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COFFEE: NEW RESEARCH ADVANCES AND APPLICATIONS OF BIOTECHNOLOGY*

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ABSTRACT

Coffee improvement programs can focus on three different areas of application: agronomy, processing industry and consumer benefits. Agronomic characteristics should focus on reducing direct and indirect farming costs. In a modern coffee farm in Brazil, direct costs are ca. 63% of the total farm costs (Medina et al., 1984). To reduce coffee farming costs, the new technologies need to address fertilizer efficiency, disease and pest resistance, and crop management aspects that will reduce labor utilization (herbicide resistance, mechanized cultivation and harvesting). The development of coffee varieties with short (3 months), mid (6 months), and long (9 months) maturation cycles would permit a more even distribution of the harvesting and processing activities at the farm level and thus increasing the efficiency of farm labor utilization. The development of a true hybrid system in coffee or a commercial micropropagation process would be highly desirable to counteract frequent disease and pest outbreaks as well as for maximizing the exploitation of agroecological niches and productivity.

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Coffee quality depends primarily on the genotype, but is also highly susceptible to environmental conditions. Plantation management, which affects plant microclimate, nutrition level, seed processing quality has a major impact on the coffee quality. Superior beverage quality is produced from Arabica cultivars grown at higher elevations. Crosses between Arabica and other coffee species confirm that Arabica genes are responsible for superior beverage quality. Postharvest processing and storage also have a major role in determining final coffee quality.

There are several characteristics of coffee that could be altered, resulting in some benefits to the coffee industry and final consumers: increased total soluble solids, larger and more uniform bean size, bean density and texture, uniform maturation, caffeine content, increased levels of compounds responsible for coffee flavor and aroma.

In coffee tissue culture, there have been reports of successful regeneration via somatic embryogenesis of several wild *Coffea* species, five *C. arabica* cultivars and two interspecific hybrids. Recovery of plants via somatic embryogenesis in such a wide range of genotypes demonstrates the potential of using in vitro methods for coffee improvement. Regeneration from embryogenic cell suspensions and isolated protoplasts of Robusta and Arabica genotypes has also been described. Transient transformation utilizing the protoplast uptake method has now been reported. Considering the repeatability of the protoplast regeneration systems available today, the utilization of useful

genes for coffee improvement is now a near- to mid-term possibility with a high degree of success. Embryo rescue and anther culture techniques need further developments. Micropropagation on a large scale is now feasible through the use of embryogenic suspensions in Erlenmeyer flasks or bioreactors. Utilization of this technique will make segregating individual plants into potential commercial candidates. Somaclonal variation as a breeding tool for coffee improvement has now been described for Arabica coffee and a few interspecific hybrids.

Stable prices, superior quality and attention to consumer needs will be the most effective long-term strategies for increasing the coffee market. Biotechnology probably can provide a more efficient way to introduce value-added coffee to the industry. The availability of certain value-added coffee cultivars would open opportunities for market niches for specialty coffee brands. Most agronomic benefits will bring quality and price stability to the commodity market. Increasing the net return to coffee farmers will also contribute to production stability and long-term success of this industry.

1. INTRODUCTION

Coffee is a beverage prepared from seeds (beans) of *Coffea* species after roasting and grinding. The history of coffee can be traced to the 13th Century when it was carried from Ethiopia to Yemen in the Arabian Peninsula. In the 17th Century the beverage became popular in Europe with the opening of the first coffee shops: Venice (1615), France (1644), Vienna (1650), and London (1652).

The genus *Coffea* has ca. 100 species but only two species are of commercial importance. *Coffea arabica* accounts for about 75% of the total green coffee production. This species is an allotetraploid (2n = 44) originated in the Southwest highlands of Ethiopia (Carvalho 1946). Arabica plantations require high altitudes, mild day/night temperatures and defined dry/wet periods. Arabica coffee has preference among consumers due to its superior aroma and flavor. *Coffea canephora* (Robusta coffee) accounts for ca. 25% of total coffee produced. It is a diploid species (2n = 22) with a wide distribution in West and Central Africa. Commercial Robusta plantations began after 1850 in the African Atlantic Coast (Gabon and Angola) and they are typical of lowlands and hot and humid areas. In a few local producing areas, *Coffea liberica*, *C. devewrei*, and *C. racemosa* are also consumed.

