sol occupée par chacun d'eux. Les paramètres obtenus sont les suivants:

- X_1 = moyenne par plante productive, du nombre de fruits résiduels perforés, sur les rameaux,
- Y_1 = moyenne par plante productive, du nombre de fruits résiduels perforés, tombés au sol,
- Y'_1 = moyenne par plante productive, du nombre de fruits prématurés perforés, tombés au sol,
- X_2 = moyenne par plante productive, du nombre de jeunes fruits perforés,
- S = surface d'une parcelle.

✏ Les échantillonnages de populations de scolytes sont effectués périodiquement sur les fruits résiduels attaqués (début mars - début mai) et une seule fois sur les nouvelles fructifications (début août). Les fruits attaqués sont pris au hasard dans chaque parcelle: 50 fruits résiduels sur les rameaux et 100 fruits résiduels au sol, pour le premier échantillonnage, 25 et 50 pour les échantillonnages intermédiaires et 200 jeunes fruits perforés pour le dernier échantillonnage. Parmi les nombreux paramètres obtenus, 4 interviennent directement dans les calculs de populations:

- a = moyenne du nombre initial de stades biologiques femelles vivants, par fruit résiduel provenant d'un rameau,[1]
- b = moyenne du nombre initial de stades biologiques femelles vivantes, par fruit résiduel provenant du sol,
- b' = moyenne du nombre de femelles vivantes, par fruit prématuré tombé au sol,
- c = moyenne du nombre de femelles colonisatrices dans les jeunes fruits [2].

Tous les autres paramètres correspondent à des moyennes de nombre de femelles jeunes, âgées, d'individus

[1] Un stade biologique femelle correspond à tout individu femelle quel que soit son stade de développement. Cependant la différenciation n'est possible que chez l'adulte. Pour calculer le nombre d'individus femelles dans une population non adulte (oeufs, larves et nymphes), il convient d'utiliser la *sex-ratio*. Chez le scolyte elle est de 10 femelles pour 1 mâle (Heargreaves, 1926; Sladden, 1934; Bergamin, 1943; Le Pelley, 1968).

[2] Le nombre de colonisatrices comprend les femelles vivantes ainsi que celles qui n'ont pas survécu après leur installation sur les nouvelles fructifications

immatures, d'adultes morts, par fruit résiduel situé sur un rameau ou bien au sol.

Le nombre de caféiers productifs par parcelle est évalué en début et en fin d'essai:

- n1 = nombre initial de caféiers productifs,
- n2 = nombre de caféiers productifs après élimination des arbres malades et replantation des manquants.

Méthode d'évaluation du piégeage

Sans bien connaître l'évolution des populations résiduelles de scolytes et surtout leur redistribution dans les parcelles après migration, il est difficile de mettre en évidence l'effet du piégeage sur ces populations. La méthode retenue a été calquée sur le principe appliqué lors des études antérieures effectuées sur les lâchers de parasitoïdes du scolyte (Dufour *et al.* 1997). Il s'agit de comparer des parcelles traitées par piégeage et des parcelles témoins, en considérant d'une part les populations résiduelles de scolytes et d'autre part, les quantités de femelles ayant finalement colonisé les nouvelles fructifications. La différence entre ces quantités correspond plus ou moins au nombre d'individus morts naturellement, tués par le parasitisme ou les maladies, ou bien ayant migré vers d'autres sites, ou encore ayant été capturés par les pièges. Si les causes naturelles sont d'égale importance dans toutes les parcelles, y compris les parcelles témoins, le piégeage demeure donc la principale source de variation, établissant ainsi une relation de dépendance entre les populations de scolytes initiales et finales. Cette relation permet d'appliquer une analyse de covariance.

La covariable (Mi) et la variable (Mf) sont des moyenne de nombre de scolytes par unité de surface. Elles prennent en compte les variations du nombre de caféiers productifs par parcelle. Pour que l'unité de surface ait une signification agronomique, nous avons choisi une aire qui correspond approximativement à celle occupée par un caféier, c'est à dire 4 m^2:

- $\mathbf{Mi = [(x_1 * a) + (y_1 * b) + (y'_1 * b']\ 4 * n_1/S}$ (moyenne du nombre de stades biologiques femelles vivants présents sur 4 m^2 de caféière, avant le piégeage).
- $\mathbf{Mf = (x_2 * c)\ 4 * n_2/S}$ (moyenne du nombre femelles colonisatrices présentes sur 4 m^2 de caféière, après leur installation sur les nouvelles fructifications).

2.6. Calcul de l'efficacité

L'expression la plus simple pour exprimer l'efficacité (E) du piégeage est le pourcentage de diminution des infestations de scolytes sur les nouvelles fructifications dans les zones de piégeage, par rapport à celles observées dans les parcelles témoins:

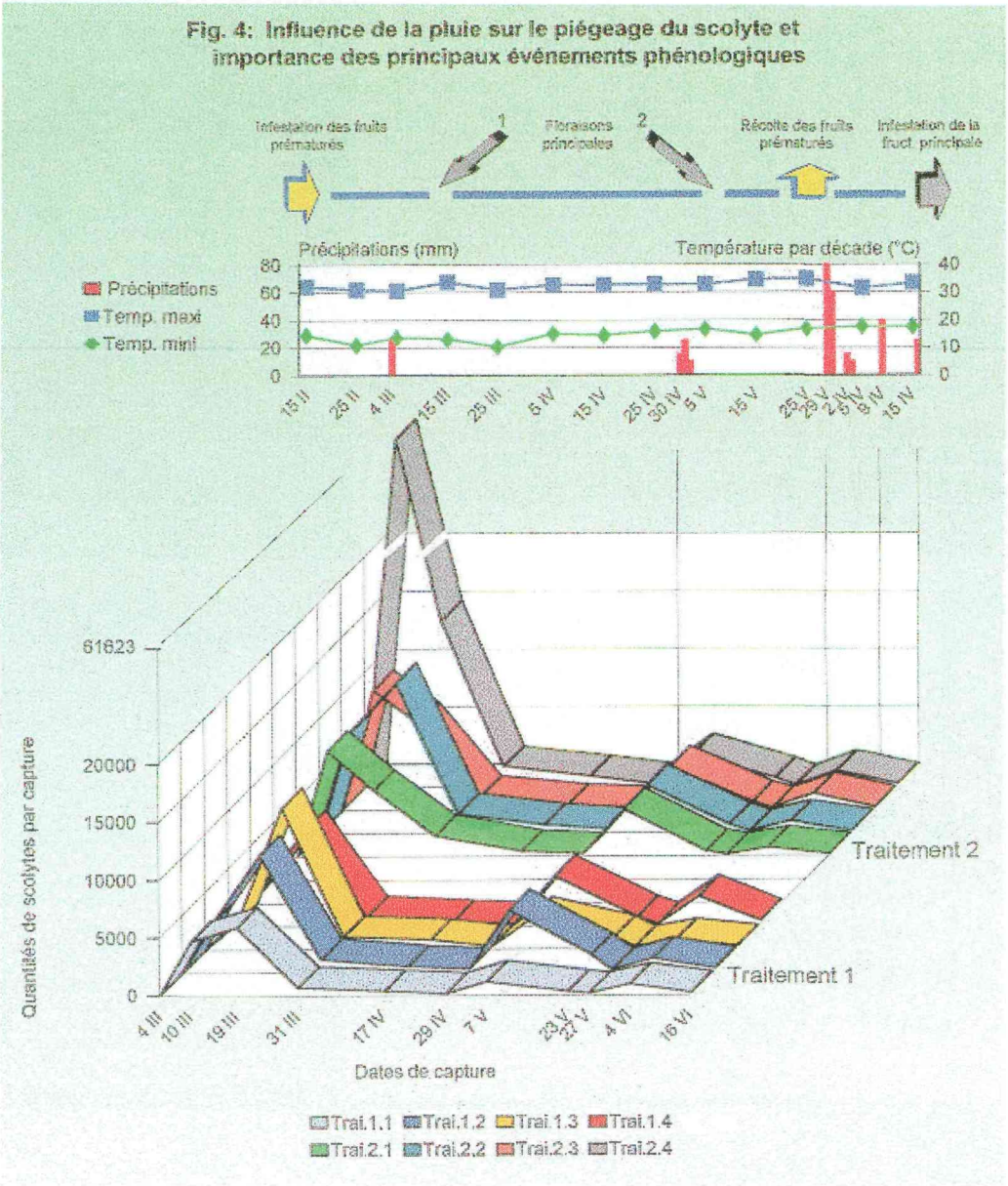
$$\mathbf{E\ (T1\ ou\ T2) = [\sum Mf\ Témoin - \sum Mf\ T1\ ou\ T2] * 100/\sum Mf\ Témoin}$$

RÉSULTATS

Importance de la pluie sur le déclenchement des migrations

La représentation graphique de l'ensemble des séquences de piégeage permet de mettre en évidence plusieurs pics de captures, chacun d'eux étant précédé d'une pluie (Fig. 4). La hauteur de ces pics est en réalité le reflet de l'intensité des différentes migrations. Le premier est le plus important en nombre de scolytes capturés et en durée: il représente donc la migration principale.

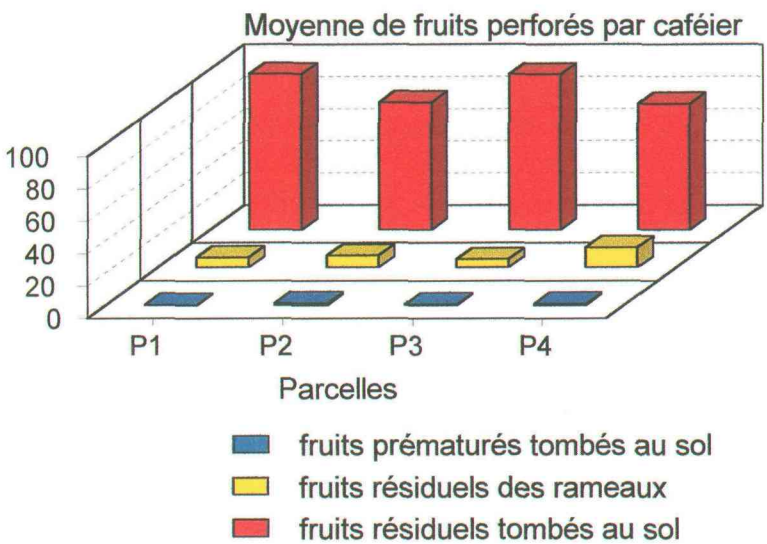
Comme le soulignent Baker *et al.* (1992), la pluie, et plus précisément l'humidité qu'elle génère au niveau des fruits résiduels, apparaît comme étant le facteur déterminant pour le déclenchement des vols. Toutefois, la quantité de pluie tombée ne semble pas agir sur l'intensité des émergences, puisque 25 mm seulement ont été suffisants pour provoquer la migration la plus forte.



Les fruits résiduels: lieu d'origine des migrations

La distribution des fruits résiduels infestés dans toutes les parcelles expérimentales montre que la plus grande proportion se trouve au niveau du sol. Dans les parcelles du traitement T1 par exemple, elle est de 6 à 18 fois plus élevée qu'au niveau des rameaux (Fig. 5). Cette différence est tout simplement le résultat d'une récolte sanitaire trés inégale entre le sol et les rameaux.

Fig 5 : Quantités de fruits perforés par caféier avant le piégeage (parcelles T1)



Evolution des populations de scolytes dans les fruits résiduels

□ **au niveau du sol:** juste avant la première pluie, le nombre de femelles matures aptes à migrer, est très élevé: pour l'ensemble des parcelles du traitement T1, la moyenne est de 766 par caféier (Fig. 6). L'émergence en masse de la plupart de ces femelles explique donc l'intensité de la première grande migration et l'importance des deux premières captures.

La période sèche située entre les 2 premières pluies, d'une durée atteignant presque 2 mois, voit se reconstituer les populations de femelles à partir des stades larvaires. Ces femelles restent dans les fruits, en attendant une nouvelle pluie pour pouvoir s'envoler.

La seconde vague de migration entraîne finalement une forte diminution des populations résiduelles et la quasi disparition des stades immatures.

□ **au niveau des rameaux:** le nombre de femelles âgées ne diminue guère au cours du temps malgré la forte diminution des stades immatures et l'effet des migrations (Fig. 7). Il semble donc exister un phénomène de réinfestation permanent des fruits résiduels situés sur les rameaux, par des colonisatrices privées d'hôte, en quête de nouveaux refuges. Ce phénomène a déjà été observé plusieurs fois, durant la même période et dans d'autres caféières (Dufour, non publié).

Fig. 6. Evolution de la structure de population de scolytes dans les fruits tombés au sol (stades/caféier)

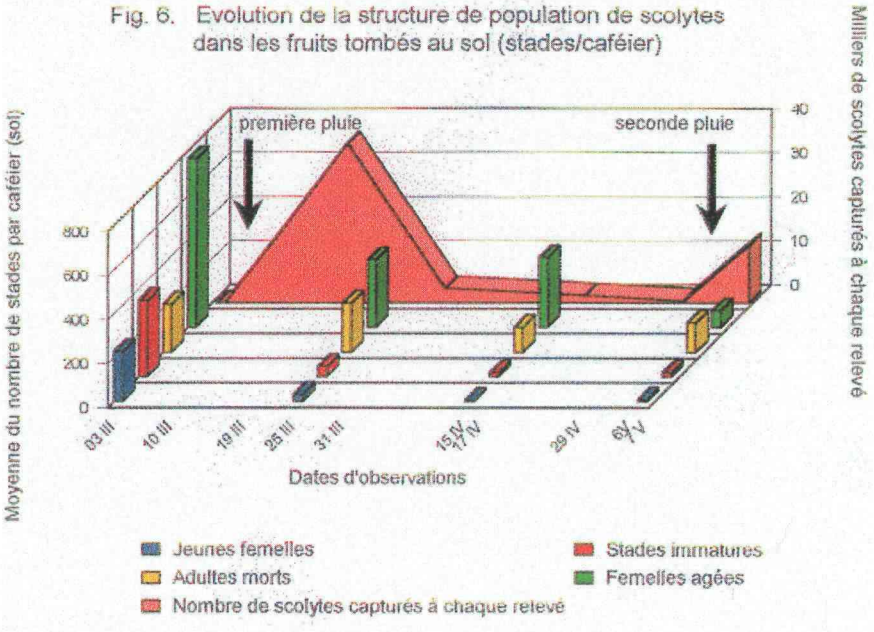
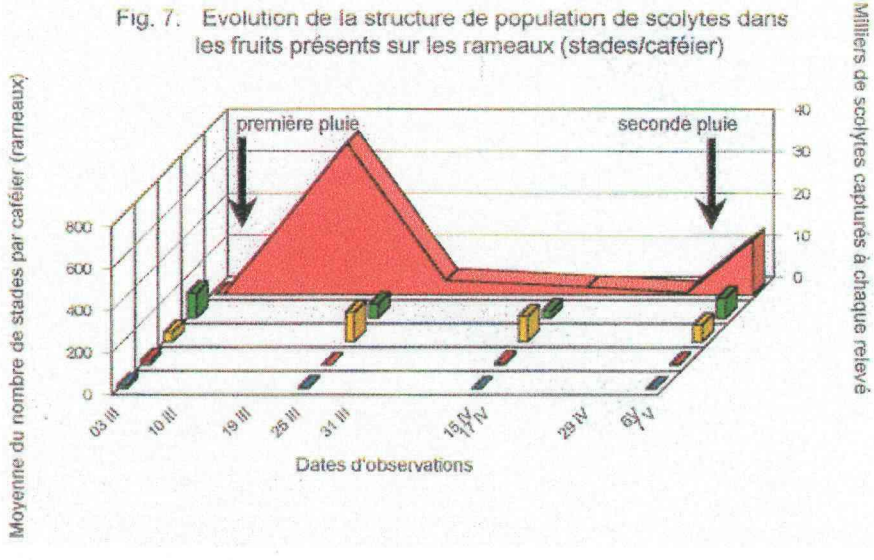


Fig. 7. Evolution de la structure de population de scolytes dans les fruits présents sur les rameaux (stades/caféier)



Mise en évidence de l'effet du piégeage sur la diminution des infestations de scolytes

Les moyennes Mi et Mf évaluées pour la réalisation de l'analyse de covariance sont présentées dans les tableaux 1 et 2. Les valeurs de Mf, beaucoup plus faibles que celles de Mi, traduisent une forte réduction des populations de femelles sous l'influence des différents facteurs de régulation. Dans les parcelles traitées,

principalement dans les parcelles T1, il faut noter une certaine homogénéité des valeurs de Mi et de Mf, qui n'apparaît pas dans les parcelles témoins. Cette caractéristique semble dépendante de l'aspect des parcelles (nombre de caféiers productifs et répartition de l'ombrage).

Tableau 1: Evaluation de Mi (avec un intervalle de confiance de 95%)

	Rep	x_1	a	y_1	B	y_1'	b'	n_1	Mi
T1	1	6.29 ± 7.10	17.90	96.88 ± 71.43	16.06	0.29 ± 0.74	1.04	651	1436.49 ± 990.54
	2	7.46 ± 10.21	20.48	79.21 ± 68.80	15.26	1.92 ± 2.11	0.94	651	1173.03 ± 962.25
	3	5.21 ± 4.56	29.68	96.58 ± 93.59	12.04	0.50 ± 1.04	1.00	737	1284.40 ± 1120.10
	4	12.42 ± 15.60	11.44	78.38 ± 70.82	16.33	0.83 ± 3.02	0.96	706	1328.18 ± 1128.74
T2	1	6.25 ± 11.85	35.62	64.38 ± 56.73	8.46	0.21 ± 0.71	1.00	658	667.74 ± 500.01
	2	11.21 ± 14.50	33.68	88.60 ± 68.11	13.28	1.63 ± 4.03	1.00	737	1516.45 ± 943.30
	3	7.50 ± 10.10	38.52	53.33 ±40.52	8.67	0.63 ± 1.38	0.96	719	714.86 ± 639.78
	4	7.29 ± 8.51	37.12	68.58 ± 60.32	13.48	0.79 ± 1.63	1.00	610	964.68 ± 726.75
Témoin	1	3.58 ± 5.72	30.10	120.75 ± 92.03	16.30	0.08 ± 0.28	1.001.	1036	2844.18 ± 2103.77
	2	7.5 ± 7.96	16.56	97.08 ± 79.76	14.72	1.71 ± 3.40	00	786	1616.15 ± 1208.72
	3	3.63 ± 5.06	11.66	66.50 ± 54.02	13.52	0.46 ± 1.47	0.98	894	1113.34 ± 879.66
	4	5.33 ± 4.44	20.70	82.54 ± 53.47	9.32	0.17 ± 0.62	0.96	417	485.15 ± 262.46

Tableau 2: Evaluation de Mf (avec un intervalle de confiance de 95%)

	Rep.	x_2	c	n_2 [3]	Mf
T1	1	38.71 ± 61.54	0.72	651	23.99 ± 38.14
	2	50.71 ± 61.73	0.69	651	30.12 ± 36.67
	3	38.29 ± 38.30	0.66	737	24.63 ± 24.63
	4	45.63 ± 56.93	0.69	706	29.39 ± 36.67
T2	1	27.00 ± 47.89	0.71	658	16.68 ± 29.09
	2	46.71 ± 102.71	0.65	737	29.64 ± 65.06
	3	12.29 ± 21.15	0.68	719	7.95 ± 13.67
	4	51.67 ± 95.77	0.66	610	27.51 ± 50.98
Témoin	1	20.46 ± 37.41	0.68	1036	19.06 ± 34.85
	2	117.08 ± 160.33	0.69	786	83.97 ± 114.99
	3	41.13 ± 97.66	0.67	894	32.57 ± 77.35
	4	79.42 ± 187.49	0.69	417	30.22 ± 71.34

L'analyse des moyennes Mi et Mf a été effectuée après élimination des données de la première parcelle du témoin. Cette parcelle s'est révélée plus ensoleillée que les autres, entrainant un dessèchement rapide des fruits résiduels, provoquant par la suite, une forte régulation naturelle des populations de scolytes et une baisse anormale du niveau d'infestation final. Les résultats indiquent qu'il n'y a pas de différence significative entre les

[3] Malgré quelques variations de productivité dues à la taille, le nombre de caféiers productifs par parcelle est resté le même au cours de l'essai, donc $n_1 = n_2$

différents traitements au seuil de 5% (F = 4.72, □ = 0.089, ddl = 2). Par ailleurs, il convient de souligner que la covariable apporte peu d'explication complémentaire (F = 3.27, □ = 0.145). Il faut rappeler que la régulation des populations de scolytes est le fait de nombreux facteurs dont il est difficile de connaître les variations et de mesurer les effets. Finalement ces variations n'ont peut-être pas la même importance dans les différentes parcelles et affecteraient donc l'application de la covariance.

