Coffee biotechnology

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In the last three decades, interest has turned to *in vitro* cell culture in different areas of coffee research. *In vitro* techniques have been applied not only for coffee improvement through genetic transformation but also to study various aspects in coffee cells such as chemical (caffeine synthesis and the production of coffee aroma), physiological and more recently, biochemical aspects. The most important advances obtained to date on *in vitro* coffee techniques in fields like biochemistry, physiology, regeneration systems and genetic engineering, are presented and discussed.
Keywords: *Coffea*, coffee, genetic transformation, *in vitro* tissue culture, regeneration, somatic embryogenesis.

Biotecnologia do café: Nas últimas três décadas tem sido dada atenção para a cultura de células *in vitro* em diferentes áreas da pesquisa com café. Técnicas i*n vitro* têm sido aplicadas não somente para o melhoramento do café, através da transformação genética, mas também para o estudo de vários aspectos, tais como químicos (síntese de cafeína e a produção de aroma), fisiológicos e, mais recentemente, bioquímicos. Os avanços mais importantes obtidos sobre técnicas de cultivo *in vitro* de café em áreas como bioquímica, fisiologia, sistemas de regeneração e engenharia genética serão apresentadas e discutidas.
Palavras-chave: *Coffea*, café, cultivo *in vitro*, embriogênese somática, regeneração, transformação genética.

INTRODUCTION

Coffee, one of the most widely traded commodities in the international markets, is an agricultural crop of significant economic importance. Coffee is grown in 80 countries around the world with 70 % being produced by smallholder farmers. Besides being a source of income for millions of people, it represents a generation of foreign currency.

Out of more than 100 coffee species only two are commercial species: *Coffea arabica* L. (arabica type coffee) and *Coffea canephora* p. ex Fr. (canephora or Robusta type coffee). Arabica type coffee is typical of the highland growing regions and is responsible for almost 75 % of world production while the remaining 25 % is occupied by the Robusta type coffee which is grown in lowland regions.

Between these two species, Arabica coffee provides a superior beverage but is quite sensitive to different pests (fungi, nematodes, and insects), whereas Robusta coffee produces a lower quality coffee but is more resistant to pests. While it would be desirable to combine these genetic traits, traditional plant breeding techniques have been largely unsuccessful since *C. arabica* is tetraploid whereas the other species are diploid. On the other hand, *C. canephora* and other non-arabica species are self-incompatible. As a result, the transfer of genetic traits from wild outbred species of the genus to the cultivated *C. arabica* cultivar is quite difficult. A further complication is *Coffea*'s lengthy period for fruit development and the bean-to-bean generation time. A breeding cycle for coffee plants takes four years, i. e., seeds to a flowering plant and back to seeds. Selections for yield are usually made from 6-8 year old coffee plants.

Several cycles of crossings and selections are required until one finds superior genotypes. In order to assure fidelity through seed propagation, six cycles of selfing are required. Considering one cycle for hybridization and selection and six cycles for seed homozygosity, a normal coffee breeding program requires 28 years. All these inconveniences make such traditional approaches costly and time consuming. Advances in plant cell culture techniques provide opportunities to shorten the time required for coffee improvement.

Fortunately, the last decades have been eyewitness of the development of new technological tools which introduce a huge potential in plant growth improvement. In addition to the conventional growth improvement, these techniques can accelerate the release of varieties with new traits. Plant biotechnology offers several possibilities for increasing productivity, diversification and production. This technology includes plant tissue culture techniques, the use of advanced molecular biology techniques for plant transformation, genomic analysis coupled with breeding and plant-disease diagnoses. This group of new techniques has been added to the toolbox to strengthen programs in order to obtain better crops. The goal is still the same as for the traditional ones: to improve traits in a way that resulting plants attain superior agronomical traits but in a faster and efficient way.

Among factors limiting coffee production, there are the different susceptibilities to several diseases, photosynthetic efficiency, water utilization and tolerance to soil acidity and to aluminum.

Plant tissue culture has allowed a mass production of plants, increasing the capacity of plant multiplication with specific agronomical traits. It has also been a major tool for basic and practical investigation in agriculture. These new developments include clonal propagation, cell suspension culture, anther/pollen culture, embryo culture, protoplast isolation and regeneration, and the development of new interspecific plantlets *in vitro*. Furthermore, these *in vitro* techniques have allowed studies at the cellular and molecular levels making the coffee plant a "model" among the woody species. This "model plant" has much potential in molecular biology, biochemical and biotechnological research.

In vitro tissue culture methods in coffee

Plant regeneration via tissue culture should be very effective for propagation and improvement of coffee plants. Although the main goal of early studies was to establish protocols to regenerate coffee species, the development of these protocols and the recent advances made it possible to apply these techniques for the study of various other aspects.