Coffee is the most important agricultural commodity generating $10-12 billion dollars per year in the green coffee trade market. It is an important source of hard currency for more than 50

countries located in tropical regions of Latin America, Africa and Asia. In average, approximately 90 million bags (60 kg) are produced per year and the top five producing countries are Brazil (30%), Colombia (14%), Indonesia (6%), Mexico (4.7%), and Ivory Coast (4.3%). Costa Rica is the leading Arabica producing country (1.538 kg/ha/yr) and typical Robusta productivity is Africa and Asia is 600-650 kg/ha/yr (Söndahl 1990).

2. COFFEE IMPROVEMENT OPPORTUNITIES

Coffee research programs can focus on three different areas for improvement: agronomy, processing industry, and final consumers.

(a) Agronomic Characteristics

The net profit of a coffee grower depends on the productivity of the plantation and his direct and indirect costs. In a typical coffee farm in Brazil, direct costs constitute about 63% of the total costs and is equally divided between agrochemicals and labor costs. To decrease coffee farming costs, coffee improvement programs need to address fertilizer efficiency, disease and pest resistance, and labor utilization. Another potential benefit to coffee farmers is to grow value-added coffee varieties that will address special market needs and permit higher profit margins to the coffee growers. A series of agronomic benefits can be

identified for coffee improvement: increase yield, disease resistance, insect resistance, drought tolerance, cold tolerance, resistance to heavy metals, herbicide resistance, early and late maturation cycles, and development of a true hybrid system.

Increased yields can be achieved by new varieties that are more efficient in carbohydrate partitioning between vegetative growth and fruit load or by new cultivars with increase efficiency for nutrient absorption and utilization. Nitrogen and potassium are the two most used elements in a coffee plantation, and thus should be targeted first. Natural resistance to disease and insects would reduce the cost of agrochemical usage. Tolerance to environmental conditions (water deficit, cold, or heavy metals) would improve coffee production in marginal areas and protect farmers against seasonal fluctuations. The use of herbicides would reduce labor costs. Herbicide resistance in coffee should focus on total biodegradable herbicides to minimize soil and underground water residues. Coffee varieties with short (3 months), mid (6 months), and long (9 months) maturation cycles will permit a more even distribution of the harvesting and processing activities at the farm level. The development of a "true hybrid system" in both Arabica and Robusta coffee would greatly help coffee producing areas. Modern hybrids would have consistent quality and yield. Adoption of diverse hybrids would permit the specificity for different ecological niches. Hybrid system would also permit and efficient method to quickly respond to disease and pest outbreaks whereby a resistant line could be combines with an elite line

without great loss in yield. An alternative to the development of coffee hybrid systems would be the development of efficient and low cost propagation methods for coffee. In this case, single plant selections would be propagated and multiclone plantations could be established. This production strategy can address all the advantages of a hybrid system within a very short period of time and potentially can be a low cost solution. The selection of individual plants well adapted to ecological regions and carrying high levels of tolerance to diseases, pests, and other adverse environmental conditions will maximize yield and stimulate multiclone use throughout diverse coffee producing areas.

(b) Processing Benefits

There are several characteristics of coffee that could be altered, resulting in some benefits to the coffee industry: increased total soluble solids, larger and more uniform bean size, bean density and texture, uniform maturation, and diverse maturation cycles.

These new traits could be incorporated into existing coffee varieties through breeding or more rapidly by cellular and molecular methods. An increase in soluble solids would provide greater yields for the soluble coffee industry. Alterations of the carbohydrate metabolism in the coffee endosperm leading to the accumulation of a higher fraction of galacto-mannans would accomplish this goal. Uniform bean size is beneficial during

roasting. Larger bean size commands a higher price in the whole bean market. Bean density and texture can affect coffee extraction yields. Uniform maturation would minimize the presence of immature beans, which gives an off-flavor to the final product. Diverse maturation cycles would also be beneficial to the coffee processor, since it would decrease the underripe and overripe bean fractions with an overall quality improvement. Another indirect benefit of this approach would be the reduction of the off-flavor "Rio Coffee". Rio Coffee develops due to cherry fermentation mainly after contact after fruit abscission. Different maturation cycles would permit the farmer to harvest (manually or mechanically) at peak maturity with minimal risks of abscission of overripe cherries or the presence of immature beans that negatively affect the beverage quality.