Evaluation de l'efficacité

Bien que la l'analyse statistique ne décèle pas de différence significative entre traitements, elle n'empêche pas l'évaluation de l'efficacité du piégeage en terme de réduction des populations. Sans aucune exclusion de donnée, nous obtenons une efficacité de 34.8% pour le traitement T1 et 50.7% pour le traitement T2. Ces résultats sont en parfait accord avec les données de capture. En effet, le meilleur piégeage a été obtenu dans le cadre du traitement T2 caractérisé par une double diffusion (éthanol-méthanol et terpènes) (Tableau 3).

Tableau 3: Quantités de femelles capturées au cours du piégeage

	5 mars-10 mars	11 mars – 19 mars	20 mars - 31 mars	1 avril - 29 avril	30 avril - 27 mai	28 mai - 16 juin	Somme des captures (Fc)
Traitement 1	**14076**	**36098**	**3345**	**2192**	**16654**	**5837**	**78202**
Traitement 2	**89692**	**40292**	**6029**	**2791**	**16595**	**4656**	**160055**
Test de Mann-Whitney appliqué sur toutes les captures (32 par traitement)	T1<T2 p<0.01	T1=T2	T1<T2 p<0.01	T1=T2	T1=T2	T1=T2	

Effet des terpènes

S'il existe un effet synergique du mélange alcoolique avec les terpènes sur l'attraction du scolyte, il semble se manifester surtout en début de piégeage, lors de la plus forte migration. Afin de vérifier que l'intensité des migrations ne soit pas plus élevée dans le traitement T2 que dans le traitement T1, on a effectué une estimation du nombre de femelles matures, en attente dans les fruits résiduels présents dans les parcelles T1 et T2, juste avant le piégeage. Les quantités de femelles aptes à migrer sont finalement un peu plus abondantes dans les parcelles T1 que dans les parcelles T2, respectivement 2 364 000 et 2 031 000. Ces données permettent donc d'écarter une éventuelle influence de l'intensité de migration sur les résultats de capture et de conforter la thèse d'un effet synergique des terpènes.

Participation du piégeage au processus de diminution des populations résiduelles de scolytes

Bien que les estimations de populations résiduelles dans toutes les parcelles expérimentales présentent d'importantes marges d'erreurs, il est intéressant d'avoir une idée sur le devenir de ces populations après migration. Elles se divisent en 3 groupes: la fraction capturée, celle qui disparaît naturellement et celle qui, finalement, survit et colonise la nouvelle fructification (Tableau 4). En réalité, peu de scolytes sont piégés par rapport à la quantité disponible. Pourtant, les 2% et 5.5% qui sont capturés, contribuent à diminuer sensiblement les niveaux d'infestation de la nouvelle fructification. Une incertitude demeure: les femelles résiduelles acquièrent-elles toutes le statut de colonisatrice?

Tableau 4: Distribution des populations de scolytes après migration

	Population initiale de stades biologiques $\sum Mi*S/4$)	Taux de scolytes atteignant leur hôte (%) $\sum Mf/\sum Mi*100$	Taux de scolytes capturés (%) $Fc/(\sum Mi*S/4)*100$	Pourcentage de scolytes disparus (%) $[(\sum Mi-\sum Mf /\sum Mi) +(4Fc/\sum Mi*S)]*100$
Traitement 1	3 949 214	2.1	2	95.9
Traitement 2	2 921 949	2.1	5.5	92.4

4. DISCUSSION ET CONCLUSION

Comme l'ont précisé Baker *et al.*, (1992), les migrations de scolytes observées en période de post-récolte sont déclenchées par les rares chûtes de pluies inégalement réparties au cours de la saison sèche et se manifestent seulement dans des conditions optimales de température. D'après nos observations, l'intensité de ces migrations est déterminée par le nombre de femelles aptes à émerger des fruits résiduels et par la quantité de ces fruits dans les parcelles. La combinaison de ces différents facteurs permet donc d'expliquer l'évolution des migrations qui, à l'échelle d'une opération de piégeage, se traduit par une succession de pics de captures. Compte tenu des variations agroclimatiques annuelles, il faut s'attendre chaque année à des changements de rythme, de durée et d'intensité des migrations, ce qui implique pour l'utilisateur du piégeage, de s'adapter à la réalité du moment: par exemple, mettre les pièges en place dès que s'annoncent les premières émergences significatives, ou encore prolonger le piégeage jusqu'à la disparition totale des vols.

Avec la technologie actuelle, on commence à percevoir les effets du piégeage de masse sur les populations résiduelles. Pour une densité d'environ 26 pièges/ha, l'efficacité évaluée en terme de réduction des infestations, atteint 34.8% lorsque l'attractif diffusé est un mélange d'éthanol et de méthanol et 50.7% lorsque le mélange est enrichi de terpènes. Ces résultats coïncident avec les données de 103 jours de piégeage: un peu plus de 78 000 femelles capturée avec le premier mélange et 160 000 avec le second. Il s'agit-là de la confirmation de l'attractivité du mélange alcoolique (Mathieu, 1995) et de la première mise en évidence du rôle synergique des terpènes identifiés par Mathieu *et al.* (1997), dans les conditions d'un piègeage de masse. Il faut remarquer que l'effet des terpènes est de courte durée: les causes sont peut-être à rechercher dans le comportement du ravageur ou le niveau de stabilité des terpènes.

Il est tout de même intéressant de constater que les scolytes capturés ne représentent qu'une infime partie des populations résiduelles (2% et 5.5%) et que cette fraction retenue par les pièges, semble jouer un rôle important dans la réduction des nouvelles infestations. Que deviennent les femelles migrantes qui ont échappé au piégeage et qui, par ailleurs, n'ont pas trouvé d'hôte (92.4% et 95.9%)? La majeure partie de ces insectes meurent sans pouvoir poursuivre leur cycle biologique. La lutte pour la survie, conduit pourtant certains d'entre eux, à rechercher un refuge temporaire notamment dans les cerises sèches encore présentes sur les rameaux. Mais combien de temps peuvent-ils survivre? Finalement, ne serait-ce pas les femelles issues des dernières pontes qui auraient le privilège d'infester les nouvelles fructifications?

Si de nombreuses questions restent encore sans réponse, il faut en revanche considérer que l'efficacité du piégeage est loin d'être négligeable et que les niveaux de capture peuvent augmenter très fortement. Techniquement, il est tout à fait possible d'améliorer les performances des pièges: par exemple, en appliquant une couleur attractive pour le scolyte, en facilitant l'accès vers la source d'effluves et en élaborant des formulations plus efficaces.

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THE ROLE OF PARASITES IN THE NATURAL CONTROL OF ANTESTIA *Antestiopsis intricata* (Ghesquiere and Carayon). POSSIBILITIES FOR FURTHER CONTROL USING EXOTIC PARASITES

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Abstract

A survey was carried out at Jimma Research Center and two sub-centers to identify the natural enemies of eggs of Antestia -*Antestiopsis intricata*. Four parasites including one secondary parasite, of the egg were collected and identified. 45-50% of the eggs were attacked by parasites, predominantly by *Asolcus surans* (Scelionodae) followed by *Anastatus Antestiae* (Eupelmidae) and *Hadronotus antestiae* (Scelionidae). A secondary parasite, *Pediobuis sp.* was reared from Metu. Possibility for classical Biological control using introduced parasites from other African coffee producing countries is discussed.

INTRODUCTION

Antestia- *Antestiopsis intricata* (Ghesquiere and Carayon) is one of the most important insect pests of coffee in Ethiopia (1). Population dynamics study have shown that there is an inverse relationship between its population and altitude making it the worst enemy of coffee grown at low (below 1500) and medium (1500-1750) altitude. Although there have been attempts to control it using Insecticides(6,7) very little information is available on the identity and role of its parasitoids. Natural enemies, especially parasitoids, play an important role in the natural control of coffee pests. This has been demonstrated by the fact that severe outbreaks of pests have resulted from the use of persistent insecticides which have killed their parasitoids and predators. It has been pointed out that D.D.T. to control other coffee pests such as coffee thrips and Antestia, has resulted in the outbreak of mealybugs and Tingid (*Habrochila ghesquierei*) previously suppressed by their natural enemies (10,11).

The different developmental stages of Antestia are attacked by several natural enemies particularly parasitoids. Records from various coffee growing countries of Africa indicate that eggs of *A. intricata* is attacked by *Acroclisoides africanus., Anastatus antestiae, Hadronotus antestiae, Asolcus seychellensis, Asolcus Mopsus,* and *Asclcus suranus. Furth*ermore three species of the genus *Aridelus, Corioxenus antestiae* and *Bogosia rubens* are listed as parasites of nymphs and adults. Four species of Reduvide and unidentified mantid are also reported as predators of Antestia *(9)*. The occurrence of *Corioxenes antesties* and *Bogosia rubens*,, in Ethiopia, attacking adults of *A. intricata*_ has already been reported (5) but nothing is mentioned about egg parasites. It was, therefore, the objective of this study to collect and identify parasites of the different developmental stages of Antestia and determine their contribution to the natural control of this pest and assess the possibility of further reducing Antestia population using introduced parasitoids from neighbouring coffee growing countries of Africa

MATERIALS AND METHODS

From past records, it has been shown that Antestia population reaches its peak during May/June period (1). Egg collection, therefore, started in March and continued until August. Antestia egg batches were collected from the main center (Jima) and two subcenters, Metu, and Mugi situated at an altitude of 1750, 1440,and 1300 masl respectively. Antestia egg batches were collected every week from Coffee plants growing under shade. Individual egg batches were then placed in a test tube, plugged with cotton, and kept in the laboratory under normal conditions. The maximum and minimum temperatures in the laboratory were also recorded. Emerging parasites were left to starve and die which were then identified and counted. Number of un hatched eggs and eggs from which nymphs emerged were also recorded. Some literature has also been reviewed to asses the possibility of classical biological control of Antestia.

RESULTS AND DISCUSSION

Table 1. Identity and relative abundance of the different species of Antestia egg parasites by location and accross location.

No.	Species	Relative Abundance (%)			
		By location			Across location
		Jimma	Mugi	Metu	
1	*Asolcus suranus*	80.5	40.0	79.5	66.7
2	*Anastatus antestiae*	11.0	54.0	11.0	25.3
3	*Hadronotus antestiae*	8.5	1.5	0.0	3.3
4	*Pediobius spp.*	0.0	4.5	9.5	4.7

The different species of parasites collected and identified, and their relative abundance is shown in table 1. At all locations, except Mugi, *Asolcus suranus*, is the most abundant followed by *Anastaus antestiae* and *Hadronotus antestiae*. Anastatus antestiae on the other hand was more common than the other two species at Mugi. *Pediobius sp.*, a secondary parasite, was collected at all location except Jimma. Both *A. orbitalis ghesquierei* and *A.intricata* are attacked by *Asolcus suranus* (13) and *Anastatus antestiae*(4). Similarly *Hadronatus antestiae* breeds on all species of Antestiopsis particularly *A.intricata* (3) which is also the finding in this study with reference to *A.intricata*. 46, 53 and 57% of the eggs collected from Jima (Melko), Mugi, and Metu respectively were attacked by the three species of parasites combined (table 1). Some studies in Kenya have shown percent parasitism of eggs ranging from 63 to 90% (9). This is very much higher than what has been observed from this study which may be due to the fact that there are more species of parasites in Kenya than are found in Ethiopia. The fact that Anastatus antestiae was more common at Mugi may have something to do with altitude since Mugi is situated at lower altitude than the other two locations

The number of egg parasites, Nymphs, and unhatched eggs recorded from eggs collected from the field at each of the three locations is shown in table 2.

Table 2. Total number of eggs, number and percent parasites, nymphs and unhatched eggs.

Location	Eggs	Parasitoids	Nymphs		Unhatched Eggs		Percent Eggs attacked
			Number	Percent	Number	Percent	
Jimma (Melko)	5169	2335	1884	36	950	18	46
Mugi	539	285	125	24	126	23	53
Metu	1875	1082	527	28	266	14	58

A. orbitalis Bechuana, *A. facefoids* and *A.intricata* are known to be attacked by *Bogosia rubens* and *C. antestiae* the latter role in reducing antestia population being through the reduction of fecundity of its host (9).In Ethiopia they attack only 5% of Antestia adults(1) They are, therefore, so rarely found that their influence on the population of Antestia is minimal. No other nymph or adult parasites exist in Ethiopia.
In most cases only one species of parasite emerged from a single egg batch. In few instances two species were reared. For example *Handronotus antestiae* and *Anastatus antestiae* were obtained from one egg batch. It has been observed that Hadronatus antestiae females do not avoid eggs which have been already parasitized (2) confirming the result obtained in this study. Three egg batches each containing both *Anastatus antestiae* and *Asolcus suranus* were also observed.
Neither parasitoids nor nymphs has hatched from some of the eggs which is shown in table 1 to be 18, 23 and 14% for Jimma, Mugi and Metu respectively. This could be due to eggs laid unfertilized or unsuccessful development of nymphs or parasites. Field collected eggs of A.orbitalis bechuana in Kenya resulted in 4 to 20% un hatched eggs(2). The specific factors and their contribution to such a level of un hatchability of eggs should be investigated to make use of it in any pest management strategy.

POSSIBILITY FOR CLASSICAL BIOLOGICAL CONTROL

There are a few instances in which natural enemies have been introduced from one African country to another or within a country. Parasites of *planococcus kenyae* introduced from Uganda in 1938 reduced the previously damaging pest into a minor pest (8). *Corioxenos antestiae* established itself after being introduced from western Uganda to Toro (12) while its establishment as a result of its introduction from Tanzania to Kenya has not been confirmed. Although the percentage of Antestia eggs attacked by parasites is high, it is still less than the 63 to 93% egg parasitism recorded in Kenya (2). Furthermore, there are now at least seven species of egg parasites recorded in African coffee producing countries compared to three in Ethiopia(Table 3) . It may, therefore, be worth introducing some of the species of egg parasites such as *Asolcus mopsus*, *Asolcus seychellensis*, *Acroclisoides africanus* and *Ooencyrtus sp* not occurring in Ethiopia. From earlier findings, the two parasites(*Bogosia rubens ans Corioxenos antestiae*) of the nymphs and adults are not effective in reducing Antestia population. On the other hand there are three species of parasites of Antestia nymphs and adults belonging to the genus *Aridelus,* found in some African countries(table 4) but absent from Ethiopia. Particular attention should, therefore, be given to specifically *Aridelus coffeae* and *Aridelus taylori* that are known to attack *A.intricata* (11) for possible introduction to Ethiopia.

Table 3. Parasites of eggs of Antestia recorded in Africa.

Species	Species of Antestia attacked **	Countries recorded from	Ethiopian situation**
1. Antastatus antestiae	A, B, C	Kenya, Uganda, Congo, Rwanda, Cameroon	P
2. Hadronotus antestiae	B, C	Kenya, Uganda, Congo, Rwanda, Cameroon	P
3. Asolcus suranus	B, C	Uganda, Tanzania	P
4. Asolcus mopsus	B, C	Uganda, Cameroon	NP
5.Asolcus seychellensis	A, B, C, D	Kenya, Tanzania, Uganda, Seychelles, Malawi, Cameroon	NP
6.Acroclisoides africanus	A, B, C, D	Kenya, Uganda, Congo, Rwanda	NP
7. Ooencyrtus sp.	A	Kenya	NP

Adapted from Lepelley 1968

- A = *Antestiopsis orbitalis bechuana*
- ** NP = Not present (B = *A.. orbitalis* Ghesquierei Carayon)
- P = Present (C = *A. intricata* (Ghesquiere and Carayon) D = *A. facetoides* Greathead)
-

Table 4. Parasites of nymph and adults of Antestia recorded in African countries.

Species	Species of Antestia attacked*	Stages attacked**	Countries reccorded from	Ethiopian situation
1. Aridelus coffea	A, B	N, AD	Uganda,Rhodesia, Tanzania, Kenya	Not recorded
2. Aridelus rafur	A,D	N	Tanzania	" "
3. Aridelus taylori	B	N	Uganda	" "
4. Bogosia rubens	A, B, C	AD, n	Kenya, Uganda, Tanzania, Rwanda	Recorded on adults
5.Corioxenos antestiae	A, B, C, D	N, AD	Tanzania, Uganda	" "

Adapted from Lepelley 1968

- A = *Antestiopsis orbitalis bechuana*, B = A. *orbitalis* ghesquierei Carayon, C = *A. intricata* (Ghesquiere and Carayon) and D = *A. facetoides* Greathead
- AD = Adult
- ** N = Nymphs (n = occasionnally nymphs)

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PRESELECTION METHODS FOR COFFEE BERRY DISEASE RESISTANCE IN ETHIOPIA

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SUMMARY

Studies were conducted to determine as to which one of the pre-selection methods was the most reliable. Field score using visual assessment and berry count techniques, detached berry test (DBT) and seedling tests were made on progenies of 1982 and 1985 selections.
In detached berry test (DBT) berries from progenies of test selections were inoculated with conidial suspension. Boxes were closed after inoculation. Nine days later, the number of diseased berries were recorded. In the seedling test six weeks old seedlings were inoculated with spore suspension. Four days later seedlings were uncovered and placed in growth room for three weeks after which number of infected seedlings were recorded. In DBT significant differences were observed between treatments and positive correlation (r=0.624) and negative correlation's were observed with visual assessment and yield respectively. In seedling test 75% were in class 4, 5% in class 1 and only 2.8% were healthy. Weak correlation (r=0.268) and (r=0.254) for berry count and visual assessment respectively was observed between seedling test and field scores.
Field score technique using visual assessment was subject to human error and tended to under estimate or overestimate the actual loss. Berry count was found to be relatively better field score technique. During DBT berries are removed from their natural habitat and hence normal metabolism of berries could momentarily be disturbed making the method unreliable. On the other hand, if temperature and humidity are properly controlled, seedling tests are not mostly biased by environmental conditions and hence together with berry count, should be used as the most reliable technique.