The first requirement to regenerate a large number of plants is to obtain a large population of cells. This is accompanied by removing a small portion of the plant (an explant) and placing it in liquid or on solid medium. This medium should contain various nutrients and hormones sufficient for the cells to divide and reproduce and eventually to form a large collection of cells (suspensions if liquid medium, calli if solid medium). After an appropriate time, when this primary culture is large enough, it can be subdivided and/or transferred to another medium depending on the type of regeneration required.

Callus Cultures: Callus formation from explants is occasionally spontaneous, but generally requires an auxin in the medium, often in combination with cytokinin. The pioneer report on coffee tissue culture was done by Staritsky (1970) who used internode segments of young orthotrophic shoots as explants. He obtained fast growing callus using a modified Linsmaier and Skoog medium (1965) supplemented with sucrose (30 $g.L^{-1}$), thiamine HCl (1 $mg.L^{-1}$), L-cysteine HCl (10 $mg.L^{-1}$), *myo*-inositol (100 $mg.L^{-1}$), kinetin (0.1 $mg.L^{-1}$), 2,4-dichlorophenoxyacetic acid (2,4-D, 0.1 $mg.L^{-1}$) or naphthalene acetic acid (NAA, 1 $mg.L^{-1}$).

Later, Sharp et al. (1973) established callus from seeds, shoots, leaves and anthers of *Coffea arabica* and used the same salt medium but increased some of the organic components to establish these cultures. They found conditions (absence of illumination and a temperature of 28°C) in which callus cells proliferate more efficiently. Monaco et al. (1974) obtained a rapid cell proliferation at the surface of explants from developing fruits with immature seeds of *Coffea arabica* and *Coffea stenophylla* that were grown in the dark at 25-28°C and in medium used by Staritsky (1970) but in the absence of 2,4-D.

They observed differences in the rate of cell proliferation and texture of cultured perisperm tissues that varied from a white friable callus to a cream colored solid callus. These early studies mentioned above were the basis for subsequent work and opened the possibility of applying these kinds of techniques in the formulation, development and improvement of procedures to induce the morphogenetic potential in other coffee species. Thereafter, Herman and Haas (1975) obtained clonal propagation of *C. arabica* from callus culture through organoid formation; and Söndahl and Sharp (1977; 1979) established conditions for formation of somatic embryos from auxin-induced leaf callus of *C. arabica* using media with different combinations of auxins and cytokinins.

Cell suspension and Protoplast Cultures: The establishment of cell suspension cultures has been used in coffee for different purposes, such as an interphase for obtaining somatic embryos, protoplast isolation, plant regeneration, etc. Although Keller et al. (1972) reported production and release of caffeine from primary callus cultures derived

from endosperm and pericarp of *C. arabica*, the first coffee suspension cultures were established with the aim to study the production of aromatic compounds from cells (Townsley, 1974). Cultures were initiated from friable callus derived from orthotropic shoots of *C. arabica*. Later, these same suspension cultures were used to analyze caffeine and chlorogenic acid contents (Buckland and Townsley, 1975) and to compare unsaponifiable lipids in green beans with those in cell suspensions (Van der Voort and Townsley, 1975). Additional studies of caffeine synthesis and biodegradation of purine alkaloids in coffee suspension cultures were made by Frischknecht and Baumann (1980), and Baumann et al. (1983).

A number of papers have been published on various aspects of the isolation and culture of coffee protoplasts. Söndahl et al. (1980), Orozco and Schieder (1984), and Acuña and de Peña (1987) isolated protoplasts from leaf callus, leaves, and cell suspensions, respectively. In all cases, cell wall formation, cell division, and callus formation was observed but in no case did regeneration of plants occur, which complicates the production and culture of protoplasts from coffee cells. Schöpke et al. (1987) reported somatic embryogenesis and plantlet regeneration in protoplasts isolated from cell suspension-derived somatic embryos of *C. canephora*. Only some of the embryos could be regenerated to plantlets when globular embryos were subcultured on a medium devoid of growth regulators.

In other studies, Schöpke et al. (1988) and Schöpke (1989) isolated protoplasts from cell suspensions of *C. arabica*, *C. canephora*, *C. salvatrix*, and *C. racemosa.* In these cases, the protoplasts regenerated cell walls and a few divisions occurred, but the cells did not survive more than 10 days in culture. After these initial successes, regeneration from protoplasts of *C. arabica* was achieved by other workers. Acuña and de Peña (1991) also reported plant regeneration from protoplasts of embryogenic cell suspension of *C. arabica* Caturra. In this report, from a total of 65 somatic embryos, 35 were transferred directly to nonsterile soil in the greenhouse. They also found that the total process of regeneration of plants from protoplasts took an average of 9 to 10 months.