(c) Consumer Benefits

Two aspects that will directly affect coffee consumers are coffee quality and price. Improved aroma and taste are always desirable in the coffee market. It has been proposed that increased levels of sulfur-containing amino acids in the coffee bean will contribute to the enhancement of aroma (Shibamato 1991). Enhancement of reducing sugars and oil content also may improve coffee flavor and aroma. Varying levels of organic acids (high to low) would address different consumer requirements. Coffee flavor is very complex (more than 400 peaks have been identified in gas

chromatography) and therefore it will be more difficult to manipulate them. The best approach to ensure premium coffee flavor is to harvest at peak maturity from trees growing in optimum growing conditions (humidity and temperature). Varying caffeine content (high, medium, low and zero) will address some of the consumer needs. Varying caffeine levels would create specialty brands without any extra cost to the processor and without any price or quality penalties to the final consumer.

Coffee quality depend primarily on the genotype, but is also highly susceptible to environmental fluctuations. Plantation management, which determines the microclimate, nutrition level, seed processing methods, and farm storage has a major impact on the final coffee quality. Superior beverage quality is produced from Arabica cultivars grown at higher elevations. Crosses between Arabica and other coffee species confirm that Arabica genes are responsible for superior beverage quality (Carvalho 1988). Postharvest processing and warehouse storage also have a major role in determining final coffee quality.

3 GENETICS AND BREEDING

(a) Genetic Analysis

The scarcity of genetic analysis data for coffee can be understood by the perennial nature of this crop requiring so many years to be accomplished. Although millions of plants have been

carefully checked in nurseries and farms, in search for new variants, only a few were found in the last decades, probably due to the tetraploid nature of Arabica coffee. Attempts to use physical and chemical mutagens to increase the number of variants in *C. arabica* were unsuccessful. Genetic analysis of morphological mutants within *C. canephora* is not available yet but several dozen of wild robusta populations originated from Ivory Coast, Cameroon, and Congo are available in germplasm collections (Charrier and Berthaud 1988).

In *C. arabica* extensive studies have been made by the Institute of Agronomy, Campinas, Brazil to better understand the inheritable variability within this species (Krug and Carvalho 1951; Carvalho 1958, 1988). Initially, the main characteristics of *C. arabica* cultivars were analyzed. Afterwards, factors affecting characters of economic importance, such as plant height and yield potential and more recently, factors related to disease resistance and adverse environmental conditions were studied. Gradually, a *C. arabica* germplasm bank was established with land races, new cultivars, and deviant types found in nurseries and farms, as well as accessions of native germplasm from Ethiopia, the center of origin or diversification of this species.

The primitive Arabica cultivar, botanically described as *C. arabica* var *typica* was used as the standard type in all genetic analysis, allowing the establishment of dominance relationships, epistasis and pleiotropic effects. The relationship of the Arabica and Bourbon cultivars was established. The dominant *T* allele is

present in the Arabica cultivar (*TT NaNa*), while the higher yielding Bourbon has the alleles *tt NaNa*. Crosses of mutants with Murta plants (*tt Nana*) indicate their relationship either to Arabica or to Bourbon primitive cultivars (Carvalho et al. 1991).

It was also studied factors affecting the color of young leaves, fruits and seeds, fasciation of the polysperma cultivar, presence and associated characteristics of mutants with developed sepals in the ripe fruit, leaf shape and size, orientation of plagiotropic branches, flowering habit, size and shape of the trees and defective seed endosperm development. Detailed investigations were done with the so called Laurina mutants (*lr lr*). Besides reducing the caffeine content of the seeds, this factor has a strong pleiotropic effect of the shape of the plant, fruit, seed and length of lateral branches.