POSTER

INTRODUCTION

Coffee berry disease can be controlled by the use of resistant varieties, spraying with recommended fungicides and by cultural practices.
Resistant varieties play an important role in combating CBD. Development of resistant varieties save the nation valuable foreign exchange spent on fungicides, fuel and spray machinery and further more avoids the hazardous effect of pesticides on the environment.
The exhaustive testing of selected materials for resistance to CBD in the mother trees and their progenies in the laboratory and in locations where the epidemic is not only severe but regularly so, is very vital.
Different pre-selection methods are employed to screen coffee types for CBD resistance. This include detached berry test, seedling hypocotyl test which are both conducted under laboratory conditions field

scoring methods which include visual assessment and berry count are also practiced to screen various coffee types for CBD resistance.
This paper reports comparison of laboratory and field methods viz. DBT, seedling test and field score based on results of the tests carried out on progenies of 1982 and 1985 selections.

MATERIALS AND METHODS

1. Field Score

Visual assessment:- to estimate the level of CBD under field conditions the percentage of diseased berries on individual trees were assessed.
Berry count:- Branches of trees to be tested were marked and the number of berries per branch was recorded. Individual branches of a tree were used as replications. Number of healthy and diseased berries were recorded every three weeks six times through out the crop season.

2. Detached berry tests (DBT)

Two hundred fully expanded berries were collected from 25 progenies of each test selection. Berries were surface sterilized in laundry bleach for five minutes and washed in distilled water. Fifty berries were arranged in a plastic box lined with tissue papers, to maintain a high level of humidity a foam plastic with hollows was used as a substrate. Inoculum was prepared from diseased berries with active lesions collected in the field. The berries were inoculated by placing a drop of suspension of CBD conidia on each berry. Inoculum density was $2.5x10^5$ conidia ml^{-1}. The boxes were hermetically closed after inoculation to maintain the high relative humidity needed for infection and symptom development. Completely randomized design with four replications was used. After nine days the plastic boxes were opened and the number of diseased berries were recorded. For calculation, the fractions of diseased berries were angularly transformed (Vander Graaf, 1982).

3. Seedling hypocotyl testes

One hundred fifty seeds were collected from each progeny of the test selections The parchment was removed and the seeds were soaked in sterilized distilled water and kept for 48 hours, after which they were planted on sterilized moist sand in plastic boxes. The design was completely randomized design (CRD) with four replications. The test was carried out with modification of the stem (hypocotyl) inoculation technique used by van der vossen et al (1976). Spore suspensions ($2x10^6$ conidia ml^{-1}) were prepared from four weeks old culture on PCA slants in universal bottles. Diseased berries were used as inoculum sources and were collected from the field.
Inoculations were done by wrapping strips of absorbent cotton wool dipped in inocula round the seedlings, behind the cotyledons. Seedlings were watered Just before inoculation, control seedlings were treated with sterile distilled water. After inoculation the trays of inoculated seedlings were covered with closely fitting plastic sheets. They were kept for four days and relative humidity was maintained at high level (100%). After four days the seedlings were uncovered and the stripes of cotton wool carefully removed before placing the trays in a cooled illuminated temperature controlled growth room at 19-20°C for the next two to three weeks (Hakiza,1985).Time taken for seedlings to develop symptoms at the point of inoculation was recorded. At the end of three weeks number of infected seedlings were recorded. Data were transformed before being analyzed.

RESULTS AND DISCUSSION

Detached berry test results field scores and yield of test selections are given in table 1
Accordingly, some individual coffee lines showed reduced level of infection, in the DBT when compared with the standard check. Significant differences were observed between the treatments and the standard control at 5% level and highly significant differences were observed between the treatments and the susceptible control at 1% level. Most selections, with low disease levels in DBT showed similar trends in the field score. Significant differences in yield both at 1% and 5% levels were also observed among the selections and the controls. The standard control and two other selections gave relatively high yield while the susceptible control gave the lowest yield level (table 1).

Table1 - Mean results of Detached berry test, field score and yield (kg/ha) on test progenies. (mean of 3 years).

Selection	DBT	VA	Yield	Selection	DBT	VA	Yield	Selection	DBT	VA	Yield
1	31.30	7.86	828.8	**10**	16.09	0.11	752.0	**19**	39.76	0.01	-
2	46.01	12.23	537.6	**11**	18.47	0.45	760.0	**20**	40.01	17.21	-
3	41.75	18.62	480.0	**12**	39.65	9.65	846.4	**21**	33.68	3.75	-
4	36.87	0.16	627.2	**13**	31.22	0.69	982.4	**22**	28.00	3.61	723.2
5	38.32	3.75	500.8	**14**	31.50	9.48	673.6	**23**	38.08	2.15	353.6
6	26.66	1.97	796.8	**15**	57.37	28.49	539.2	**24**	28.76	6.75	534.4
7	27.26	1.03	724.8	**16**	24.60	9.18	982.0	**25**	39.67	3.35	694.4
8	39.54	3.99	768.0	**17**	29.82	1.03	761.6	****26**	59.55	17.86	299.2
9	33.38	24.22	484.8	**18**	24.30	4.02	1011.2	***27**	33.42	0.00	957.6

STATISTICS	Mean	LSD 0.05	LSD 0.01	C.V%
DBT (detached berry test)	**34.45**	**10.22**	**13.72**	**16.59**
VA (: visual assessment)	**7.02**	**12.17**	**16.25**	-
Yield	**692.90**	**152.00**	**203.20**	**23.69**

Positive correlation (r=0.624) was observed between detached berry test and visual assessment while a negative correlation (r=-0.596) was observed between DBT and coffee yield at the same time, a negative correlation (r=-0.582) was observed between visual assessment and coffee yield. (Table.2).

Table 2. r-values between DBT , field scores and yield on test selections.

	V.A	Yield
DBT	0.624	-0.596
Yield	-0.582	

Frequency distribution analysis for the DBT indicated that most selections lie between 26-31 and 38-43% CBD infection (fig. 3) like wise, for visual assessment the distribution shows that quite a good number of selections are between 0 and 2 % disease level (fig.4) on the other hand, three selections gave yields between 900-1010 k.g/ha (fig.5).

Percent infected seedlings on a 0-4 score scales is given in Table 3. Accordingly, about 75% of the selections were in class 4 in which all seedlings were dead, 17% of the selections were in class 2 and 3 score scales infected seedlings under these classes showed deep black lesions covering a large proportion of the stem. Most seedlings died when the lesions coalesced 5% of the selections were in class 1 and showed scabs and brown superficial lesions on the hypocotyl only 2.8% of the seedlings were healthy and showed no symptoms on hypocotyls.

Table 3. Infection level of selections on 0-4 score scales in seedling test (% infected seedlings)

Score scale	1996	1997	1998	mean	sd
0	1.76	3.28	3.29	2.77	0.77
1	5.70	3.72	6.40	5.27	1.39
2	7.78	6.43	10.74	8.32	4.46
3	3.67	9.97	12.29	8.65	4.46
4	81.09	76.6	67.28	74.99	7.04

In the field score using visual assessment frequency distribution analysis indicated that most selections showed CBD level between 1 and 7 and only two selections showed relatively high level of CBD which was between 53 and 70% (fig. 1). In the berry count test the highest frequency of selections showed disease levels between 0 and 1 %. On the other hand only one selection showed between 26-29% level of CBD infection (fig.2). In the seedling test, differences between seedlings obtained from the different selections were significant both at 1% and 5% levels, 19.31 and 14.61 respectively. At the same time low coefficient of variation (13.11%) was also observed in the test. Correlation analysis showed very week positive correlation between seedling test and berry count (r=0.268) and between seedling test and visual assessment (r=0.254). On the other hand reasonably fair level of positive correlation was observed between VA and BC in the field score (Tab. 4).

Table 4. r-value between seedling tests and field scores in test selections

	VA	BC
Seedling test	0.254	0.268
VA	-	0.631

BC- berry count

Inoculating seedlings by wrapping strips of absorbent cotton wool dipped in inocula round the seedlings Just below the cotyledons, has been observed to give better results than inoculating seedlings by spraying, using a hand sprayer. However, care should be taken in maintaining the uniformity in size of the absorbent cotton wool. If variation occurs, the amount of inoculum absorbed will vary which may consequently affect the resistance level of seedlings. An increase in the amount of inoculum could be wrongly interpreted and may give false impression of susceptibility.

In conclusion During the DBT berries are removed from their natural habitat and hence normal berry metabolism could momentarily be disturbed. This may require the use of techniques which could make the detached berries alive during the process of testing. Which, unfortunately is not currently practiced. This makes the DBT a non viable option. Consequently, the use of DBT as a pre-selection method is controversial.

Seedling hypocotyl tests which are not mostly biased by environmental conditions enable quite a good number of coffee types to be screened in the lab. However, care should be taken in controlling temperature and humidity Hence, together with berry count technique in the field they should be used extensively. This could result in the obtention of significant amount of CBD resistant coffee types selected from various regions and which could be provided to coffee growers in many parts of the country.

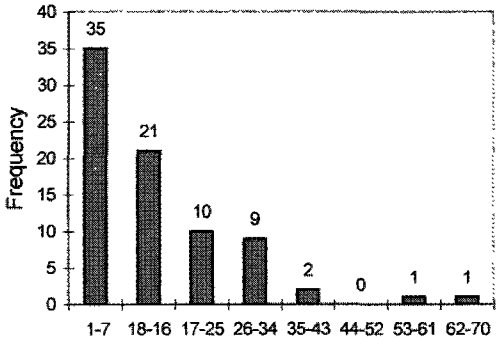

Fig. 1.Frequency distribution of 1985 Sidamo selections in visual assessment in the field.

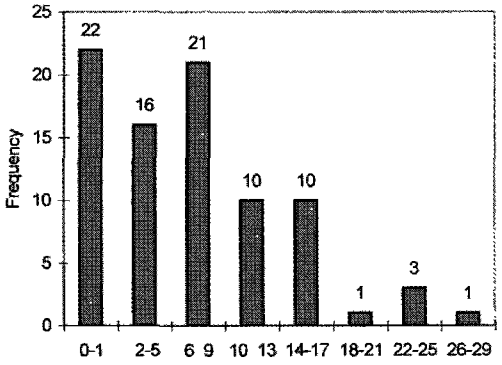

Fig. 2. Frequency distribution of CBD infection on 1985 Sidamo selections in berry count in the field.

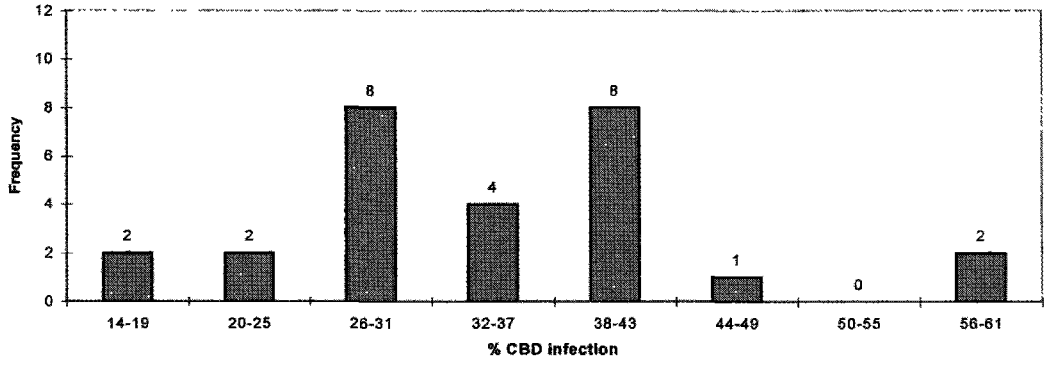

Fig.3 Frequency distributin of 1982 selections in DBT in the laboratory

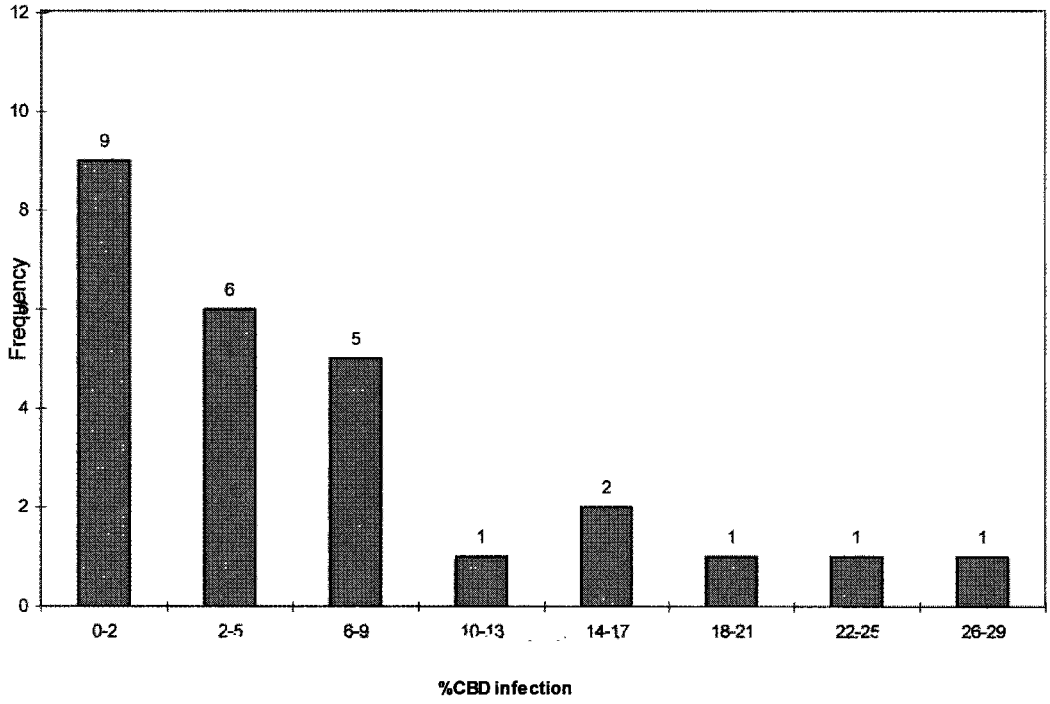

Fig.4 Frequency distribution of 1982 selections in visual assessment in the field at Gera

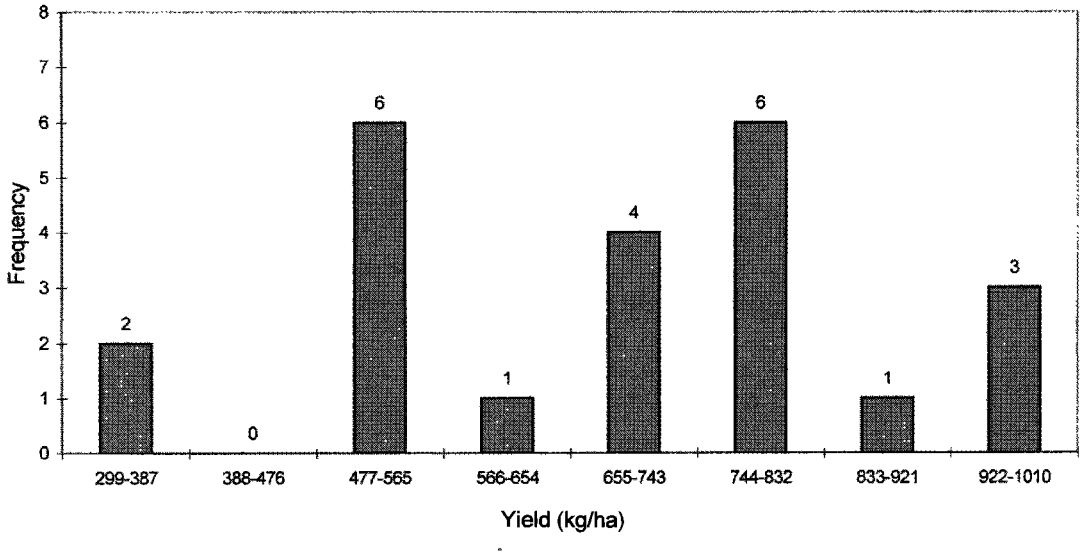

Fig.5 Frequency distribution of 1982 selections in yield

ACKNOWLEDGEMENT

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PROTEOLYTIC ENZYME ACTIVITY IN *Coffea arabica* VARIETIES VARYING IN RESISTANCE TO COFFEE BERRY DISEASE

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SUMMARY

Proteolytic enzyme activity of tissue homogenates and intercellular fluids of six Arabica coffee varieties was investigated using cupplate assay method with gelatin agar medium. Tissues used were hypocotyls, attached and detached green berries. Inoculation increased the activity in all tissues except in attached berries of varieties Catimor, Rume Sudan and Ruiru 11 which exhibited resistance. Increase was however less in resistant varieties.

POSTER

INTRODUCTION

Coffee berry disease (CBD) is one of the most serious Arabica coffee disease in Africa [1]. The processes that lead to susceptibility or resistance to disease need be understood in order to optimise their control [2]. On aspect in CBD pathogenesis is the role of degradative enzymes and the role of this study was to study the role of proteolytic enzymes.

Proteolytic enzymes degrade proteins and are the equivalent to peptide hydrolase [3] and can degrade materials like gelatin. These together with other cell wall degrading enzymes may be instrumental in infection of plants by pathogens [4] because proteins are found in plant cell walls, middle lamellae and plasma membranes. They may also inactivate plant enzymes or enzyme inhibitors of protein nature which are involved in resistance [5]. If of plant origin, these enzymes may inactivate pathogen enzymes or deaminate free amino acids to release ammonia which may be toxic to invading agent [6].

The objective of this study was to determine the changes in proteolytic activities in susceptible and resistant CBD reactions.

MATERIALS AND METHODS

Arabica coffee varieties of varied CBD resistance i.e. susceptible (SL 28, SL 34) medium resistance (K7, Catimor) and resistant (Rume Sudan, Ruiru 11) were used. Seedling hypocotyls, detached and attached green berries were inoculated with a single spore isolate of *C. kahawae* by the methods of Van der Vossen et al [7].

Hypocotyls and attached berries were sampled weekly and detached berries at 2 day intervals. Controls were treated with distilled water.

Tissue homogenate extracts were prepared from berry pulp and top half of hypocotyls by crushing 2g in 2 ml and 1 ml respectively of Tris-HCl buffer (0.05 M, pH 7.5), using prechilled mortars and pestles. The crushed material was centrifuged at 18,000 g for 30 min at 4°C. The supernatant was stored in deep freezer (20°C) until used. Intercellular fluids were prepared by infiltrating 7 mm pieces of

hypocotyls with sterile distilled water *in vacuo,* blotter drying and centrifuging them at 9000 g for 10 minutes at 4°C [8]. The fluids were stored at –20°C until used.

Proteolytic activity was assayed by the method of Dingle et al [9]. The extracts were sterilized by filtration using 0.22pm filter membranes, 50pl aliquotes placed in wells in 1% gelatin agar medium and incubated at 370C for 20 hours. Digestion zones were developed by flooding with mercuric chloride. Where gelatin was totally, partially or undigested, the zones around the wells, appeared clear faint and opaque white respectively. Trypsin (20 mg/ml) was used as standard and its digestion zone diameter assigned 100 enzyme units. Sterilized water and buffer were used as non-enzymatic controls. The digestion zone diameters of the samples were converted into enzyme units in relation to those of trypsin per experiment.

RESULTS

There was an increase in proteolytic activity in hypocotyl homogenates of all varieties after inoculation but the increase was greater in susceptible varieties observed as larger and clear zones (Table 1). Aging also caused slight increase in noninoculated samples. The activity in intercellular fluids was observed only in susceptible varieties (Table 2) and was less than in tissue homogenates. No activity was observed in noninoculated samples. In attached berries, proteolytic activity was observed in varieties SL 28, SL 34, and K 7 but in K7 (medium resistant) it occurred one week later (Table 3). In detached berries, the pattern was similar but there were faint zones in non-inoculated and resistant varieties starting between the 6th and 8th days. There were significant differences (P = 0.05) enabling differentiation of susceptible from susceptible varieties.