Spiral and Pétiard (1991) regenerated plants from protoplasts of *C. arabica, C. canephora* and from the interspecific hybrid Arabusta. Grèzes et al. (1994) optimized conditions for the isolation of protoplasts from non-embryogenic cell suspensions of *C. arabica*. They described experiments to improve the yield of viable protoplasts, by analyzing the different parameters (enzymatic treatments, plasmolysis, physiological state of the cells or temperature) influencing the release of coffee cell protoplasts. A number of viable protoplasts ranging from 3.5 x 10^6 to 4.6 X 10^6 protoplasts per gram of fresh weight was obtained corresponding to a 10- to 15-fold increase of the protoplast yield obtained by Acuña and de Peña (1991). Tahara et al. (1994) reported a simplified method for preparing and culturing protoplasts from embryogenic callus induced from young leaves of mature *C. arabica* trees, and subsequent formation of somatic embryos from these protoplasts with cytokinin as the only plant growth regulator.

Among the several procedures reported for the regeneration of coffee protoplasts, there are great differences, especially with regard to the growth regulators and the cultured media used. Flexibility towards growth regulators can be seen in protoplast cultures that resulted in the regeneration of plantlets. The embryogenic tissue used for protoplast isolation can be induced either by an auxin/cytokinin mixture (Schöpke et al. 1987; Schöpke, 1989; Spiral and Pétiard, 1991) or by a cytokinin alone (Yasuda et al., 1986; Acuña and de Peña, 1991; Tahara et al., 1994). Correspondingly, the media used for protoplast culture contain an auxin/cytokinin mixture or a cytokinin, except for the medium used by Acuña and de Peña (1991) who induced embryogenic tissue with 6-benzylamino purine (BAP) alone, and then cultured protoplasts isolated from this tissue in a medium containing a mixture of 2,4-D, NAA, and kinetin.

Somatic embryogenesis: Somatic embryogenesis is a valuable tool for the clonal propagation of *Coffea* species and for the breeding for disease resistance, stress tolerance, low-caffeine content, etc. Early studies have demonstrated that the presence of an auxin is critical for embryo initiation and decreasing the auxin concentration or its complete absence promoted maturation. Thus, in general terms, the established basic protocol involves culture in a primary medium with an auxin source and subculturing in a secondary medium devoid of growth regulators, both containing a substantial supply of reduced nitrogen (Ammirato, 1983).

In coffee, there are a number of reports on *in vitro* plant regeneration through somatic embryogenesis from stem, leaf tissues and perisperm tissue (Staritsky, 1970; Söndahl and Sharp, 1977; Dublin, 1981; Yasuda et al., 1985; de García and Menéndez, 1987; Raghuramulu et al., 1987; Neuenschwander and Baumann, 1992; Söndahl and Lauritis, 1992; Sreenath et al., 1995). The majority of culture

media used for the induction of somatic embryogenesis in coffee contained a mixture of an auxin and a cytokinin. However, Dublin (1981) showed that it is possible to induce embryogenesis in leaf explants with a cytokinin (BAP) alone. This observation was confirmed by Yasuda et al. (1985) and Hatanaka et al. (1991), who reported that cytokinin is very important and auxins have inhibitory effects towards *Coffea* somatic embryogenesis.

Yasuda et al. (1995) established somatic embryogenesis in *C. arabica* and *C. canephora* from leaf explants of mature trees using cytokinin as a sole plant hormone. Both species reacted in different ways. In *C. canephora*, somatic embryos formed from the cut edges of cultured young leaf explants in contact with cytokinin of the medium. Addition of auxin with cytokinin inhibited embryo formation. Somatic embryos were grown to young plants on the cytokinin medium. In *arabica*, embryogenic callus was induced after prolonged culture with cytokinin and then somatic embryos formed on the embryogenic callus.

Leaf pieces of *arabusta* coffee trees can be induced to form embryos directly when cultured on a basal MS medium devoid of auxin and containing high levels of cytokinin. By this means, somatic embryogenesis of coffee has been obtained in single-step (Dublin, 1981; Yasuda et al., 1985; Hatanaka et al., 1991; Yasuda et al., 1995) and double-step procedures (Söndahl and Sharp, 1977; Dublin, 1984; Zamarripa et al., 1991; Neuenschwander and Baumann, 1992). The single-step procedure has the advantage of rapid embryo formation and high embryo germination rate. The double-step procedure yields high frequency somatic embryogenesis (HFSE) which has clear implications with regard to large scale production of somatic embryos. In addition, for some genotypes, the HFSE process may be the sole procedure for obtaining somatic embryogenesis.