Some factors such as maragogipe, mokka and fasciation affect various characteristics. Others seem to have the effect restricted to a single trait like the allele cera which, in triple dosage, changes the normal green endosperm to waxy colored, or erecta that determines an upright orientation of the lateral branches. The four main short structure factors reduce internode length and as a consequence plant height. Caturra (*Ct*), São Bernardo (*Sb*), Vila Lobos (*Vt*) and San Ramon (*Sr*) are dominant, independently inherited and interact with each other in dihybrid combinations in a typical

duplicate dominant epistasis, the presence of one dominant allele at one locus masking the effect of the other. Caturra (*Ct*) was transferred to high yielding cultivars to reduce costs of labor for harvesting (Carvalho 1988).

The cera factor proved to be an invaluable marker to determine the ontogenetic origin of the tissue that forms the seeds as true endosperm. Also due to the xenia effect, it allowed fast and convenient evaluation of the degree of cross pollination under natural conditions. The erecta factor, by reducing the canopy diameter, permits close spacings in the field, allowing for higher plant densities.

Factors controlling resistance to leaf rust disease (*H. vastatrix*), bacterial blight (*Pseudomonas syringai pv garceae*), coffee berry disease (*Colletotrichum coffeanum*), nematodes (*Meloidogyne exigua*) and leaf miner (*Perileucoptera coffeella*) were also investigated, emphasizing their importance in the breeding program. Genetic factors controlling the resistance to leaf rust disease were mainly found in *C. arabica* accessions and in selected *C. canephora* lines. They have been transferred to commercial cultivars, resulting in new resistant germplasms such as Icatu, Catimor Colombia and others. Icatu coffee is of particular value to Brazilian coffee producing regions due to its rusticity, high yield and non-specific resistance to the pathogen.

The determination of the genetics of resistance to coffee berry disease has provided the basis for the development of resistant cultivars, of utmost importance to consumers since

chemicals are normally sprayed throughout the fruit development cycle for controlling the disease. Resistance to nematodes and to leaf miner present respectively in *C. canephora* and *C. racemosa* are being transferred to *C. arabica* and such interspecifically derived germplasms look very promising (Medina et al. 1984).

Though not many coffee genetic factors have been characterized so far, their study has proved to be of great value for assisting coffee breeding programs and undoubtedly provide a significant improvement in our knowledge of the coffee tree.

(b) Breeding

Commercial production of coffee has been greatly improved by plant breeding during the past 50 years. New coffee varieties have been released with high yield, disease resistance (mainly leaf rust and coffee berry disease), insect resistance, nematode resistance and compact stature. In Arabica coffee for instance, the variety Mundo Novo is 300% more productive than the original variety Typica (Carvalho et al. 1952). However, conventional breeding has limitations for coffee improvement because of the genetic barrier of chromosome number (diploids vs. tetraploids), auto-incompatible alleles (diploid species) and the long-term breeding cycles. The release of a new coffee variety has been estimated to require a minimum of 24 years of continuous breeding.

Several breeding methods are being used for *C. arabica* improvement programs which include: (a) plant introduction; (b) artificial crosses from cultivated populations; (c) pedigree method; (d) backcross method; and (e) interspecific crosses (polyploidy and triploid breeding). So far, mutation breeding through seed mutagenesis and pollen irradiation have not been successful.

Introduction and selection were effective for developing commercial cultivars in many Latin American coffee producing countries; Brazil ('Bourbon', 'Caturra', 'Maragogipe', etc.), Costa Rica ('Hybrid Tico', 'San Ramon', 'Vila Sarchi'), Guatemala ('San Bernardo'), El Salvador ('Pacas'), Mexico ('Garnica'), Jamaica ('Blue Mountain') and Puerto Rico ('Columnaris'). Examples for Africa and Asia include the following cultivars: 'Amphillo', 'Geisha', 'Harar', 'Java', 'Kents', 'Mysore', 'SL selections', 'K7', 'Padong', and 'Sudan' (Carvalho 1988).

The artificial crossing method is being utilized to transfer traits of interest, including disease resistance and short stature, to high yielding cultivars. 'Red' and 'Yellow Catuai' are two compact stature cultivars developed by artificial crosses between 'Mundo Novo' (high yielding and tall) with 'Caturra' (compact).