Table 1: Proteolytic enzyme activities of tissue homogenate extracts of coffea arabica varieties inoculated and non-inoculated with colletotrichuin kahawae. Digestion zone diameters were converted as fractions of those of Trypsin which were assigned arbitrary values of 1 00 enzyme units.

Variety	Treatment	Weeks after inoculation					Variety Means
		0	1	2	3	4	
SL34	N	0	0	0	35.99 F	35.99 F	14.67 F
	I	0	0	47.58 C	48.87 C	58.75 C	31.04 C
SL28	N	0	0	0	0	40.73 F	8.15 F
	I	0	0	48.23 C	50.15 C	69.04 C	33.56 C
Catimor	N	0	0	0	39.85 F	40.05 F	15.98 F
	I	0	0	34.25 F	45.02 F	40.73 F	24.00 F
K7	N	0	0	0	0	30.72 F	6.14 F
	I	0	0	35.51 F	44.37 F	41.37 F	24.25 F
Rume Sudan	N	0	0	0	0	33.36 F	6.67 F
	I	0	0	0	42.44 F	46.74 F	17.84 F
Ruiru 11	N	0	0	0	0	32.04 F	6.41 F
	I	0	0	0	36.65 F	38.73 F	15.08 F
Time means	N	0	0	0	12.64	35.71	
	I	0	0	27.60	44.58	49.29	
Trypsin	100 C	100 C	100 C	100 C	100 C	100 C	

Key : C=clear, F=faint, N=non-inoculated, I=Inoculated

LSDs (P=0.05)

	Non-inoculated	Inoculated
Variety	1.041	2.301
Time	0.950	2.101
Variety x Time	2.327	5.145

Table 2: Proteolytic enzyme activity of intercellular fluids of hypocotyls Coffea arabica varieties inoculated with C. kahawae. Digestion zone diameters were converted to fractions of those of Trypsin which were assigned 100 enzyme units.

Variety	Weeks after inoculation					Variety Means
	0	1	2	3	4	
SL34	0	0	34.48 C	36.78 C	36.78 C	21.61
SL28	0	0	35.65 C	37.39 C	36.53 C	22.00
Catimor	0	0	0	0	0	0
K7	0	0	0	0	0	0
Rume Sudan	0	0	0	0	0	0
Ruiru 11	0	0	0	0	0	0
Time means	0	0	11.67	12.45	12.22	
Trypsin	100 C	100 C	100 C	100 C	100 C	

Key : C=clear zone
LSDs (P = 0.05)

Varieties	0.433
Time	0.396
Variety x Time	0.969

Table 3:Proteolytic enzyme activity of pulp (pericarp) homogenates of attached green *Coffea arabica* benies inoculated with *Colletotrichum kahawae.* Digestion zones were converted as fractions of those Trypsin which were assigned 1 00 enzyme units.

Variety	Weeks after inoculation					Variety Means
	0	1	2	3	4	
SL34	0	0	38.63 C	46.47 C	34.63 C	32.18
SL28	0	0	40.39 C	46.82 C	42.27 C	32.37
Catimor	0	0	0	0	0	0
K7	0	0	0	35.14 C	38.68 C	18.50
Rume Sudan	0	0	0	0	0	0
Ruiru 11	0	0	0	0	0	0
Time means	0	0	13.17	21.41	20.79	
Trypsin	100 C	100 C	100 C	100 C	100 C	

Key : C=clear zone
LSDs (P = 0.05)

Varieties	1.022
Time	0.835
Variety x Tirne	2.045

DISCUSSION

There was limited proteolytic activity in non-inoculated tissues and this increased with age of hypocotyls. This has been reported in other crops as well (6). Inoculation of the coffee varieties increase their proteolytic activity and more so in susceptible reactions thus exhibiting a direct relationship with disease severity and this occurs in other diseases also (6). Proteolytic enzymes therefore play a role in the infection of *C. arabica* by *C. kahawae.* more studies are required to define the source of the enzymes (fungal or host), characterization, active role and the possibility of their use in tests for CBD resistances.

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TECHNIQUES FOR SCREENING RESISTANCE TO COFFEE BERRY DISEASE (*Colletotrichum kahawae* Waller & Bridge)

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INTRODUCTION

In Tanzania Coffee Berry Disease is a major problem. Thus screening coffee hybrids for CBD resistance is essential for research. The efficacy of various techniques is presented.

MATERIALS AND METHODS

The standard suspension used for inoculations is 2.0 x 10^6 conidia/ml of sterile water. Double inoculations are made at 48 hours interval.

Pre-selection: hypocotyl tests

The best stage for seedling inoculation is 5 - 6 weeks after germination. Temperature is maintained at 20-21°C and relative humidity at 100%. Reaction is assessed after 21 days using a 0-4 scale (Table 1). Disease Index Reaction (DIR) is calculated from this assessment.

- Spraying method: A double inoculation is applied through atomisation. Reaction appears after 10 days.
- Dipping method: The seedlings are uprooted then dipped twice in the spore suspension. They are laid in plastic boxes and kept in the dark for 48 h. The roots are installed between two layers of paper tissue to minimise drying. (Fig.1). Symptoms on hypocotyl appear on the 6th - 7th day.
- Disc method: The seedlings are arranged in plastic boxes as for the dipping method. Each seedling is inoculated by placing a disk of mycelium on the hypocotyl.

Tests on mature plants

Branches with 4 - 6 clusters of soft green berries (right stage for inoculation) are selected.

- Attached Berries (AB): When the weather is conducive for infection each branch is enclosed in a polythene sleeve. Inoculation is done twice, in the evening to allow the infection process. Scores are taken after 14, 21 and 28 days by recording active and scab lesions.
- Semi-Detached Berries (SDB): Branches are cut in the field. Each branch is inserted in a flask with tap water. The fruits are inoculated twice. Reaction is assessed from 10 days after the second inoculation at 4 days interval.
- Detached Berries (DB): Surface sterilised berries placed on moist sterilised sand in plastic boxes are inoculated twice. Reaction is recorded 7 days after the second inoculation.

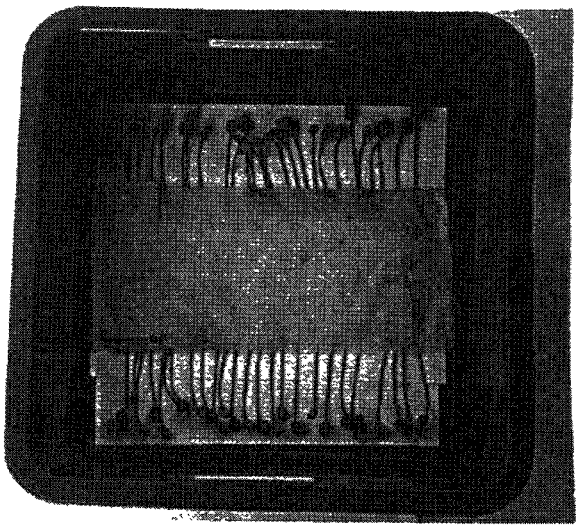

Figure 1: Seedlings under Dipping test

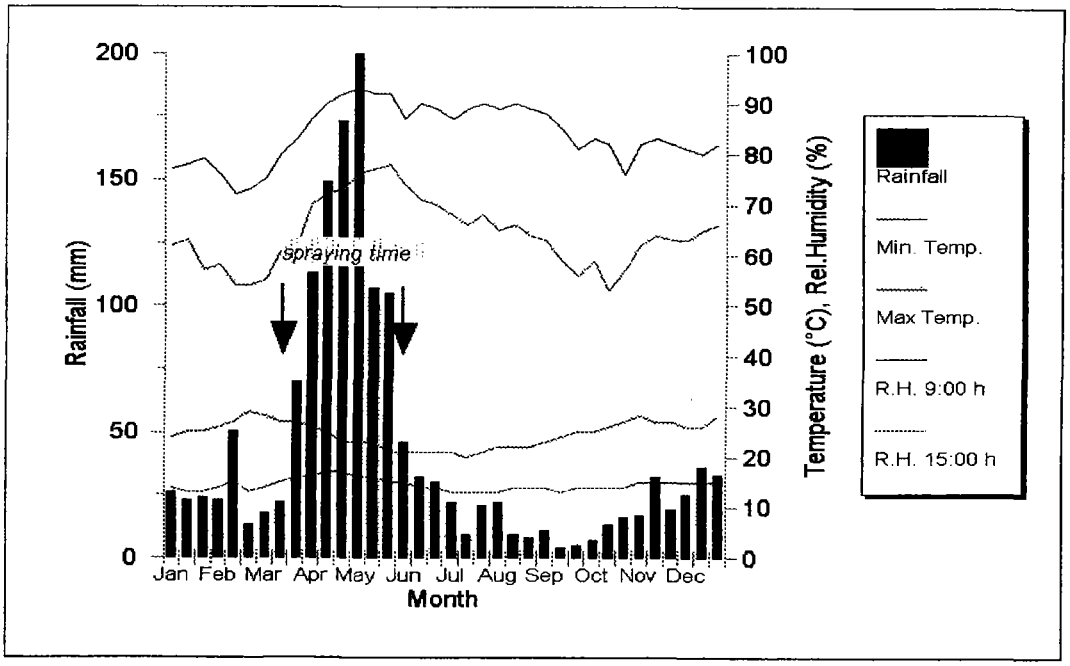

Figure 2: Meteorological data, Lyamungu

- Natural infection: Sixteen hybrid progenies and a susceptible control were established in a major coffee zones. CBD resistance was assessed using a 1-6 scale, according to the proportion of dominant lesions (scabs and active).

RESULTS AND DISCUSSION

Results are presented in Table 2

Pre-selection tests

Spraying method allows to screen large numbers of seedlings. The amount of inoculum per seedling is difficult to control. The selection pressure is not very high. Dipping method requires smaller numbers of seedlings. Reaction is quick and creates much higher selection pressure. Disc method has an impact similar to spraying method. Reaction is slow. It also requires small numbers of seedlings.

Mature bearing plants

- Attached Berries (AB): Reflects true conditions. Weather and berry susceptible stage should coincide (Fig. 2). Therefore flowering dates have to be recorded. More effective than natural infection.
- Semi-Detached Berries (SDB): Used provided there are enough berries at susceptible stage. Can accommodate large quantities. Efficiency comparable to AB technique.
- Detached Berries (DB): Berries deteriorate fast. Results are not consistent with other techniques.
- Natural infection: The weather has to be conducive for CBD. Disease escapes may happen. However meaningful results can be obtained after several years. The control variety clearly scores higher than all hybrids.

RECOMMENDATIONS

Integrating several techniques allows getting reliable information on CBD resistance. DB technique appears the least reliable. Further investigation is still needed before choosing the most efficient techniques.

Table 1: Assessment scale

0: Resistant, no symptoms
1: Moderately Resistant, small greenish/brownish lesions < 0.5 mm
2: Moderately Resistant, lesions > 0.5 mm
3: Susceptible, large brown/black lesions, tip remains green
4: Susceptible, black lesions around stem, brown tip

Table 2: Comparison of CBD screening techniques

Parents/Variety	Pre-selection Technique DIR *					Artificial Inoculation ** Mean % Infected berries						Natural infection *** Scale 1 - 6					
	Dip. 1	Spr.1	Dip.2	Spr.2	Disc	AB		SDB		DB		LARI		South		North	
RS x HDT	78	23				2,2	s	3,8	s	0,0		1		1		1	
RS x HDT	58	16				0,4	s	0,5	s	13,8	s	1		1		2	s
RS x HDT						0,4	s	3,9	a	8,6	s	1		2	s	2	s
Kaffa x KP423						3,7	s	3,7	a	7,5	a	1		1		1	
N39 x HDT	57	30	66	9	10	3,8	s	4,2	s	5.0	a	1		1		1	
N39 x RS	63	48				4,8	a	0,5	s	3,3	a	1		1		1	
H66 x HDT						3,1	s	0,0		0,0		1		1		1	
KP423 x HDT						3,4	s	0,9	a	12,5	a	1		1		1	
(N39 x HDT) x RS	66	28				0,7	s	1,1	a	1,3	a	1		1		1	
(N39 x HDT) x Illubabor						2,2	s	0,0	s	1,3	a	1		2	s	1	
(N39 x Kaffa) x (RS x HDT)	67	40															
(N39 x {N39 x Geisha}) x N39	73	72															
Kaffa x (N39 x {N39 x Geisha} x HDT)						0,3	s	0,0		6,3	a	1		1		1	
({N39 x Kaffa} x {RS x HDT}) x HDT						0,5	s	0,0	s	22,5	a	2	s	1		1	
(N39 x {N39 x Geisha}) x (HDT x Illubabor)	62	23				1,1	s	0,0	s	0,0		1		1		1	
([N39 x {N39 x Geisha}] x HDT) x N39						1,9	s	0,1	s	0,0		2	s	1		1	
([N39 x {N39xGeisha}] x HDT) x KP423						0,0		0,0		0,0		1		1		1	
(HDT x N39) x ({N39x [N39xGeisha]}xHDT)	68	52				4,2	s	0,1	s	18,8	a	1		1		1	
N39 Bourbon type	100	93	83	87	43	35,9	as	50,2	a	47,5	a	4	as	3	a	3	a
K7 (VCE966)			93	1	29												
Hibrido de Timor CIFC 1343 (HDT)						0,0		4,8	s	4,4	s	-		-		-	
HDT (VCE1589)			25	9	8												
HDT (VCE1591)			55	5	16												
HDT (VCE1594)			50	1	1												
Rume Sudan (RS)						0,0		3,7	s	1,3	s	-		-		-	
RS (VC298)			33	6	7												
RS (VC506)			28	3	13												
RS (VC510)			36	2	12												
Mean	69,2	42,5	52,0	13,7	15,5	4,0		4,1		8,7		1,3		1,2		1,2	

Correlations: Dipping1/Spraying1. r = 0.76, Dipping2/Spraying2: r = 0.45, Dipping2/Disc: r = 0.74
AB/SDB: r = 0.97 AB/DB: r = 0.81 SDB/DB: r = 0.80

Dipping1: CIRAD; Dip.2, Spraying1 & 2, Disc: ARI Lyamungu (LARI) s = scabs, a = active lesions

* Disease Index Reaction: calculated from 0 - 4 assessment Key:0 - 25 Resistant, 26 - 50 Moderately Resistant 51 -75: Moderately Susceptible, 76 - 100: Susceptible

** Key (Fernie, 1963, Lyamungu): 0 - 5 Resistant, 6 - 20 Partially Resistant, 21 - 39 Moderately Susceptible, 40 – 100: Highly Susceptible

*** Key (Ngulu & Kilambo, 1993): %branches, types of lesions: 1 Nil infection, 2: <25%, scabs only, 3: <25%, active predominant, 4: <50%, actives and scabs, 5: <75%, actives and scabs, 6: >75%, actives and scabs

EVALUATION EN CHAMP DE L'EFFICACITE DE CERTAINES FORMULATIONS DE FONGICIDES VIS-A-VIS DE L'ANTHRACNOSE DES BAIES DU CAFEIER ARABICA

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INTRODUCTION

A cause de sa grande adaptation à un climat d'altitude, le caféier arabica est cultivé au Cameroun dans les hauts plateaux des provinces de l'Ouest et du Nord-Ouest entre 1000 m et 2000 m d'altitude dans des exploitations de type familial. Dans cette zone à forte pluviométrie (3000 mm d'eau par an) et notamment sur une altitude comprise entre 1900 et 2000 m. cette culture est sujette à des attaques d'une grave maladie, l'anthracnose des baies. autrement appelée en anglais, coffee berry disease (CBD) due *à Colletotrichum coffeanum Noack-* qui occasionne des pertes de récolte considérables pouvant atteindre 80 à 90 % de la production totale des vergers en absence de tous traitements chimiques.

Cette situation est courante depuis que le pays est en crise économique caractérisée par la baisse des prix des matières premières dont le café. la dévaluation du franc CFA, et l'augmentation des prix des intrants agricoles qui font que les planteurs ne parviennent plus à acheter les fongicides pour protéger leurs récoltes. Ceci explique en grande partie la diminution de la superficie du verger au profit des cultures vivrières plus rentables et par conséquent la chute de la production d'année en année.

Ces grandes pertes de rendement sont dues au fait que dans la majorité des exploitations familiales de la région, le cultivar couramment rencontré est la variété Jamaïque reconnue pour sa sensibilité au CBD. La variété Java proposée aux planteurs par la recherche et qui est moins sensible à la maladie que la variété Jamaïque ne parvient pas encore à remplacer totalement cette dernière sur le terrain. Cette absence de variétés résistantes ou tolérantes en milieu paysan fait encore de la lutte chimique le principal et l'unique moyen connu des planteurs pour contrôler cette maladie.

Compte tenu de cette situation, le laboratoire de Phytopathologie du Centre Régional de Recherches Agricoles de Nkolbisson tout en menant des travaux sur la recherche de variétés de caféier arabica résistantes au CBD se préoccupe du screening en champ de nouvelles molécules fongicides pouvant permettre un contrôle plus efficace de la maladie.

Après une période morte. de plus de cinq ans due à la crise économique qu'on a évoqué plus haut, en 1997/1998 et en 1998/1999 ce laboratoire testé les formulations fongicides suivantes :

- campagne 1997/1998 Le Delan 700 WG (deux doses) et le FORUM R.
- campagne 1998/199 Le CHEM-COPP 50 et le CHEM-COBRE PLUS.

MATERIEL ET METHODES

Matériel végétal

Les essais étaient installés à SANTA dans l'Arrondissement du même nom, Département de la MEZAM. Province du Nord-Ouest. Ce site se trouve à 1900 ni d'altitude avec 3000 mm de pluies par an et a été retenu en raison de la très forte intensité de la maladie qu'on y a toujours observé. Le travail

s'est fait dans les exploitations familiales où le caféier arabica est en association avec les cultures vivrières et fruitières.

Dispositif expérimental

Le dispositif expérimental utilisé est la randomisation totale arbre par arbre avec 100 répétitions par objet ou traitement; chaque arbre étant une parcelle élémentaire. Chaque objet est identifiable dans le champ par des pancartes de même couleur. En 1997/1998 il y avait 5 objets soit au total 500 arbres en observation; en 1998/1999 4 objets soit 400 arbres en observation. Sur chaque arbre en observation on choisit cinq branches portant suffisamment de jeunes baies pour pouvoir de façon hebdomadaire conduire les observations pendant plus de vingt (20) semaines de la 6' semaine après la floraison jusqu'à la récolte (d'avril à décembre).

Afin de différencier d'une semaine à l'autre les chutes de nature mal connue (chutes mécaniques, physiologiques on dues aux piqûres d'insectes... etc) des chutes imputables au CBD, les baies malades sont marquées autour de leur pédoncule par un fil et suivies jusqu'à leur chute.

Fongicides testés

- Liste des formulations fongicides testées en 1997/1998

Nom du fongicide	Matière (s) active (s)	Dose
DELAN 700 WG	Dithianon (700g/lkg)	339g de pc/15 1 d'eau
DELAN 700 WG	Dithianon (700g/Ikg)	509g de pc/15 1 d'eau
FORUM R.	Dimetomorphe (6%) plus cuivre (40%)	250g de pc/15 1 d'eau
DACOBRE *500*	Daconyl Plus cuivre (25 % Chlorot. + 25% Oxychlor. Cu).	60g de pc/15 1 d'eau

- Liste des formulations fongicides testées en 1998/1999

Nom du fongicide	Matière (s) active (s)	Dose
CHEM-COPP 50	Oxyde de cuivre (50% Cu)	75g de pc/l5 1 d'eau
CHEM-COBRE PLUS	Oxyde de cuivre Plus Zinc	75g de pc/15 1 d'eau
DACOBRE 500	Daconyl plus cuivre (25% Chlorot. + 25% Oxychlor. Cu.)	60g de pc/15 1 d'eau

Ces formulations fongicides ont été comparées au Dacobre 500 pris comme fongicide de référence et à un témoin non traité.