Interspecific hybridization with artificial or spontaneous tetraploids of *C. canephora* has been successfully used by different research groups. In Brazil, a new cultivar is being released ('Icatu') after more than 30 years of breeding and selection. 'Icatu' was developed from a cross between a colchicine-induced

tetraploid of *C. canephora* and *C. arabica* cv. Bourbon, followed by successive backcrosses to high yielding Arabica varieties ('Mundo Novo', and 'Catuai'). The breeding work in Oeiras (Portugal) capitalized on a spontaneous cross between *C. arabica* and a 4X diploid individual ('Timor Hybrid'). This natural hybrid was backcrossed to a high yielding *C. arabica* variety ('Caturra') and the interspecific hybrid population was named 'Catimor'. Seeds from the S3 and S4 generations of 'Catimor' were distributed to coffee producing countries. Locally adapted cultivars ('Catimor' derivatives) are being released after several cycles of selfings and selections. In the Ivory Coast, many interspecific hybrids were produced between *C. arabica* and 4X Robusta and the resulting hybrid population was called Arabusta. Attempts have been made to cultivate selected F_1 Arabusta individuals utilizing several vegetative propagation methods (Charrier and Berthaud, 1988).

The use of triploid breeding strategy has been another alternative to capture existing variability among diploid individuals for Arabica coffee improvement. In Colombia, Orozco (1989) has described excellent advances utilizing 90 *C. canephora* selected trees in crosses with the Caturra variety (*C. arabica*). One benefit of triploids is to increase the probability of genetic recombination during meiosis. At the 3rd generation (F_3 of triploids or S_3 or BC_1), self-compatible, productive, and rust

resistant progenies have been selected. Embryo culture has been used for the recovery of the above interspecific crosses. These results demonstrate the potential success for this breeding approach, especially when combined with embryo rescue techniques.

Breeding strategies for *C. canephora* improvement have to rely on methods for perennial allegamous species where individuals are strongly heterozygous and give rise to heterogenous progenies. The self-incompatibility genes present in Robusta and the life-cycle make very difficult self and sib matings methods for character fixation. The following breeding methods have been proposed for *C. canephora* by Charrier and Berthaud (1988): (a) synthetic and hybrid varieties; (b) clonal selection; (c) reciprocal recurrent breeding; (d) haploidisation; and (e) interspecific hybridization.

4. CELLULAR AND MOLECULAR BIOLOGY

(a) Regeneration via Somatic Embryogenesis

Coffee tissue culture was first reported by Staritsky (1970), who described the presence of somatic embryos from orthotopic shoots of *Coffea canephora*. Herman and Hass (1975) reported the development of organoides from *C. arabica* callus cultures. High and low frequency somatic embryogenesis from coffee leaf explants were first reported by Söndahl and Sharp (1977) in *C. arabica* cv. Bourbon. Further studies with mature leaves of *C. canephora*, *C. congensis*, *C. dewevrei* cv. Excelsa, and *C. arabica* cvs. Mundo Novo,

Catuai, Laurina and Purpurascens demonstrated the occurrence of the high frequency somatic embryogenesis pathway in a wide range of coffee germplasm (Söndahl and Sharp, 1979).

A histological study of somatic embryogenesis from Arabica coffee leaf explants demonstrated that somatic embryos originated from mesophyll cells (Söndahl et al., 1979b). The development of low-frequency somatic embryos (after 70 days in culture) and high-frequency somatic embryos (after 90-120 days in culture) were further characterized by scanning electron microscopy (Söndahl et al., 1979a). The high frequency embryos developed from distinct friable embryogenic tissue composed of small, rounded, cytoplasmically dense cells measuring about 20 μm in diameter. In contrast, neighboring callus cells appeared as long vacuolated cells about 150 μm in length (Söndahl et al. 1979a).