Traitements

Le DACOBRE 500, produit de référence et le CHEM-COPP 50 est utilisé en sept (7) traitements annuels conformément à un calendrier des traitements observé au Cameroun dans la lutte contre le CBD et qui commence après la nouaison soit six semaines après la floraison des caféiers et prévoit trois traitements d'un mois d'intervalles, un traitement à trois semaines d'intervalle et trois traitements à deux semaines d'intervalle. Le DELAN 700 WG, le FORUM R. et CHEM-COBRE ont été utilisés en cinq (5) traitements annuels à la fréquence d'un traitement tous les trente (30) jours conformément aux recommandations des firmes phytosanitaires chargés de la promotion de ces produits.

Les traitements sont effectués à l'aide d'un pulvérisateur à dos à pression entretenue de manière à couvrir la totalité de l'arbre notamment les branches porteuses de fruits.

Les Observations
Elles sont hebdomadaires et précèdent les traitements. Elle portent sur le comptage du nombre des baies malades atteintes de CBD, le nombre de baies tombées des suites de la maladie, le nombre de baies saines et le nombre total de baies sur chacune des branches marquées.

Récolte
On a tenu aussi compte du poids de la récolte qui est un indice non négligeable de l'efficacité du fongicide utilisé vis-à-vis de la maladie. les récoltes ont été faites en deux ou trois fois. A chaque fois on a enregistré le poids de cerises récoltées dans les 100 arbres de chaque traitement.

RESULTATS

Mesure de l'efficacité des fongicides testés
L'efficacité des fongicides expérimentés est estimée par le pourcentage cumulé des baies malades atteintes du CBD (pourcentage de perte). Ce pourcentage est calculé par la formule suivante :

$$\% \text{ de perte} = \frac{BT_1 - BT_n \times 100}{BT_1}$$

BT_1 = Nombre de baies total au 1er comptage
BT_n = Nombre de baies total au dernier comptage.

Les résultats statistiques sont obtenus par une analyse de variance du pourcentage de baies malades. Les fongicides sont classés selon leur efficacité par le test de comparaison multiple des moyennes de *Newman et Keuls*

Résultats obtenus en 1997/1998
Ils sont illustrés par les tableaux 1, 11, III, IV ci-dessous.

Tableau I Pourcentage de perte en Fonction des traitements

Traitement	% brut	Ecart type de la moyenne
Témoin non traité	84.42 (1.20)	2.505
Delan WG(33g/15 l d'eau	67.81 (0.99)	2.518
Dacobre 500	63.76 (0.96)	2.505
Delan 7 OWG (50g/l51d'eau)	63.47 (0.95)	2.505
Forum	49.09 (0.77)	2.505

Les données entre parenthèse dans le tableau ci-dessus sont obtenues après transformation angulaire en arcsinus.

Tableau II : Analvse de variance du pourcentage de perte après transformation angulaire des données

Source de variation	SCE	DDL	cm	F	p
Traitement	9.29996448	4	2.32499112	24.28	0.001
Erreur	46.06353557	493	0.09343516		
Total	55.36350005	497	-		
CV %	31.19 %				

L'analyse de variance du pourcentage de perte montre une différence hautement significative entre les traitements. Le test de comparaison multiple des moyennes de ***Newman et Keuls*** permet de les classer par groupe homogène.

Tableau III : Classement des fongicides testés en fonction de leur efficacité.

Traitement	**% perte**	**Groupe homogène**
Témoin non traité	84.42	A
Delan 700 WG (33g/15 l d'eau)	67.81	B
Dacobre 500	63.76	B
Delan 700 WG (50g/l5 l d'eau)	63.47	B
Forum	49.09	C

Au seuil de probabilité P = 0.05, le test de comparaison multiple des moyennes de *Newman et Keuls* fait ressortir trois (3) groupes. Le témoin non traité forme un groupe distinct par rapport au reste des traitements. Les deux doses de Delan 700 WG et le Dacobre 500 (fongicide de référence) appartiennent tous les trois à un même groupe homogène, ce qui indique que ces trois traitements ne sont pas significativement différents entre eux. Le Forum présente le plus faible pourcentage de perte de fruits. Le test de comparaison de moyennes le classe dans un groupe distinct par rapport aux autres fongicides. Il montre par conséquent une efficacité significativement différente des autres traitements.

Tableau IV : Poids (en gramme) de cerises récoltées sur la totalité des 100 arbres mis en observation par traitement.

Traitements	**lère récolte**	**2ème récolte**	**3e récolte**	**Total**	**Moyenne**
Forum	19.900	23.200	50.700	93.800938	
Delan 7OOwG (5Ogll5 1)	15.000	14.200	39.800	69.000	690
Dacobre 500	16.700	12.900	28.500	58.100	581
Dela.n 70OWG (33g/15 1)	15.500	13.500	21.400	50.400	504
Témoin non traité	8.200	5.000	12.200	25.400	254

Ce tableau montre que les arbres ayant subi les pertes de fruits les plus importantes sont ceux du témoin non traité avec seulement 25,4 kg de cerises fraîches récoltées sur 100 arbres. Les pertes sont relativement réduites sur des arbres ayant reçu les traitements fongicides au cours de la campagne. C'est avec le Forum que les pertes ont été les plus faibles (93,8 kg de cerises fralches/100 arbres).

Sur le Dacobre 500 (fongicide de référence) et le Delan 700 WG utilisé à la dose de 33g/15 1 d'eau, on a récolté deux (2) fois plus de café que sur le témoin. île Delan 700 WG utilisé à la dose de 50g/l5 1 d'eau a produit 2,7 fois plus de café que le témoin non traité. La meilleure récolte en café a été obtenue avec le Forum qui a donné 3,6 fois plus de café marchand par rapport au témoin non traité.

Résultats obtenus en 1998/1999

Ils sont illustrés par les tableaux V, VI, VII et VIII.

Tableau V : Pourcentage de la maladie en fonction des traitements.

Traitements	**% brut**	**Ecart type de la moyenne**
Témoin non traité	65.95	2.746
CHEM-COBRE Plus	63.72	2.502
CHEM-COPP 50	35.36	2.259
DACOBRE 500	21.01	1.416

Tableau VI Analyse de variance du pourcentage de perte après transformation angulaire des données.

Source de variation	SCE	DDL	cm	F	p
Traitement	14.48612172	*3*	4.82870724	92,34	0.0001
Erreur	20.70831187	396	0.05229372		
Total	35.19443359	399			
CV %	49.16 %				

L'analyse de la variance montre une différence hautement significative entre les traitements. Ceux-ci sont classés par groupe dans le tableau suivant par le test de comparaison multiple des moyennes de *Newman et Keuls.*

Tableau VII : Classement des fongicides testés en 1998/1999 en fonction de leur efficacité

Traitement	% de la maladie	Groupe homogène
Témoin non traité	65,95	A
CHEM-COBRE Plus	63,72	A
CHEM-COPP 50	35,36	B
DACOBRE	21,01	C

Au seuil de P = 0,05 le test de *Newman et Keuls* a permis de distinguer trois groupe de fongicides. Le témoin non traité et le CHEM-COBRE se retrouvent dans le même groupe distinct par rapport aux deux autres traitements qui sont chacun dans un groupe distinct l'un par rapport à l'autre. Le Dacobre 500, fongicide de référence est significativement différent du CHEM-COPP 50. Ces deux fongicides sont significativement différents du témoin non traité et du CHEM-COBRE Plus.

Tableau VIII : Poids (en kg) de cerises récoltées sur la totalité des 100 arbres mis en observation par traitement

Traitement	ler récolte	2e récolte	Total	Moyenne
DACOBRE 500	81,5	80	161,5	1,615
CHEM-COPP 50	74	83.5	157,5	1,575
CHEM-COBRE Plus	35	29	64	0,640
Témoin non traité	25	19	44	0,4

Ce tableau montre que le témoin non traité a été le plus attaqué par la maladie et a perdu le plus de fruits avec seulement 44 kg de cerises sur 100 arbres. Le CHEM-COBRE Plus a permis de récolté 64 kg de fruits-, le CHEM-COPP 50 157,5 kg. Le DACOBRE 500, produit de référence a permis d'obtenir la meilleure récolte avec 161,5 kg sur 100 arbres. Ce résultat confirme celui obtenu par analyse statistique.

CONCLUSION

La pression infectieuse de la maladie est très forte dans l'écologie arabicole de Santa avec respectivement 84,42 % de perte en 1997/1998 et 65,95 % en 1998/1999.

Les résultats obtenus ont permis de retenir les fongicides suivants pour l'épreuve de prévulgarisation en vue de leur utilisation dans la lutte contre le CBD au Cameroun. Ce sont : Le FORUM R., le CHEM-COPP 50 et pourquoi pas le DELAN 700 WG (50g) eu égard à la forte intensité de la maladie en absence de tout traitement chimique à condition que ces fongicides soient utilisés dans les doses et schémas de traitements prescrits et qui ont fait leur preuve.

METHOD OF REARING LARVAE AND SOME ASPECTS OF THE BIOLOGY AND CONTROL OF COCOA STEM BORER *Eulophonotus myrmeleon* (Felder)

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Abstract

Difficulty of rearing the larva of the Cocoa stem borer-*Eulophonotus myrmeleon*(Felder) and thereby getting the adult insect for proper identification had remained a problem for many years. Early instar larvae of the cocoa stem borer, collected from field at Jimma research center, were laboratory reared using freshly cut coffee branches for determining the duration and dimension of the different developmental stages. The identify and role of larval parasites was also determined. The result indicates that the average larval, pupal and adult stage is 202.8 $\pm$ 12.4, 16.4 $\pm$ 0.93 and 6.1 $\pm$ 0.5 days respectively. Two parasites, namely Bathyaylax sp and an unidentified species were recovered. The earlier attacked 24% and the latter 2.6% of the larvae collected. Method of rearing larva, some features including dimension of the different stages of the insect is described. The integration of parasites and a physical means in the control of the insect is also described.

POSTER

INTRODUCTION

There are now over 45 species of Arthropod pests recorded on coffee in Ethiopia (1). The Cocoa stem borer- Eulophonotus myrmeleon (Felder) is one of eight species of coffee stem/branch boring insects known to exist in Ethiopia. It was first recorded in this country in 1973 (2) and is now found in most of the coffee growing areas of Ethiopia. It belongs to the family Cossidae whose larvae is known to bore trunks and branches of trees and occur on coffee in the west indian islands, Africa and Asia (4). Several species in the genus Xyleutes and Zeuzera have been recorded to attack coffee (4). Early instar larva of the Cocoa stem borer attack tiny tertiary branches and gradually move to side branches until it finally bores through the top main stem of the Coffee plant resulting in a considerable crop loss. It was not possible to get it expertly identified because of the difficulty of getting the adult. It was commonly known as cossid stem borer for a long time since the general appearance of the larva and type of damage resembles that of family cossidae. Since any further study on the insect depends upon its exact identity, it was necessary to obtain the adult insect in order to get it expertly identified. This study was, therefore, carried out in the laboratory to rear the larva to an adult by continuously feeding it with freshly cut coffee branches. Because of the dangers associated with the use of Pesticides, alternative means of controlling pests should be given top priority. This includes making use of natural enemies (parasites and predators) and other non-chemical means. While rearing larva additional information on the role of the combination of a physical means and parasites in the control of Cocoa stem borer was also collected.

MATERIALS AND METHODS

Rearing of Larva

From previous observations, Cocoa stem borer damage reaches its peak in the field around March. It was therefore, decided to start field visit in December to make sure that the early stage of the first instar larva is detected as early as possible. The Five hectares coffee farm was visited every two days during December and January and any wilting tertiary branch was examined for the presence of the larva by bending the branch in different directions. A branch, already attacked by a larva, breaks at the point of entrance of the larva. Such branches were brought to the laboratory, each larva was removed by splitting open the branch with a knife and its length measured. Newly cut branch, larger in diameter than the one from which the earlier stage larva has been removed, was used in the feeding process. A hole, nearly the diameter of the stem and 5-10 mm in depth is made using a knife. A piece of paper is wrapped and tied around the hole with a sewing thread. The larva is then placed inside the paper with its ends folded inwards to prevent the larva from escaping. The branch is placed in a horizontal position inside a cylindrical glass cage. The larva makes its way through the branch and seals the hole behind it using its excreta. The paper is usually removed after 24 - 48 hrs depending on how well the larva has sealed the entrance hole. Branches are changed every 5 - 10 days, depending on length of the branch and stage of larva, since the branch looses moisture and turns dry sooner than in the field condition where it would still be partially attached to the unaffected part of the branch. As larva gets larger then a close follow up on daily and later hourly basis was made by continuously removing the excreta to make sure that the larva does not change to pupa without being noticed. The length of the larva was measured when it ceased feeding. The larva is then left to pupate. This time the branch is kept in a ventilated cage made out of mesh wire to ensure emerging adults remain within the cage

Parasitoids

In order to determine the identity of larval parasites and the role they play in controlling the Cocoa stem borer, attacked branches were collected from early period of infestation (January) up to August which is about the time when no infestation occurs. Branches were then split open in the laboratory. Those larvae that were found already attacked by parasites were kept in cages for parasite emergence. Active and seemingly healthy larvae were fed in a similar manner described above for 3-5 days. A healthy larva would actively bore through the branch and produce excreta continuously where as parasitized larva will cease both feeding and producing excreta. The earlier is then recorded as healthy and the latter as parasitized after split opening the branch and making sure that the larva has ceased feeding because of being parasitized. The emerged parasites were counted, identified and recorded.

RESULTS AND DISCUSSION

Life cycle

➔ **Egg :** Attempt to have the adults lay their eggs inside caged branches was not successful. Therefore, nothing is known about the appearance, size, shape, where it is laid on the coffee tree, its duration etc. Insects belonging to the family cossidae are known to lay their eggs in crevices in the bark (3). It is believed that eggs of Cocoa stem borer are laid in a similar manner mentioned above.

➔ **Larva :** The smallest larva measured 2mm. The length of the first instar larva is expected to be smaller than 2mm.This is because the field collected larva were probably already a few days old when brought to the laboratory. The last instar larva had a length ranging from 30 to 40 mm. The duration of the larval stage was 202.8 +12.4 days

➔ **Pupa :** It is a typical lepidopteran pupa having a reddish brown color and with an average length of 24 mm. Average duration of the pupal stage is 16.4 + 0.93

Identity and role of parasites.
Two species of parasites were recovered out of a total of 789 larvae collected from the field. 26.6% of the larvae were found attacked by these parasites of which Bathyaulax killed 24 % and the unidentified species 2.6 %.

The first sign of attack by the insect was observed to start in early January. The first instar larva starts feeding on tertiary branches and gradually progresses towards boring in to larger size branches, as it grows older. Consequently the top main stem, up to 1 meter and above, can be attacked resulting in heavy loss of crop. It can also damage a number of branches before reaching the main trunk. In both instances, in addition to crop loss, the coffee tree is deformed and unnecessary growth is initiated at the point of entrance of the larva.

As indicated above 26.6 % of the larvae were killed by parasites. The contribution of these natural control agents needs to be given serious consideration while control is envisaged. As mentioned earlier, the larva begins by attacking very small and tiny tertiary branches. Any control measure should therefore focus on limiting the progress of the larval attack to larger size branches, which usually bear fruits. The effective and environmentally friendly method of control is to integrate the contribution of the two parasites with a physical means. The integrated control requires scouting a coffee field starting from early January and collect any tertiary branch attacked by the larva. Any wilting branch should be located and bent in different directions. It will break at the point of entrance if it had been attacked by a larva. Such branches should be collected and split open to assess what is inside. Branches with actively boring larva should be put together and burned while branches housing weak looking larva or already parasitized larva should be left on the tree to give way to the emergence of the parasites. Such practice reduced damage to the top part of the main stem by 75%. This method is particularly applicable to small coffee farmers with small land holding where the family members can walk within the farm and collect damaged branches. The insect can be kept under control if weekly scouting of the field is carried out for the first six weeks after the first sign of damage is observed.

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CAFFEINE DOES NOT PROTECT COFFEE AGAINST THE LEAF MINER *Perileucoptera coffeella*

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Summary

The larvae of *P. coffeella*, a leaf miner, is one of the main pests attacking coffee plantations. Caffeine is the major alkaloid in this crop and may have a role in insect resistance. Since there is genetic variability in both resistance to the coffee leaf miner and in caffeine content among different coffee species, we investigated the role of this alkaloid as an anti-herbivory compound. Coffee plants containing different levels of caffeine were exposed to oviposition of the insect and the caffeine content and damaged leaf area were evaluated. The same procedure was carried out with interspecific hybrids between *C. arabica* and *C. racemosa*, varying in resistance against the leaf miner. In addition, plants were exposed to the insect but one leaf of each pair was protected from oviposition with paper bags. In another experiment, leaf disks from plants with known susceptibility to the attack of the leaf miner were infiltrated with caffeine aqueous solutions, from 0 to 2%, and exposed to oviposition. None of the experiments established a significant correlation between reduction of leaf damage and caffeine content of the tissue. In the experiment in which one leaf of the pair was protected of the insect, there was an increase in caffeine in the infested leaves, particularly in younger leaves. It is suggested that caffeine does not have a protective role in coffee plants but that its increase due to different stresses, as indicated in several reports and observed here, might be a consequence of changes of the size of the adenine pool, which is its main primary precursor, as the general metabolism is affected.

Poster

INTRODUCTION

Purine alkaloids such as caffeine belong to a distinct group of alkaloids since they are derived from purines instead of amino acids. Different species contain these alkaloids and many are not genetically related. These plants have a worldwide distribution, from *Camelia* in China, *Coffea* in Africa, *Ilex* in South America (Suzuki et al. 1992), up to the *Erodium cicutarium* (Eijk 1952) in the Scandinavian countries. Caffeine concentration varies in the tissues of these plants as well as throughout their organs. The most argued role for alkaloids has been as a chemical defense against herbivory (Harborne 1988) and several works have reported that alkaloid synthesis, accumulation and storage are controlled at the genetic level (Kutchan 1995). However, few reports have given complete understanding of the real function of alkaloids in nature from an ecological point of view (Brown and Trigo 1994).

A biological role for caffeine has long been considered.Recently it was shown that seeds of selections *Coffea arabica* from Ethiopia and Kenya with a higher caffeine content were more resistant against *Colletotrichum coffeanum*, the causal agent of the coffee berry disease - CBD (Biratu et al. 1996). However, in this case, only seven plants were studied. Medeiros et al. (1990) observed an increase of caffeine in infected leaves of coffee selections displaying resistance against the leaf rust *Hemileia vastatrix*. However, this was also observed in susceptible interactions but at later stages of infection.Regarding plant-insect interactions, Mazzafera (1991) observed in a field experiment for coffee yield selection, that among 2,500 plants, a few plants were preferred by leaf-cutting ants.

Analysis showed that one noticeable difference in leaves of these plants was their lower caffeine content. They were identified as progeny from only two crosses which were randomly distributed in the field. However, since attack of coffee plants containing normal caffeine content were previously observed, the data were discussed cautiously. There are two main pests attacking coffee, the leaf miner *Perileucoptera coffeella* and the berry borer *Hypothenemus hampei*. Since it is known that there is a genetic variability for caffeine content in fruits and leaves (Clifford et al. 1989; Mazzafera and Magalhães 1991) and for resistance against these pests, we have tried to establish a relationship between them using published results in the literature (Mazzafera et al. 1996). Although no significant correlation was found, we could not discard any protective biological role for caffeine, since a limited number of species was considered. Therefore, in this paper we show data obtained from experiments performed with several coffee species and interspecific hybrids in order to study caffeine as a chemical defense against the coffee leaf miner *P. coffeella*.

MATERIALS AND METHODS

Caffeine analysis

Caffeine from leaves or leaf disks was extracted with 80% methanol at 60°C and analyzed in a high performance liquid chromatography system with a C_{18}-reversed phase column and detection at 280 nm (Mazzafera et al. 1997).

Insect rearing

Coffee plants were exposed to insect oviposition inside of wooden cages covered by a voile net Katiyar and Ferrer (1968). After one day exposure, the infested plants were taken out of the cages and kept at 27°C $\pm$ 2 °C, 75% $\pm$ 5% humidity and 14h of light (laboratory fluorescent light) until the development of the chrysalides. Then the leaves were removed from the plants and placed inside the cages.

Resistance evaluation of coffee species and interspecific hybrids

Resistance against coffee leaf miner was evaluated in thirteen species of *Coffea* (*C. arabica* cv Mundo Novo, *C. arabica* cv Catuaí Vermelho, *C. canephora* cv Robusta, *C. canephora* cv Kouillou, *C. canephora* cv Guarini, *C. dewevrei, C. eugenioides, C. kapakata, C. stenophylla, C. racemosa, C. salvatrix, C. congensis, C. brevipes, C. liberica, C. sessiflora and C. humilis*) and several hybrids between *C. arabica* and *C. racemosa*, belonging to a population of the fourth backcross for the first specie (H13660, H13685, H13376, H13465, H14060, H14066 and H14126). Leaves of the third or fourth pairs were removed from one year old coffee seedlings and the petioles immediately immersed in water in glass tubes. The tubes were fixed in a rack which was transferred to the cages. After 24h of exposure to the insects the abaxial blade of the leaves were observed under stereomicroscopy and disks were taken from places containing eggs. The disks were obtained with a cork borer (1.8 cm diameter) and using a small brush, only two eggs were left in each disk. The disks were transferred (with the abaxial blade facing up) to plastic boxes containing a foam moistened with distilled water and kept at the same laboratory conditions as the cages. The resistance/susceptibility was evaluated by determining the size (cm^2) and the lesion type (scale 1-4) (Guerreiro Filho 1994).

Variation of the caffeine content in leaves of infested coffee seedlings

Seedlings of *C. arabica* cv Catuaí Vermelho, *C. racemosa, C. stenophylla* and three hybrids (H14060, H14066 and H14126) between the first two species were evaluated in this experiment. One year old seedlings with, on average, 7-8 leaf pairs were maintained in the cages for 24h. One leaf of each pair was protected from the oviposition with a transparent paper bag closed with adhesive tape around the petiole. The eclosion of the larvae was followed with stereomicroscopy, occurring after 5-6 days of the exposure to the insects. One day after eclosion, which could be

detected by the depression on the top of the egg, both leaves of each pair were collected and analyzed separately for caffeine as described before.

The effect of caffeine on the development of *P. coffeella*

Seedlings of *C. arabica* cv Catuaí Vermelho, *C. racemosa* and *C. canephora* cv Robusta were used in this experiment. In addition, susceptible hybrids between the first two species were also evaluated. The seedlings were exposed to the insects and the eclosion checked every day until the fifth day. If it had not occurred by this day, leaf disks were taken from the leaves, leaving two eggs per disk, and infiltrated with aqueous caffeine solutions (Mazzafera and Eskes 1987). Water infiltrated leaves were used as controls. The disks were kept in plastic boxes and the insect damage was evaluated by the size and type of the lesion as described previously (Guerreiro Filho, 1994).

RESULTS

Evaluation of the resistance of coffee species and interspecific hybrids

There was variability among plants of *C. racemosa*, one of them showing ten times more damaged leaf area than the others. Genetic segregation was also observed for *C. eugenioides*. This species has been considered as resistant against *P. coffeella* (Guerreiro Filho et al. 1991). Two plants showed 5-10 times more damaged area. The same was observed for *C. stenophylla*, which has been considered immune against the coffee leaf miner. The plant with 1,30 cm^2 of damaged area would be classified as moderately susceptible according to the scale proposed by Guerreiro Filho (1994). On the other hand, the cultivar Bangelan of *C. congensis*, considered as susceptible (Guerreiro Filho et al. 1991) showed four plants classified as resistant. A similar situation was observed with *C. canephora* cv Guarini. Two plants would be considered as moderately resistant according to the lesion type. We believe that these differences occurred because, except *C. arabica,* all other coffee species are allogamous. Since the seedlings were obtained from seeds and there was not any control of the crossings occurring naturally, some segregation could be expected. Higher caffeine contents were observed in *C. arabica* and *C. canephora*. The variability observed in the last specie had been reported by Mazzafera et al. (1997) in fruits and by Silvarolla et al. (1998) in leaves. Correlation analysis between the caffeine content and response to *P. coffeella* showed no significance when the plants were considered altogether. The data of damaged leaf areas and caffeine contents of *C. arabica* and *C. racemosa* hybrids indicate that all progenies were segregating for *P. coffeella* resistance. The proportion between resistant and moderately resistant individuals, and susceptible and moderately susceptible individuals is 1:3. Caffeine content also showed segregation. Interestingly, in most of the cases the concentration was higher than those values found for the parentals. It was frequently observed that individuals with similar caffeine content had a different reaction against *P. coffeella*. There was no correlation between caffeine content and leaf damage.

Variation of the caffeine content in leaves of infested coffee seedlings

Coffee seedlings were subjected to leaf miner infestation, but one of the leaves of the leaf pair was protected with paper bags. As already reported in the literature, the caffeine content is reduced with leaf aging. This was observed with plants with high and low caffeine content, such as *C. arabica* and *C. racemosa*, for example. Similar results were also observed in the hybrids between these two species. The results indicate that caffeine consistently increased in these leaves, but not in the protected ones. In general, the higher increases were observed in the younger leaves of the first and second leaf pairs. In older leaves caffeine increased less, except for *C. stenophylla*. It is interesting to observe that among the hybrids the percentage increase in caffeine was lower than that observed for coffee species.

The effect of caffeine on the development of *P. coffeela*

Leaf disks carrying *P. coffeela* eggs were infiltrated with caffeine solutions and the damaged area estimated. The plants of *C. canephora* cv Robusta and the hybrids between *C. arabica* and *C. racemosa* were susceptible and *C. racemosa* were resistant. There was significant reductions of the damaged area in the two hybrids only when 1 or 2% caffeine solutions were used. However, the damage caused was still within the classification range of moderately susceptible plants. Significant change of the lesion type was observed only for *C. arabica* and H14066 with the same solutions, however, they were still typical of susceptible interactions. There was not any change of lesion type and damaged leaf area of C. racemosa.

The data presented here are not conclusive to determine that caffeine is not an anti-herbivory compound, since it may have played a role during coffee evolution. However, convincing proof for this statement is still lacking and we may consider that conclusions solely on the basis of its concentration in plant tissues is a matter of speculation.

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INSECTS ASSOCIATED WITH COFFEE BERRIES IN GHANA

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Summary

In a study aimed at identifying the natural enemies of the coffee berry borer, *Hypothenemus hampei* (Ferrari), coffee berries were collected from several localities in Ghana within the period January 1997 - February 1998. The berries were thoroughly inspected for insects before being kept in lampshades covered with fine nylon mesh for the emergence of insects. Insects recorded were of the orders *Hymenoptera* (families *Braconidae, Bethylidae, Pteramalidae* and *Eupelmidae*); *Diptera* (families *Cecidomyiidae, Ceratopogonidae, Chironomidae, Drosophilidae, Muscidae, Phoridae, Sciaridae, Tephritidae*); *Coleoptera* (families *Anthribidae, Corylophidae, Dermestidae, Laemophloeidae*) and *Lepidoptera* (families *Lycaenidae, Gelechiidae*). Among these, the berry borer *Araecerus coffeae* (Coleoptera: Anthribidae) and a suspect parasitoid *Opius* sp. (Braconidae: subfamily Opiinae) are new records to Ghana. Other insects included an unidentified berry feeder of the family Gelechiidae ((*Lepidoptera*), *Leptophloeus* sp. (*Coleoptera: Laemophloeidae*) and an unidentified *Diptera* both of which might be predatory. Other insects associated with the berries were *Antherigona orientalis* (Diptera: *Muscidae*), *Attagenus* sp. (*Coleoptera*: *Dermestidae*) and unidentified Sciaridae which were probably mere scavengers.

The majority of insects, *H. hampei* and the *Drosophilidae* being the exception, occurred in very low numbers. Thus, the proportional representation of insects recorded during the study period were, *H. hampei* (59.1%), *Drosophilidae* (32.5%), *Gelechiidae* (3.1%), *Virachola lorisona* (2.1%), *Araecerus coffeae* (0.3%), *Thrirhithrum coffeae* (0.3%), *Antherigona orientalis* (0.2%), *Attagenus* sp. (0.4%), *Opius* sp. (0.3%), *Leptophloeus* sp. (0.2%) and 0.5% for the remaining insects. The study is being continued to confirm the roles of identified insects, particularly the suspect parasitoids and predators, with the aim of manipulating them for biological control of *H. hampei*.

INTRODUCTION

The coffee berry borer, *Hypothenemus hampei* Ferrari (Coleoptera: Scolytidae) is the most notorious pest of coffee in many of the world's chief coffee producing areas. It is endemic in Central Africa and is the most important pest of coffee in Ghana (Padi, 1985, 1996). Both adults and larvae feed on coffee berries and as many as 21 adult and larval beetles have been recorded in one berry (Padi, 1984). Damage to berries starts when the
berry is young and soft. Attacked young berries usually fall prematurely. In older berries, the entire content may be eaten and the berries rendered unsaleable. Infestation levels recorded from three localities in the Eastern Region ranged between 10.7 and 71.0% (Owusu-Manu & Ampomah, 1991; Owusu-Manu *et al.*, 1993), depending upon the level of shade and general farm sanitation. In a nation-wide survey, thè percentage infestation of dry berries left on trees from the previous season ranged between 12 and 100% (Padi, 1984). Another berry borer, *Virachola lorisona* Hewitson (Lepidoptera: Lycaenidae) recorded in Ghana in recent times (Padi, 1984) is of minor importance.

The main method for the control of *H. hampei* in Ghana has been by good cultural practices. This involves regular weeding and pruning of trees. It also involves removing all bad berries during harvesting, soaking the berries in a container of water and decanting the bad floating berries, which are

then burnt or buried. It also involves picking all fallen berries from the ground after harvesting, as well as removing all berries remaining on trees at the end of the cropping season and destroying them. Although effective, this method is tedious and time consuming. It has, therefore, become necessary to develop alternative control measures.

This paper reports on one year's results of a study ultimately aimed at developing a biological control method to be incorporated in an Integrated Control strategy for the control of coffee berry borers. The immediate objective of the study, initiated at the Cocoa Research Institute of Ghana (CRIG), is to identify the indigenous natural enemies of berry borers in Ghana, evaluate their effectiveness with the view to manipulate those found promising for control purposes.

MATERIALS AND METHODS

Coffee berries were collected from farmers farms, once every two months, from ten localities (Table 1) within five of the coffee growing geographical regions in Ghana. The Western Region was not included in the study because of the then poor condition of motor roads in the area. The berries were collected from as many trees as possible in each locality and sub-sampled to obtain 200 berries per locality. The sampled berries from each locality were incubated in a glass lampshade covered with fine nylon mesh for a period of four months during which period the lampshades were inspected daily for the presence of insects. All insects found were identified as far as possible and recorded. They were later matched against specimens identified by the Plant Protection Institute of Pretoria, South Africa for more detailed identification. At the end of the four months, the berries were dissected and all insects found, both dead and alive, were recorded.

RESULTS

The berry borer *Hypothenemus hampei* (Ferrari) was recorded in high numbers from all localities (Table 1). Other insects recorded were of the families Braconidae, Bethylidae, Pteromalidae and Eupelmidae (Order Hymenoptera); Cecidomyiidae, Ceratopogonidae, Chironomidae, Drosophilidae, Muscidae, Phoridae, Sciaridae and Tephritidae (Order Diptera); Anthribidae, Corylophidae, Dermestidae, Laemophloeidae (Order Coleoptera) and Lycaenidae and Gelechiidae (Order Lepidoptera). Of these, Araecerus coffeae (Coleoptera: Anthribidae), *Leptophloeus* sp. (Coleoptera: Laemophloeidae), *Attagenus* sp. (Coleoptera: Dermestidae), *Antherigona orientalis* (Diptera: Muscidae), *Trirhithrum coffeae* (Diptera: Tephritidae) and *Opius* sp. (Hymenoptera: Braconidae) were identified to the genus or species level.

H. hampei was the most abundant species (Table 1) and constituted 59.1% of all insects recorded. It was most prevalent at Assin Foso (Total: 1800) in the Central Region, followed by Fumso (1783) and Jamasi (1494) in the Ashanti Region. Next in abundance was the unidentified fruit fly (Diptera: Drosophilidae) which constituted 32.5% of the insects collected and, like *H. hampei*, was most abundant at Assin Foso (1106) followed by Fumso (1074) and Jamasi (956). The other berry borer *Virachola lorisona* (Lepidoptera: Lycaenidae) previously record in Ghana (Padi, 1984) occurred in very low numbers (2.1%) in all localities. Other insects which occurred in very low numbers were the Gelechiidae (3.1%), *Araecerus coffeae* (0.3%), *Thrirhithrum coffeae* (0.3%), *Antherigona orientalis* (0.2%), *Attagenus* sp. (0.4%), *Opius* sp. (0.3%) and *Leptophloeus* sp. (0.2%). The remaining unidentified insects of the families Corylophidae (order Coleoptera), Chironomidae, Cecidomyiidae Ceratopogonidae, Phoridae, Sciaridae (order Diptera), Pteromalidae and Eupelmidae (order Hymenoptera) constituted only 0.5% of the total number.

DISCUSSION AND CONCLUSIONS

The present study has confirmed previous findings that *H. hampei* is the most widespread coffee berry borer in Ghana (Padi, 1984; Padi & Ampomah, 1996). *Thrirhithrum coffeae* , identified in this study, is known elsewhere to feed on coffee berries (Le Pelley, 1968) and is considered to be of minor importance. It is, nevertheless, reported to be widespread from West to East Africa where it has been

recorded in Ghana, Sierra Leone, Nigeria, Camerouns, the Congo and Uganda (Le Pelley, 1968). In Kivu, Congo, it is reported to be associated with six trypetid parasitoids as well as with a *Xanthomonas*-type bacterium which produces a characteristic flavour in arabica beans. An *Araecerus* species, *A. fasciculatus* is reported as being one of the few really important "stored product" pests of coffee in South American countries (Le Pelley, 1968) and also as a storage pest of cocoa (Entwistle, 1972). It is uncertain whether *A. coffeae* recorded in the present study is synonymous with A. *fasciculatus*. If it is, then the present finding indicates that infestation of coffee berries by this storage pest starts from the field and that this is the first record of the pest on coffee in Ghana.

A number of species of the family Drosophilidae (fruit flies) occur world-wide, in ripe and over-ripe fruits of coffee but none is considered a pest. The Mexican fruit fly, however, is reported to be important enough to merit control with insecticides viz. Lebaycid and Dipterex. In Kenya in East Africa, the fruit fly *Ceratitis capitata* is considered as troublesome since the larvae tend to make the skin of the fruit stick to the seed, making pulping imperfect, but this is generally considered unimportant. Based on the relatively high numbers of fruit flies recorded in the present study, it is necessary to identify the species involved and to identify their role, especially since this is the first documented record of their presence in coffee berries in Ghana.

The families Braconidae, Bethylidae, Pteromalidae, Eupelmidae (order Hymenoptera); Phoridae, Cecidomyiidae, and Chironomidae (Order Diptera) and Laephloedae (Order Coleoptera) recorded in the present study are well known world-wide, to be parasitic or predatory on insect pests on different crops including coffee and cocoa (Le Pelley, 1968; Entwistle, 1972). For example, *H. hampei* is known to be parasitised by two Bethylids, *Prorops nasuta* and *Cephlalomia stephanoderes*. Moreover, *P. nasuta* was introduced to Java, Brazil and Ceylon and was reported to be of considerable value in the control of *H. hampei* in Brazil. Its importance in Africa as a control agent, however, does not appear to have been a subject of any interest (Le Pelley, 1968). C. *stephanoderes* is reported to be the most important parasite of *H. hampei* in Cote d'Ivoire where almost 50% of the broods of the borer in black berries are parasitised. A Braconid *H. coffeicola*, on the other hand, feeds on the eggs and larvae of *H. hampei* and its action is reported to complement that of *P. nasuta*. Hargreaves (1924), however, found that the larvae fed on larvae of other parasites, thus reducing their efficacy. It is present naturally in most of the coffee areas of Africa where H. hampei is present. Based on their being so widespread in Africa, it is likely that the unidentified Bethylids and Braconidae encountered in the present study may include these three parasitoids.

In Brazil, an ant, a species of *Crematogaster,* is reported to destroy appreciable numbers of immature stages of *H. hampei* in berries (Le Pelley, 1968). Although queens of the ants *Oecophylla longinoda* and *Crematogaster clariventris* have been found inside coffee berries in previous studies (Padi, 1984) they were not found feeding on *H. hampei* and neither species were encountered in the present study.

Of the species identified, the storage pest *Araecerus coffeae* and the suspect parasitoid *Opius* sp. (Braconidae: subfamily Opiinae) are new records in Ghana. Other insects which emerged from the berries were an unidentified berry feeder of the family Gelechiidae ((Lepidoptera), *Leptophloeus* sp. (Coleoptera: Laemophloeidae) and an unidentified Diptera both of which might be predatory. *Antherigona orientalis* (Diptera: Muscidae), *Attagenus* sp. (Coleoptera: Dermestidae) and unidentified sciaridae which were recorded in very low numbers are probably mere scavengers.
The study is being continued and efforts continue to fully identify all insects encountered. The efficacy of natural enemies, both parasites and predators, will be evaluated. The role of ant species found associated with berries in the field also requires further scrutiny since some of them might be predatory on *H. hampei* or other berry borers as observed in Brazil. Studies on fungal pathogens associated with the berries have also been initiated.