A novel type of somatic embryogenesis has been proposed by Neuenschwander and Baumann (1991) using a modified version of Söndahl and Sharp (1977) two-step method. Leaf callus is produced on the original "conditioning medium" (MSI) containing 2,4-D (4.5 μM) and kinetin (18.4 μM) and then transferred to a liquid "induction medium" (MSII) supplemented with half of the original Söndahl and Sharp (1977) plant growth regulator concentrations (0.23 μM NAA and 2.7 μM Kinetin). The somatic embryos produced after 18-24 weeks in this liquid medium displayed a highly synchronized pattern called Self-controlled Somatic Embryogenesis and the resulting embryos germinated at a rate of 94.5% (Neuenschwander and Baumann, 1991). Data from a total population

of 135,000 embryos produced by the original protocol (Söndahl and Sharp, 1977) yielded a combined recovery rate of 29% for both maturation and germination phases. The above modified process provides a greater improvement for the conversion rate of coffee somatic embryos and so it could be relevant for the development of bioreactor micropropagation methods.

The histogenesis of callus induction and embryogenesis from *C. canephora* stem explants was described by Nassuth et al. (1980). Parenchymatous cells of the cortex contribute to the formation of callus tissues. Somatic embryo-like structures were present in the callus after 14 days of culture. A histological study of somatic embryo differentiation from leaf discs of Robusta was presented by Pierson et al. (1983). Somatic embryos developed within 90 days from leaf explants of Arabusta, an F_1 hybrid of *C. arabica* x *C. canephora* (4X), cultured on a medium rich in cytokinin and without auxins (Dublin, 1981). Later, Hatanaka et al. (1991) described the effect of cytokinins and auxins on somatic embryogenesis of Robusta leaf cultures. Auxins inhibited embryo formation and 5 μM was the optimum cytokinin (2-ip, 6-benzyladenine, kinetin) concentration. The pattern of embryo development described for Arabusta was similar to the low frequency somatic embryogenetic pathway reported from leaf cultures of *C. arabica* (Söndahl and Sharp, 1977). Somatic embryos have also been induced from egg cells of *C. canephora* (35 days following fertilization) after 90 days of primary culture (Lanaud, 1981).

b) Cell Suspension Cultures

Coffee suspension cultures were established with the aim of producing aromatic compounds from suspension cells (Townsley, 1974). Cultures were established from friable callus derived from orthotopic shoots of *C. arabica* cv. El Salvador. These suspension cultures were also used for analyzing caffeine and chlorogenic acid content (Buckland and Townsley, 1975) and to compare unsaponifiable lipids in green coffee beans with cell suspensions (Van der Voort and Townsley, 1975). The maximum caffeine content of a 20 to 26 day-old suspension culture was 0.04% on a dry weight basis, which is substantially lower than the 1.2% caffeine content of Arabica beans (Carvalho, 1988). The low caffeine content should be interpreted with caution, since caffeine increases as the cell density increases (culture aging). In addition, caffeine is water soluble and diffuses into the liquid medium. In vitro caffeine synthesis from coffee cells was considered relatively high when compared with the synthesis of other secondary plant metabolites (Frischkuecht et al., 1977). Additional studies of caffeine synthesis and biodegradation of purine alkaloids in coffee suspension cultures were made by Frischknecht and Baumann (1980), Baumann and Frischknecht (1982) and Baumann et al. (1983). A recent study on purine alkaloid synthesis from *C. arabica* cell suspension found an increase in the production of caffeine (twofold), theobromine (tenfold), and 7-methylxanthine (twentyfold) when cultures were grown under a light (13 h)/dark (11 h) regime in

the presence of adenine (1 mM) and ethylene (5 mM ethephon) (Schulthess et al., 1991). The maximum chlorogenic acid content in *C. arabica* beans ranges from 7.13 to 8.17%. In diploid species, chlorogenic acid ranges is 2 to 70% (*C. salvatrix*) and 10.30% (*C. canephora*) (Carelli et al., 1974). Chlorogenic acid synthesis in *C. arabica* suspension cells is induced by the presence of light (3.25 mM), but it is completely inhibited by the presence of 1.0 to 10.0 mM ethephon (Schulthess et al., 1991). Cell suspension cultures have been established from embryogenic tissues of *C. arabica* cv. Mundo Novo and plantlets suitable for clonal propagation were recovered (Peña, 1984).

(c) Culture of Embryos, Endosperm, Anthers and Peristem Tissues

Embryos of *C. canephora* x *C. dewevrei* were successfully established by Colonna et al. (1971). Callus from endosperm tissues of *C. arabica* was induced by Keller et al. (1972) with the objective of studying caffeine synthesis. Abundant friable callus from perisperm tissues of *C. arabica* and *C. stenophylla* was induced by Monaco et al. (1977). Perisperm callus proliferated rapidly in the absence of auxin, suggesting that this tissue was not auxin dependent.

Sharp et al. (1973) cultured somatic and haploid tissues of *C. arabica* and obtained callus growth from petioles, leaves and green fruits, proembryo formation from anthers and shoot development (orthotropic shoots). Anthers from several *Coffea* species have been cultured. Friable callus development was observed only with *C. liberica* (Monaco et al., 1977). Anther culture can facilitate the production of homozygous plants (dihaploids) in one single generation, and can provide access to new recombinant forms (gametic array). The availability of dihaploid coffee lines would shorten the time for the development of a "true hybrid seed system" in coffee (7 years vs. 24 years).

(d) Protoplast Culture

Protoplasts have been isolated from leaf-derived friable callus of *C. arabica* cv. Bourbon. Microcolonies were recovered following wall regeneration and cell division in about 30% of the cultures (Söndahl et al., 1980). Small colonies from coffee protoplasts isolated from young leaves have also been reported (Orozco and Schnieder, 1982), but these microcolonies did not survive the first subculture onto semi-solid medium.

Protoplast isolation and embryo development from the Robusta genotype was reported by Schoepke et al. (1987). More recently, protoplast regeneration from coffee embryogenic suspension cultures has been described by several groups (Acuna et al., 1991; Barton et al., 1991; Spiral and Petiard, 1991). Acuna and Peña (1991)

described the production of microcolonies from isolated protoplasts after several subcultures on medium containing 0.5 mg/l 2,4-dicholorophenoxyacetic acid (2,4-D), 6-benzladenine (BA) and naphthaleneacetic acid (NAA). Upon transferring protoplast derived calli to hormone-free medium, somatic embryos were recovered which developed normally and germinated. Spiral and Petiard (1991) successfully utilized embryogenic suspensions of Arabica, Robusta, and the interspecific hybrid Arabusta for protoplast isolation and somatic embryo regeneration. Microcolonies were obtained on modified Blaydes medium with 2,4-D, kinetin, and NAA (Blaydes, 1966). Somatic embryos differentiated 8-9 months later on Yasuda medium with BA (Yasuda et al., 1974). It is clear that embryogenic coffee cell suspensions have been essential for coffee protoplast regeneration. The availability of a repeatable protoplast regeneration system for commercial Arabica and Robusta cultivars will be critical for future coffee improvement via somatic hybridization and DNA uptake.

(e) Transformation

Coffee protoplasts were co-cultivated with *Agrobacterium* tumefaciens carrying NPT II and ß-glucuronidase marker genes under control of CaMV 35s promoter. Transient transformation was demonstrated by the GUS histochemical assay on callus tissues derived from treated protoplasts (Spiral and Petiard, 1991). Protoplasts from Arabica suspension cultures were treated by

electroporation in the presence of a plasmid carrying the NPT II marker gene. Callus, embryos, and plantlets were derived from the electroporated protoplasts. Experiments are currently being done to confirm the incorporation of the NPT II marker in the coffee tissues (Barton et al., 1991).

(f) Bioreactor Micropropagation

Initial attempts to develop micropropagation protocols for coffee plants were based on the development of arrested orthotropic buds present at the main axis. Cultures of nodal orthotropic explants of *C. arabica* were reported to regenerate an average of 2.2 plantlets (Custer et al., 1980). In vitro axillary bud development of *C. arabica* cv. Mundo Novo and two interspecific hybrids (*C. canephora* x *C. eugenioides*; *C. congensis* x *C. eugenioides*) produced 4.5 plants per node after 16 weeks in culture (Söndahl, 1982; Söndahl and Nakamura, 1980). Studies on vegetative propagation of Arabusta led to isolation of shoots following the development of arrested buds (Dublin, 1980). A long-term program has been devoted to apply nodal culture method for large scale coffee propagation (Berthouly et al. (1987). It has been reported a yield of 7-9 shoots per cultured node explant of *C. arabica* cv. Catimor every 12-14 weeks. The culture medium consisted of MS salts supplemented with BA (5 μM), indolebutyric acid (0-2.5 μM), citric acid (50-250 mg·litre^{-1}), ascorbic acid (100-300 mg·litre^{-1}) and cysteine (100-300 mg·litre^{-1}). Apical meristems of *C. arabica*

have been cultured with the aim of germplasm cryopreservation (Kartha et al., 1981) or as an alternative propagation method (Zok, 1985).