Table 1. Number of insects reared and dissected from coffee berries collected from different localities in Ghana (January 1997-February 1998)

REGION/LOCALITY	INSECTS (ORDER / FAMILY / GENUS / SPECIES)											
	HYMENOPTERA			DIPTERA			COLEOPTERA				LEPIDOPTERA	
	Braconidae	Eupelmidae	Bethylidae	Drosophilidae	Miscidae	Terphri tidae	Scolytidae	Demitidae	Anthribidae	Laeophloeidae	Gelechiidae	Lycaenidae
	Opius sp	unidentified	unidentified	Unidentified	*Antheri gona*	*Trirh ithrum* sp	*H..hampei*	*Attagenus coffeae*	*Araecerus*	*Leptophloeus*	Unidentified	*Virachola lorisona*
EASTERN REGION												
Tafo	2	0	0	94	1	0	152	1	0	0	8	11
Afosu	1	0	0	162	3	0	269	2	0	2	5	30
ASHANTI REGION												
Jamasi	3	5	0	956	6	8	1494	10	1	7	88	10
Fumso	3	1	0	1074	4	1	1783	14	8	6	95	42
BORONG -AHAFO REGION												
Bechem	4	0	0	47	1	9	92	1	12	0	49	18
Wamfie	1	0	0	12	1	0	143	0	4	0	15	15
CENTRAL REGION												
Assin Foso	6	0	0	1106	3	15	1800	11	9	5	99	34
Breman-Asikuma	13	0	0	43	2	4	785	5	0	2	10	43
VOLTA REGION												
Kpeve	3	0	1	254	3	0	340	0	3	2	8	23
Akaa	1	0	1	252	1	0	420	3	0	1	5	32
TOTAL	37	6	2	4000	25	37	7278	47	37	25	382	259

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CHEMISTRY AND FOOD ENGINEERING SESSION

Otto G. Vitzthum

CHEMISTRY

o In the first plenary paper **W.Grosch** reported on the development of the key aroma compound concept for coffee. He stressed the release of impact odorants from coffee in the first hours after roasting and discussed experiments, mimicking the coffee smell by addition and substraction of compounds in an artificial mix. He further underlined the role of 2-furfurylthiol as an important aroma contributor.

o **R. Liardon** and colleagues stressed the difficult issue of sensory interrelationship between various odorants in coffee aroma. By new techniques of sensory profiling the relative importance of 26 key aroma compounds with low and high sensory thresholds and how they may affect each other was reported; cleaning-up procedures for chemical characterisation and GC-sniffing were described. 3 trained persons were actively doing "CHARM"analyses. A special chart for comparison of sensory vs. chemical data was shown.

o Defective Arabica green beans that can be separated by electronic sorting were investigated by **G. Full.** Off flavor causing compounds were identified as esters of C4- to C6-chain acids and Z-4-heptenal; three of them were reported first time in coffee. Best illustration of differences between light and dark green in comparison with normal beans could be shown by "radar plot" demonstration. A sensory confirmation was given.

o **C.Yeretzian** and colleagues also reported on off flavors stemming from dry post-harvest handling. By two-dimensional GC and GC-mass spectrometry as well as confirmation by GC-sniffing they characterized more precisely the three already known compounds geosmin, 2-methylisoborneol and 2,4,6-trichloroanisol as main culprits causing sometimes the earthy/mouldy off-taints in coffees.

o Again 2-furfurylthiol was the aim of investigations carried out by **E.C. Pascual and coll**. Its degradation in solution was examined by reacting OH radicals with FFT; OH radicals that may be formed from hydrogenperoxide, which is present during preparation of coffee brews - may react to short-living RS-SR radicals. The tracking of these intermediates by electron spin resonance spectrometry and the identified degradation products were reported. (Comment: this study may have an impact for investigating the coffee roast process).

o **Steinhart and Bücking** looked for the retardation effects of various milk products on the release of coffee aroma compounds from beverages. They applied dynamic and static headspace techniques. GC-sniffing was used for characterisation of the aroma profiles by checking
50 aroma impact odorants. The volatiles were analyzed by GC-MS. The intensity reduction of the key flavors by different additives to the beverage was reported.

o **K.Speer and coll.** reported on new degradation products of the diterpenes kahweol and cafestol generated by roasting. These were the aldehydes cafestal and kahweal.

o **A. Bradbury and coll.** investigated atractyligenin glycosides in green coffees. Glycosides in general were considered also as flavor precursors for roast coffee. - Here the authors characterized 3 carboxyatractyligenins linked with glucopyranosyl and isovaleryl groups by means of multiple MS and NMR. Quantitation was made by liquid chromatography/mass spectrometry; the content of these compounds in soluble coffees is in the order of magnitude of 0,5 %.

o **F. Serra and coll.** used the technique of stable isotope analyses to characterize the quality of coffee coming from different origins. This approach succesfully had been applied to other food products before. - In first experiments it could be shown that the ratio of the isotopes ^{15}N to ^{13}C showed - especially for coffees from ASIA - statistically significant different data. Improvements are expected by introducing sulfur stable isotopes.

o In his plenary paper on nonvolatile compounds **S.Homma** concentrated on new developments for the characterization of Maillard reaction compounds. Microorganisms' decolorating effects and Fe(II) chelating properties were discussed. A Zn(II) chelating compound was isolated and characterized as brown-colored polymer;

chemical degradation yielded phenolics, sugars and amino acids. Compounds obtained by alkaline fusion of the polymer were described as well.

o A highly interesting contribution on the carbohydrates in green coffee was brought by **M.Fischer et coll.**

Polysaccharides containing cell wall material - isolated at various stages of bean growth 12, 17, 29 weeks after flowering - were purified in three different ways. The obtained polymer was characterized by compositional and linkage analysis. Special emphasis was given on the determination of cellulose, arabinogalactan and
the - late appearing - mannan.

o **M.Petracco and colleagues** isolated a foaming fraction from coffee, that is representing the surface foam complex in espressos. - Hot water extracts were cleaned up by precipitation and dialysis; the obtained product was characterized by spectroscopic means after further fractionation; two subfractions were found being responsible for foamability and foam stability.

POSTERS

In a poster **Bücking and coll.** studied the retention of coffee volatiles in the oral cavity by fats, proteins and carbohydrates. By "oral vapor" gas chromatography the breath of volunteers was analyzed for four characteristic coffee aroma compounds. These amounts decreased with increasing fat content of milk additives. Furthermore coffee volatile release from human saliva was investigated as well.

o An interesting approach to the assessment of coffee acidity and bitterness was presented by **A.Kazuyoshi**. A special sensory ratio scale for taste (gust scale) was used for evaluation. Some correlated physico-chemical properties like correlation with luminosity were described.

o A new method for the determination of 16-O-methylcafestol was introduced by **I. Koelling-Speer and coll.** for the discrimination between Arabica and Robustas in corresponding blends, thus replacing an older DIN method. Important was the introduction of the time-saving Extrelut extraction.

o **C.Sanz and coll.** optimized the headspace techniques for analyses of the coffee aroma volatiles. They showed the characterization of 120 compounds in the head space and described the optimal temperature and equilibration time conditions.

FOOD ENGINEERING

o In a very comprehensive and promising study **S.Schenker and coll.** investigated the basics of coffee roasting. A better understanding of the process was obtained by examining the changes occuring in the microstructure of the beans. Phenomena like influence of inner surface, oxygen accessibility, role of cell wall micropores, oil migration, gas desorption during storage and bean volume expansion were examined and discussed.

o Another approach for the determination of the origins of coffee was explored by **D.Jaganyi**. Kinetic extraction data of

caffeine, kations and anions for Arabica and Robusta coffee infusions were determined. Caffeine and sodium could be used for characterisation of the coffee origin.

o The formation of phenolic compounds during the roasting process was studied by **R.Zimmermann and coll.**, using the highly sophisticated laser induced resonance-enhanced multiphoton ionization (REMPI) technique in combination with the very fast time-of-flight mass spectrometer (TOFMS) in the headspace. It allows the real time on-line monitoring of off gasses from food processes. - Here the degradation products from quinic and ferrulic acid during roasting were shown. The pyrolytic formation of phenols was in fairly good correlation with the roasting time. Other trace compounds in roast gasses may be investigated as well, if other laser wavelengths are used.

o A new precise moisture meter for green and roast coffee was presented by **G. Wiseman.** An in-line microwave moisture sensor from TEWS yielded reduced standard deviation data. Thus a greater conformance to manufacturing specifications was achieved. Calibration was done against an atmospheric oven method. There are some limits however for roasted beans directly after roast and quench.

o A mathematical model for volatile aroma retention during freeze-drying of coffee extracts was developed by **J.M. Pardoe. and coll.** In model studies the majority of coffee volatiles first was removed by steam distillation from the coffee solution; five selected aroma compounds were added and their release during freeze-drying was quantified.

o **B. Zeller and F. Saleeb** reported on an improvement of the batch decaffeination process, using solvents like methylene chloride, ethyl acetate or soy bean oil. From lab-scale experiments it was found that for the final aqueous isolation of the caffeine from solvents the addition of caffeic acid stimulated the formation of caffeine-caffeic acid complexes. The decaffeination efficiency thus could be improved.

POSTERS

In the poster session **R. Cappuccio and F. Suggi-Liverani** presented the computer simulation of a coffee brewing model. They found that four parameters basically influence the flow rate: water pressure, water temperature, particle size distribution and void ratio within the coffee bed. An experimental model of the water flow rate as a function of time, pressure and temperature has been developed. This is useful for a better understanding of the solubles' extraction, that characterizes espresso coffees' body and aroma.

o **E.E.M. Mori and colleagues** tried to map the different Brazilian coffee qualities, based on a method developed by the International Coffee Organization ICO. Sensory, physical and chemical analytical methods of coffee were used to certify qualities and origins.

o **R.Zimmermann and colleagues** showed the useful application of their REMPI method in combination with Chemical Ionization Mass Spectrometry (PTR) for direct on-line analysis of coffee.

18th ASIC Symposium (Helsinki, August 1999)

WORKSHOPS :

EFFECTS OF COFFEE ON BRAIN AND BEHAVIOUR

ENHANCEMENT OF COFFEE QUALITY BY REDUCTION OF MOULD GROWTH

Gerrit van der Stegen

On the Wednesday morning was the ***Workshop on Effects of Coffee on Brain and Behaviour: What does coffee consumption bring in daily life?***
From the contributions of Prof. Smith, Dr. Snel and Dr. Van Boxtel there were following conclusions:

With respect to **Everyday life situations:**

- Low doses of caffeine improve mood and enhance processing of new information;
- Caffeine has beneficial effects on real-life performance, such as driving and performance over the working day;
- The effects of caffeine are more obvious when alertness is reduced by factors such as a minor illness, working at night and sleep deprivation;
- Low doses of caffeine improve visual task performance and information processing related to a higher overall arousal level;
- Caffeine enlarges the information that can be processed and focusses attention to increase selectivity;
- Caffeine improves memory performance and is able to alleviate the age-related performance deficit to some extent.

Fredholm, Zwartau, Di Chiara and Nehlig went in their contributions into the difficult subject of habitual coffee and caffeine consumption. A lot of the attention went to the underlying neurochemical mechanisms, with the following conclusions:

With respect to **Caffeine and dependence:**

- Caffeine antagonizes adenosine at the A1 and A2a receptors and thus stimulates motor activity by affecting the activity in the basal ganglia;
- Tolerance to caffeine does not reflect increased metabolism but may relate to a decrease in adenosine A2a receptor number;
- Caffeine can act as a reinforcer, possibly involving the A1 receptor;
- Calcium channel agonists and antagonists modulate the reinforcing action of caffeine;
- Caffeine (0.5-10 mg/kg) does not lead to any release of dopamine or increase in functional activity in the shell of the nucleus accumbens (which is the brain structure relevant to dependence) which is consistent with its lack of addictive potential
- Caffeine stimulates dopamine release in the prefrontal cortex which is consistent with its psychostimulant and reinforcing properties

With the overall conclusion that caffeine has reinforcing effects, being experienced as pleasant. However the neurochemistry results clearly demonstrate the caffeine does not activate the brain structures involve in addiction. Or in more daily terms. The effects of daily caffeine consumption are experienced as pleasant and do not induce addiction. Thus coffee is a beverage which can be enjoyed, without concerns.

On Thursday morning was the ***Workshop on Enhancement of Coffee Quality by Reduction of Mould Growth*** as a follow-up to the Workshop in Nairobi in 1997.

Highly valuable experiences were exchanged in the contributions of Dr. Frank, Dr. Frisvad, Dr. Vega, Mrs. Taniwaki and Dr. Naidu, from an impressive amount of work already done. The most important moulds for OTA in coffee are *A. Ochraceus* and *A. Carbonarius*. As mould infection routes for coffee where presented soil contact and insect damage. Mould growth is most risky during drying of the coffee and at rewetting of green coffee. The critical wateractivity range is from 1 down to 0.8.
Dr. Boutrif updated the meeting about recent international development in mycotoxin regulations. In contributions of Mr. Dubois, Dr. Boutrif, Dr. Viani and Dr. Ngabirano, the ICO/FAO project for prevention of mould growth and its first experiences in Uganda were presented. All other coffee producing countries will follow in the coming years of this 5 year project.
The train has started and is running now. And recent results for OTA in roasted coffee indicate that since Nairobi there might already be a downward trend in mean OTA levels.
With the currently about 5% of green coffee having a potential OTA problem and 95% of the green coffee being clean, there is full confidence that this project will eliminate OTA as a potential problem for coffee.

G.van der Stegen
August 6, 1999

18th ASIC Symposium (Helsinki, August 1999)

PHYSIOLOGY SESSION

Rinantonio Viani

The main themes of this session were the question of OTA contamination and the cardiovascular effects of coffee consumption, both addressed by Prof. Ron Walker in his introductory plenary conference.

OTA

Food contamination by OTA is widespread with cereals and pulses contributing more than one half of human consumption and coffee coming third at around 10%. All sources of OTA in the diet have been identified and Dr. Olsen estimates the intake of Scandinavian populations at below 5 ng/kg b wt/d, by correlating food consumption habits with blood levels.

The question if OTA is genotoxic still divides the scientific community, as explained by Prof. Dekant: if a substance is non genotoxic a threshold level of intake can be fixed, taking an appropriate safety factor. This is the conclusion reached by JECFA, as explained by Prof. Walker, which has fixed a maximum tolerable weekly intake of 100 ng/kg b wt/wk. If a substance has genotoxic effects no threshold level can be established and a quantitative risk assessment must be made.

The SCF of the EU has taken an intermediate position, indicating a limit of 5 ng/ kg b wt/d «because of concern about (its) toxic and pharmacokinetic properties». According to Prof. Dekant, most of the data available and, in particular, his and Nestlé's latest results, point to a non-genotoxic mechanism, so that a safe threshold consumption level could be fixed.

Cardiovascular questions

Now that cafestol has been identified as the major cause for the increase in LDL cholesterol in people consuming high quantities of «boiled» coffee, a new dietary risk indicator, homocysteine, is becoming popular. According to Prof. Walker available data do not indicate any direct link between coffee consumption and hyperhomocysteinemia. Seasonal and nutritional effects are larger than any hypothetical effect of coffee and these have not been controlled in epidemological or experimental studies on coffee. Further studies are needed to verify if homocysteine is really an independent risk factor.

Dr. Happonen has studied the possible link between coffee consumption and myocordial infarction. His results indicate different patterns in people without coronary heart disease, with a U shaped curve and lowest risk at 4 cups/ day, and a decreased risk of acute events with increasing consumption in people with coronary heart disease. Apparently, consumption of 2 cups/ day by such population could even counteract the effect of habitual smoking. Such data probably just show that we should look away from habitual parameters and that further work is needed.

Tanskanen has characterized heavy coffee drinkers, consuming 7+ cups/ day, in an adult Finnish population as male current smokers, living in rural areas. Minor mental health characteristics cannot be independently associated with heavy coffee drinking without large scale prospective studies, not yet available. His data also indicate that moderate coffee consumption is safe.

Other questions

Lücker has compared the link of coffee consumption to gastrointestinal symptoms, such as heartburn. Consumption of heated coffee in a single dose cross-over study, reduced lower stomach mucosal irritation, particularly in people testing positive for Helicobacter pylori.

The posters presented by Brice and Smith on coffee as an index of the measure of caffeine content of saliva, showed that saliva caffeine levels are good indicators of total consumption, is independent of a person's psychological characteristics, but not of the behavioral effects produced by a given dose.

18ème Colloque ASIC (Helsinki, août 1999)
SESSION AGRONOMIE

André Charrier et Daniel Duris

La session d'Agronomie comprenait un exposé de synthèse, 27 communications orales et une vingtaine d'affiches. Les principales avancées de la recherche ont été rapportées sous 5 rubriques différentes.

Biotechnologies végétales

Dans son exposé introductif, R. NAIDU (CCRI, Inde) a fait une revue des progrès des recherches engagées en Inde sur l'ensemble des biotechnologies appliquées aux caféiers et des perspectives. Les principaux résultats concernent la micropropagation d'une vingtaine de variétés sélectionnées, la régénération *in vitro* à partir d'explants variés, la culture d'embryons zygotiques et la cryoconservation, les marqueurs RAPD et AFLP...

A. DESHAYES (Nestlé, France) a présenté un protocole efficace et simplifié de production des embryons somatiques et d'obtention de jeunes plantes. Son application à différentes souches de caféiers cultivés a en particulier permis l'installation d'essais de comportement au champ de *C. canephora* dans différents pays producteurs, manifestant un développement normal. Les coûts de production de plants issus de micropropagation et de bouturage horticole sont du même ordre. La réduction du coût de la micropropagation est à rechercher dans l'amélioration de l'efficience du protocole actuel.

V. LOYOLA-VARGAS (CICY, Mexique) a obtenu l'induction préférentielle de l'embryogenèse somatique directe de *C. arabica* par modification du protocole expérimental de YASUDA pour les différentes sources d'azote. Ce protocole modifié réduit le temps d'obtention des embryons somatiques et fournit un modèle intéressant pour l'étude de la variation somaclonale.

M. CARNEIRO (CIFC, Portugal) a réussi à régénérer des embryons au stade " torpille " par culture d'anthères et de microspores isolées de catimor en optimisant les prétraitements et différents facteurs de la culture *in vitro*.

S. HAMON (IRD, France) a présenté les progrès significatifs obtenus dans le développement de la technique de cryoconservation des semences de *C. arabica*. La faisabilité de la conservation à long terme du germoplasme de la collection au champ du CATIE (Costa-Rica) est testée par la déshydratation des graines, leur immersion directe dans l'azote liquide et un emploi limité de la culture *in vitro*.

G. GRAZIOSI (Université de Trieste, Italie) a réalisé la caractérisation moléculaire du cultivar Bourbon L.C. dérivé par variation somatique naturelle de la variété Laurina. En vue d'accroître le nombre de locus polymorphes, il a criblé 2 banques d'ADN génomique de *C. arabica* et identifié de nouveaux locus microsatellites.

C. KY (IRD, France) a étudié l'hérédité des composants biochimiques du café vert impliqués dans la qualité à la tasse dans la descendance Back-cross d'un croisement interspécifique entre une espèce de caféier sauvage sans caféine et *C. liberica* var. *dewevrei*. Les acides chlorogéniques manifestent une hérédité multigénique additive tandis que l'absence de caféine est contrôlée par un gène majeur. Une carte génétique de l'ordre de 200 marqueurs génétiques, essentiellement AFLP, a été construite et utilisée pour la détection de QTL des principaux caractères morphologiques et biochimiques.

J. STILES (ICT, Hawaï) a présenté 2 communications sur la physiologie moléculaire des caféiers. La 1ère aborde la caractérisation et le clonage de 2 enzymes (ACC synthase et ACC oxydase) contrôlant la caractérisation et le clonage de la Xanthosine N7 metyltransférase (XMT), la 1ère enzyme de la chaîne de biosynthèse de la caféine. Les clones cDNA ont été séquencés, insérés en position antisens dans des vecteurs de transformation en vue d'évaluer l'expression de ces gènes dans les caféiers génétiquement modifiés.

T.LEROY (CIRAD, France) a réalisé la transformation génétique de *C.canephora.* et *C.arabica* avec un gène synthétique Cry 1 Ac de *Bacillus thuringiensis*. Plus de 100 plantes OGM ont été obtenues pour différents génotypes de *C.canephora* et montrent un degré variable de résistance à la mineuse des feuilles (*Leucoptera* spp) en test au laboratoire. Son évaluation et la gestion au champ de ces caféiers OGM ont reçu l'agrément de la commission française de génie biomoléculaire (CGB)

Sélection et amélioration génétique

Les communications et posters ont concerné d'une part les stratégies d'amélioration et de sélection par les principaux caractères agronomiques et la qualité du café, d'autre part l'évaluation et la diffusion des variétés améliorées chez les producteurs.

P. JAGORET (CIRAD, France) a présenté l'évaluation préliminaire de 84 hybrides naturels entre *C. arabica* et *C. canephora* en Nouvelle Calédonie. La distinction de 2 groupes principaux pour leur quantité d'ADN (3C et 4C) affecte la plupart des caractéristiques étudiées.

P. CHARMETANT (CIRAD, France) a rapporté le programme d'hybridation réalisé en Tanzanie pour exploiter la résistance à la rouille orangée (CLR) et à l'anthracnose des baies (CBD) apportée par les lignées Geisha, Rume Sudan et Hybrido de Timor. Seize hybrides complexes sont en cours d'évaluation en essais multilocaux pour leur adaptation et leur valeur agronomique et qualitative, en vue de la vulgarisation des meilleurs hybrides par voie clonale.

C.AGWANDA (CRF, Kenya) a présenté les performances d'une population de lignées élites de *C. arabica* améliorée au Kenya sur les mêmes bases, avec un niveau de résistance au CBD comparable à la variété Ruiru 11, mais dont la diffusion pourra être réalisée par semences. Un poster a complété notre information sur les sélections les plus avancées du Kenya et leurs caractéristiques de production, de qualité et de résistance au CBD. En outre, cet auteur s'est focalisé sur la recherche de critères de sélection de *C. arabica* pour la qualité à la tasse ; la notation de la flaveur est efficace pour différencier les génotypes sur ce caractère qualitatif. Pour la stabilité de la production, la mesure de caractères de croissance pendant la 2ème année de culture au champ est un bon prédicteur du comportement futur des lignées.

C.CILAS (CIRAD, France) a analysé un essai diallèle réalisé sur *C. arabica* au Cameroun. Classiquement, la vigueur hybride des hybrides F1 et l'héritabilité de la production totale se manifestent ; l'origine spontanée éthiopienne Et3 s'avère être un géniteur intéressant pour la résistance au CBD.

H.P.MEDINA-FILHO (IAC, Brésil) a présenté en poster l'expansion potentielle de la zone de culture du Robusta dans l'état de SAO PAULO (200.000 ha) et les facteurs agronomiques et sociaux favorables à cette expansion. En outre, L.C. FAZUOLI (IAC, Brésil) a résumé sous forme de tableaux les principales caractéristiques des variétés de *C. canephora* et de *C. arabica* recommandées au Brésil et adaptées aux différentes conditions agro-écologiques.

Les pratiques agronomiques

Différents exemples de systèmes agronomiques et de pratiques ont été présentés pour illustrer les situations variées rencontrées dans les différents pays producteurs de café.

P.H.HOMBUNAKA (CRI, Papouasie-Nouvelle-Guinée) a simulé l'effet du déficit hydrique sur la croissance et la production du caféier grâce à un modèle basé sur la teneur en eau disponible du sol. La prédiction de la production sous différents niveaux de stress hydrique n'est pas aisée, mais ce type d'approche contribue à une meilleure compréhension de l'agrophysiologie du caféier.

M.P.H.GATHAARA (CRF, Kenya) a expérimenté des moyens d'irrigation d'appoint simples, peu onéreux, à la portée des petits paysans, donnant un accroissement spectaculaire de la production de café dans leurs conditions socio-économiques.

R.J. ONZIMA (Ouganda) a présenté les résultats d'une étude comparative de longue durée sur la fertisation minérale et organique des clones de Robusta.
Les amendements organiques s'avèrent les plus profitables pour la production de café et la conservation des sols en Ouganda. De même, MPH GATHAARA (CRF, Kenya) a comparé différents types d'engrais verts au Kenya et préconisé de les substituer aux engrais minéraux.

D.SNOECK (CIRAD, France) a rapporté une étude originale de transfert de l'azote des légumineuses associées aux caféiers en utilisant l'abondance de l'isotope 15N.
Le tranfert d'azote dépend de la fertilité du sol et de la densité de la culture associée, et peut couvrir 25% des besoins du caféier.

K.OSEI- BONSU (CRI, Ghana) a résumé dans un poster les différentes espèces de légumineuses plantées en haies pour l'implantation des cultures de Robusta au Ghana.

Les autres posters ont abordé d'autres pratiques agronomiques comme :

- le contrôle des mauvaises herbes par des cultures intercalaires de maïs, bananier plantain, manioc...(K.OPAKU-AMEYAW, CRI, Ghana)
- les méthodes de taille en fonction de la densité de plantation des variétés de *C. arabica* de grande taille (PAMENDA TALOPA, CRI, Papouasie- Nouvelle Guinée)
- l'emploi d'acides humiques à faible dose pour la transplantation des jeunes plants de caféiers (C.R.OBATOLU, CRI, Nigéria)
- la muliplication végétative par bouturage horticole (L. AKPO, Benin)

Technologie après-récolte

A.TEXEIRA (Assicafé, Brésil) a examiné les moyens d'éviter le développement de moisissures sur le café dans les pays producteurs, en considérant les conditions écologiques variées des régions de production au Brésil. Il a formulé 2 recommmandations fortes fondées sur sa propre expérience :

1) il est nécessaire d'aborder scientifiquement ce problème (OTA) avec des protocoles adéquats.
2) il faut absolument éviter toute exposition de café à des humidités élevées au cours des opérations de séchage, stockage et transport.

J.M.FRANK (Université Surrey, Grande-Bretagne) a présenté une approche globale de la prévention des moisissures, considérant tous les stades possibles d'infection du champ aux manipulations post récolte et les espèces incriminées dans la contamination. Les taux de

contamination ne sont pas significativement corrélés avec les infestations de borer des baies (CBB).

S. AVALLONE (CIRAD, France) a abordé la composition, la structure et les caractéristiques biochimiques des parois cellulaires de la baie du caféier, avant et après fermentation, principalement des polysaccharides.

Maladies et parasites

Les différentes présentations et posters ont confirmé l'attention portée au borer des baies (CBB) et à l'anthracnose des baies (CBD).

Tous les pays producteurs sont touchés par le CBB. Les contraintes et les limites d'emploi des insecticides chimiques conduisent à développer de nouvelles méthodes de contrôle. B.DUFOUR (CIRAD, France) a présenté une méthode expérimentale de capture de masse basée sur des substances attractives. Son développement, si l'efficacité se confirme, devrait être favorisé par sa facilité de mise en oeuvre et son faible coût.

Toutes les équipes de recherche travaillant sur le CBD de *C.arabica* recherchent des sources de résistance au CBD et étudient la variabilité du pathogène responsable, *Colletotrichum kahavae*. Le réseau africain de recherche sur le café (ACRN) a engagé pour cette maladie majeure un programme coopératif de recherche associant le CRF (Kenya), le CIFC (Portugal) et le CIRAD (France).

D.BERRY (CIRAD, France) a présenté une étude sur l'influence des conditions agro-écologiques sur le développement du CBD dans la zone de production de *C.arabica* au Cameroun. Sur cette base, des propositions concrètes sont faites pour la gestion du CBD et son contrôle rationnel par voie chimique. L'évaluation au champ de différents fongicides a été rapportée par poster (J.BAKALA, IRAD, Cameroun).

RODRIGUES (CIFC, Portugal) a montré l'importante variabilité de l'agressivité des souches de C.kahavae, en particulier du Cameroun. L'interaction hôte-parasite et les mécanismes de résistance sont mieux compris grâce aux observations histo-chimiques réalisées.

C.AGWANDA et K.GICHURU (CRF, Kenya) ont montré la relation physiologique observée entre la sévérité de la maladie et les activités protéolytiques de variétés de *C.arabica* présentant différents niveaux de résistance.

E.DERSO (EARO, Ethiopie) et KILAMPO ont présenté en poster des études complémentaires sur les méthodes de sélection pour la résistance au CBD.

G.J. HAKIZA (CRC, Ouganda) a étudié les différentes espèces de Fusarium associées à la trachéomycose de C.canephora, une autre maladie en cours de développement en Afrique orientale.

M.ABEBE (EARO, Ethiopia) a rappelé les risques associés à l'emploi abusif des pesticides. Pour le contrôle des *Antestiopsis*, trois hyperparasites de cette punaise ont été détectés en Ethiopie et pourraient être évalués en lutte biologique.

Enfin, 3 posters concernant les insectes ont complété notre information sur :
- la biologie et le contrôle sur caféier du foreur des tiges du cacaoyer (M.ABEBE, EARO,Ethiopie)
- l'absence de protection contre la mineuse des feuilles en relation avec leur teneur en caféine (GUEREIRO, IAC, Brésil)
- l'inventaire des insectes associés aux borers des baies (PADI, CRI, Ghana)

Nous tenons à remercier l'ensemble des auteurs de communications et d'affiches de la session d'Agronomie pour l'intérêt scientifique de leurs présentations ainsi que les animateurs des différentes sub-sessions.

André CHARRIER et Daniel DURIS

18th ASIC Symposium (Helsinki, August 1999)
AGRONOMY SESSION

André Charrier and Daniel Duris

The agronomy session included a summarized report, 27 paper presentations and around twenty posters. The main advances made in research were grouped under 5 different headings.

Plant biotechnologies

In his introductory speech, R. NAIDU (CCRI, India) reviewed the progress made in research conducted in India on the range of biotechnologies applied to coffee trees, and future prospects. The main results involve micropropagation of twenty or so selected varieties, *in vitro* regeneration from various explants, zygotic embryo culture and cryopreservation, RAPD and AFLP markers.

A. DESHAYES (Nestlé, France) presented an efficient and simplified protocol for producing somatic embryos and obtaining young plants. In particular, its application to different strains of cultivated coffee trees has enabled the planting of *C. canephora* performance trials revealing normal development in different producing countries. Plant production by micropropagation or from horticultural cuttings costs about the same. Reducing micropropagation costs is one of the aims in improving the efficiency of the current protocol.

V. LOYOLA-VARGAS (CICY, Mexico) has achieved preferential induction of direct somatic embryogenesis in *C. arabica* by modifying YASUDA's experimental protocol for different nitrogen sources. The modified protocol reduces the time taken to obtain somatic embryos and provides an interesting model for studying somaclonal variation.
M. CARNEIRO (CIFC, Portugal) has succeeded in regenerating embryos at the "torpedo" stage by culturing anthers and microspores isolated from Catimor, optimizing the pretreatments and various *in vitro* culture factors.

S. HAMON (IRD, France) described the significant progress made in developing the cryopreservation technique for *C. arabica* seeds. The feasibility of long-term storage for germplasm from the CATIE (Costa Rica) field collection is being tested by dehydrating the seeds, direct immersion in liquid nitrogen and limited use of *in vitro* culture.

G. GRAZIOSI (University of Trieste, Italy) has carried out the molecular characterization of the Bourbon L.C cultivar derived by natural somatic variation from the Laurina variety. He has screened 2 *C. arabica* genome DNA banks and identified new microsatellite loci, with a view to increasing the number of polymorphic loci.

C. KY (IRD, France) has studied the heredity of the biochemical components of green coffee involved in cup quality in the back-cross progenies of an interspecific cross between a caffeine-free wild species and *C. liberica* var. *dewevrei*. Chlorogenic acids reveal additive multigenic heredity, whilst the absence of caffeine is controlled by a major gene. A genetic map with around 200 genetic markers, primarily AFLP, has been constructed and used to detect QTLs of the main morphological and biochemical traits.

J. STILES (ICT, Hawaii) presented 2 papers on the molecular physiology of coffee trees. The first dealt with the characterization and cloning of 2 enzymes (ACC synthase and ACC oxidase) controlling the characterization and cloning of Xanthosine N7 methyltransferase (XMT), the 1st enzyme in the caffeine biosynthesis chain. cDNA clones were sequenced and inserted into the antisense position in transformation vectors with a view to evaluating the expression of these genes in genetically modified coffee trees.

T.LEROY (CIRAD, France) has carried out the genetic modification of *C.canephora and C.arabica* with a synthetic gene Cry 1 Ac of *Bacillus thuringiensis*. More than 100 GM plants have been obtained for different *C.canephora* genotypes and they reveal a variable degree of resistance to the leaf miner (*Leucoptera* spp) in laboratory tests. Its evaluation and field management of GM coffee trees have been approved by the French Commission for Biomolecular Genetics (CGB).

Selection and genetic improvement

The papers and posters covered strategies for breeding and selection by main agronomic traits and coffee quality on the one hand, and the evaluation and distribution of improved varieties to growers on the other hand.

P. JAGORET (CIRAD, France) presented the preliminary evaluation of 84 natural hybrids between *C. arabica* and *C. canephora* in New Caledonia. The distinction of 2 groups based on their DNA quantity (3C and 4C) affects most of the traits studied.

P. CHARMETANT (CIRAD, France) reported on the hybridization programme being implemented in Tanzania to exploit resistance to coffee leaf rust (CLR) and coffee berry disease (CBD) provided by the Geisha, Rume Sudan and Hybrido de Timor lines. Sixteen complex hybrids are currently being assessed in multi-site trials for their adaptation, and their agronomic and qualitative value, with a view to extending the best hybrids through clones.

C.AGWANDA (CRF, Kenya) described the performance of a population of elite *C. arabica* lines improved in Kenya on the same bases, with a level of resistance to CBC similar to the Ruiru 11 variety, but whose dissemination could be carried out by seed. A poster completed the information on the most advanced selections in Kenya and their production, quality and CBD resistance traits. In addition, this author focused on the search for *C. arabica* selection criteria for cup quality: flavour scoring is effective for differentiating between genotypes for this qualitative trait. For production stability, the measurement of growth traits during the 2nd year after planting in the field is a good predictor of the future performance of the lines.

C.CILAS (CIRAD, France) analysed a diallel trial involving *C. arabica* in Cameroon. Classically, the hybrid vigour of the F1 hybrid and the heritability of total production appeared; the wild Ethiopian origin Et3 proves to be a worthwhile parent for CBD resistance.
H.P.MEDINA-FILHO (IAC, Brazil) presented a poster on the potential expansion of the Robusta growing zone in SAO PAULO state (200,000 ha) and the agronomic and social factors in favour of such expansion. In addition, L.C. FAZUOLI (IAC, Brazil) summed up in tables the main characteristics of the *C. canephora* and *C. arabica* varieties recommended in Brazil and adapted to the different agro-ecological conditions.

Agricultural practices

Different examples of farming systems and practices were presented to illustrate the varied situations found in the different coffee producing countries.

P.H.HOMBUNAKA (CRI, Papua New Guinea) simulated the effect of a water deficit on coffee tree growth and yields using a model based on the soil's available water content. Forecasting yields for different levels of water stress is not easy, but this type of approach contributes towards a better understanding of coffee tree agrophysiology.
M.P.H.GATHAARA (CRF, Kenya) has tested simple, cheap supplemental irrigation methods that are affordable to smallholders and result in spectacular coffee yield increases under their socio-economic conditions.

R.J. ONZIMA (Uganda) presented the results of a long-duration comparative study of mineral and organic fertilization on Robusta clones.

Organic applications prove to be the most beneficial for coffee yields and soil conservation in Uganda. Likewise, MPH GATHAARA (CRF, Kenya) compared different types of green fertilizers in Kenya and recommended using them to replace mineral fertilizers.

D.SNOECK (CIRAD, France) reported on an original study examining nitrogen transfer from intercropped legumes to coffee trees using the abundance of isotope 15N.
Nitrogen transfer depends on soil fertility and the intercrop density, and can cover 25% of coffee tree requirements.

K.OSEI- BONSU (CRI, Ghana) summarized in poster form the different legume species planted as hedges in Robusta crops in Ghana.

The other posters covered other agricultural practices, such as:

- controlling weeds by intercropped maize, plantain banana, cassava, etc. (K.OPAKU-AMEYAW, CRI, Ghana)
- pruning methods depending on the planting density of large *C. arabica* varieties (PAMENDA TALOPA, CRI, Papua New Guinea)
- using low doses of humic acids for young coffee tree transplanting (C.R.OBATOLU, CRI, Nigeria)
- vegetative propagation by horticultural cuttings (L. AKPO, Benin)

Post-harvest technology

A.TEXEIRA (Assicafé, Brazil examined ways of preventing mould development on coffee in producing countries, considering the various ecological conditions in the Brazilian production regions. He made two strong recommendations based on his own experience:

1) it is necessary to take a scientific approach to this problem (OTA) with appropriate protocols.

2) it is essential to avoid any exposure of coffee to high moisture levels during drying, storage and transport.

J.M.FRANK (University of Surrey, U.K.) presented an overall approach to mould prevention, considering all possible stages of infection from the field to post-harvest handling, along with the species involved in contamination. Contamination rates are not significantly correlated with coffee berry borer (CBB) infestation.

S. AVALLONE (CIRAD, France) covered the composition, structure and biochemical characteristics of coffee berry cell walls before and after fermentation, primarily polysaccharides.

Diseases and parasites

The different poster presentations confirmed the attention paid to the coffee berry borer (CBB) and to coffee berry disease (CBD).

All producing countries are affected by the CBB. The constraints and limitations of chemical insecticide use have led to the development of new control methods. B.DUFOUR (CIRAD, France) presented an experimental mass trapping method based on attractants. If proved to be effective, its development should be favoured by its ease of use and low cost.

All the research teams working on *C.arabica* CBD are seeking sources of resistance to the disease and are studying the variability of the pathogen involved, *Colletotrichum kahavae*. The African Coffee Research Network (ACRN) has launched a major research programme on this disease in cooperation with CRF (Kenya), CIFC (Portugal) and CIRAD (France).

D.BERRY (CIRAD, France) presented a study on the effect of agro-ecological conditions on CDB development in the *C.arabica* zone of Cameroon. Concrete proposals were made on this basis for CBD management and its rational chemical control. A poster covered the assessment of fungicides in the field (J.BAKALA, IRAD, Cameroon).

RODRIGUES (CIFC, Portugal) showed the substantial variability in the aggressiveness of *C. kahavae* strains, especially in Cameroon. More has been learnt about host-parasite interactions and resistance mechanisms through the histo-chemical observations carried out.

C.AGWANDA and K.GICHURU (CRF, Kenya) showed the physiological relation found between the severity of the disease and the proteolytic activities of *C.arabica* varieties revealing different levels of resistance.
E.DERSO (EARO, Ethiopia) and KILAMPO presented a poster concerning additional studies on methods of selection for CBD resistance.

G.J. HAKIZA (CRC, Uganda) studied the different species of *Fusarium* associated with tracheomycosis in *C.canephora*, another disease developing in eastern Africa.

M.ABEBE (EARO, Ethiopia) reiterated the risks associated with the excessive use of pesticides. For *Antestiopsis* control, three hyperparasites of the bug have been detected in Ethiopia and could be assessed in biological control.

Lastly, 3 posters on insects completed our information on:
- the biology and control on coffee of the cocoa stem borer (M.ABEBE, EARO,Ethiopia)
- the absence of protection against leaf miners in relation to their caffeine content (GUEREIRO, IAC, Brazil)
- the inventory of insects associated with coffee berry borers (PADI, CRI, Ghana)

We should like to thank all the authors of papers and posters during the Agronomy session for the scientific interest of their presentations, along with the leaders of the different sub-sessions.

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