Recent progress has been made for the development of a coffee mass propagation method via somatic embryogenesis in liquid cultures. Embryogenic cell suspensions of Arabica and Robusta coffee were established in Erlenmeyer flasks. Somatic embryogenesis was highly dependent on cell density. High density was inhibitory to growth, but an optimum density of 1.0 g f.w. $litre^{-1}$ yielded 460,000 somatic embryos per litre after 7 weeks. The projected yield for a 3 litre bioreactor charged with 3 g f.w. embryogenic cells is approximately 600,000 embryos every 2 months of culture (Zamarripa et al. 1991).

Micropropagation of a high-value perennial species like coffee can be an efficient method for propagating individual trees from a segregating population. Micropropagation reduces the time for varietal development and preserves heterozygosity and plasticity in coffee plantations. Micropropagation in coffee must be accomplished via somatic embryogenesis to be competitive in time and cost.

(g) Somaclonal Variation

Somaclonal variation explores the naturally occurring or in vitro-induced variability of somatic cells following plant regeneration (Larkin and Scowcroft, 1981). Most of this

variability is due to chromosome alterations, e.g., breakages, translocations, deletions, aneuploidy, polyploidy, gene amplification, transposons, somatic crossing-over and point mutations (Evans and Sharp, 1983). Somaclonal variation is an excellent method for shortening breeding programmes, since it can provide access to genetic variability within existing cultivars (Evans and Sharp, 1986). Somaclones carry few genetic alterations, such that the genetic integrity of the commercial variety is preserved.

Different agronomically important genotypes of coffee are being utilized for a somaclonal variation study including tall stature varieties ('Yellow Bourbon', 'Mundo Novo', and 'Icatu') and short stature varieties ('Red' and 'Yellow Catuai', 'Caturra', 'Catimor', 'Aramosa', and mixed genotypes). Somaclones were derived via high frequency and low frequency somatic embryogenesis pathways (Söndahl et al., 1984; Söndahl and Sharp 1977). Plantlets with 6-8 pairs of leaves were adapted to a field nursery and 6 to 9 months later were transplanted to the field. Control plants of donor genotypes were established at the beginning and end of each somaclone row and as borders. So far, screening for variability is being made at the R_0 generation, during the first harvest. Progenies of different classes of variability are being made for genetic analysis of the most interesting somaclonal mutations.

More than 16,000 somaclones and controls were established in the field. To date, ca. 12,000 (78%) of these coffee somaclones have been screened for variability after 2 to 4 years under field

conditions. Any visual deviation from the normal control genotype was tabulated and a total of 1,196 variants (10%) were recorded. Among the several classes of variability observed, the highest frequencies were found for cherry color (red to yellow 42.3%) and tall to compact stature (3.8%). Cherry color is a trait controlled by a semi-dominant gene (Carvalho, 1958): deep red (X_cX_c), pale red (X_cx_c), and yellow (x_cx_c). This example illustrates the tendency to detect changes from recessive to a semi-dominant or dominant state. However, traits described as dominant or semi-dominant also shifted to the recessive phenotypic expression, e.g., compact stature (dominant) shifted to tall stature (1.3%), red (semi-dominant) to yellow cherry color (1.2%) and normal green leaves (dominant) to purpuracens leaves (0.3%). One maragogype mutation (Mg - characterized by large beans and leaves) was observed among somaclones derived from 'Yellow Catuai'. One sectorial chimera was observed in one somaclone from 'Catimor'. Another group of somaclones were observed with variability for traits like maturity, susceptibility to ants, susceptibility to *Cercospora*, resistance/susceptibility to leaf rust and bean size.

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