High frequency somatic embryogenesis (HFSE) from coffee leaf explants was first reported by Söndahl and Sharp (1977) in *C. arabica* cv. Bourbon. HFSE is characterized by the abundant appearance of friable, highly embryogenic callus. The experimental procedure describes two successive media that were necessary: "the conditioning medium" and the "induction medium". Successful induction of high frequency embryogenic calluses was found to be dependent on the 2,4-D and kinetin concentrations in the first medium (conditioning medium).

The specific nature of HFSE-callus permits its use in liquid culture systems. Neuenschwander and Baumann (1992) described a protocol for somatic embryogenesis in liquid culture. They proposed the term self-controlled somatic embryogenesis (SCSE) because the new somatic embryos germinated at a rate of 94.5 % without the need of a maturation step. Yields and germination rates of HFSE were markedly lower as compared to SCSE. The production of somatic embryos in liquid medium has been applied as an approach for a rapid mass propagation of coffee plants (Zamarripa et al., 1991; Ducos et al., 1993; Noriega and Söndahl, 1993; van Boxtel and Berthouly, 1996).

Zamarripa et al. (1991) reported that the production of somatic embryos is highly dependent on the inoculum density, the production and development of embryos being inhibited when the inoculum density is high. This inhibition is partially suppressed when the medium is periodically renewed. At a low inoculum density of 1 g $FW.L^{-1}$, 460,000 embryos were produced in 7 weeks in Erlenmeyer flasks; in a bioreactor, the production was about 4,000 embryos per liter per day. Ducos et al. (1993) achieved 600,000 coffee somatic embryos per liter using a stirred bioreactor, and Zamarripa (1993) described the feasibility of scale-up of somatic embryogenesis in *C. canephora* and Arabusta reporting yields of 400-500 x 10^3 embryos after 7 weeks. The plantlet regeneration capacity was measured. Starting with 1 g of callus per liter of medium, 56,000 plantlets could be regenerated (Ducos et al., 1999).

Berthouly et al. (1995) and Etienne et al. (1997) have developed a somatic embryo mass production system using a temporary immersion bioreactor. This system includes the use of immersion techniques with specially adapted culture vessels ($RITA^R$) which allows the direct regeneration, in the same container and without subculture, of plantlets from cell suspensions. They obtained a high frequency of embryogenic tissues, cell suspensions and regeneration of plants. Using germinated somatic embryos of *Coffea arabica*, direct sowing resulted in a highly successful conversion of embryos into plants. A culture density above 1600 embryos per liter in a bioreactor positively affected embryo morphology by causing higher embryonic axis elongation (Barry-Etienne et al., 1999). Such embryos showed better plant conversion rates after direct sowing in the nursery.

Nevertheless, during the first months in the nursery, the plantlets derived from embryos produced under temporary immersion exhibited a low and slow growth when compared to seedlings (Barry-Etienne et al., 2002). Recently, mass regeneration of *C. arabica* L. somatic embryos using a temporary immersion bioreactor was improved by optimizing the immersion cycles. It was demonstrated that increasing

the frequency of short immersions (1 min immersions every 24, 12 and 4 h) stimulated embryo production (480, 2092 and 3081 embryos per liter bioreactor, respectively) and improved quality (60, 79 and 85% of torpedo-shaped embryos, respectively) (Albarrán et al., 2005).

Direct embryogenesis can be induced on certain explants. Direct somatic embryogenesis is the formation of somatic embryos from the explant without the formation of an intermediate callus phase (Raghavan and Sharma, 1995). In most plants, direct somatic embryogenesis is difficult to obtain. Loyola-Vargas et al. (1999) have reported direct somatic embryogenesis from explants of leaves in *Coffea arabica* and it was supported by histological evidence. They modified the protocol described by Yasuda et al. (1985). Browning of the tissues, caused by an excessive accumulation of phenolic compounds, is necessary for the somatic embryogenesis process in coffee (Quiroz-Figueroa et al., 2002). Similar observations have been reported by other authors (de García and Menéndez, 1987; Neuenschwander and Baumann, 1992; van Boxtel and Berthouly, 1996; Menéndez-Yuffá and de García, 1997).

Somatic embryogenesis in coffee has been the subject of several histological studies (Söndahl et al., 1979a, b; Nassuth et al., 1980; Michaux-Ferrière et al., 1987, 1989; Nakamura et al., 1992; Tahara et al., 1995; Menéndez-Yuffá and de García, 1997; Quiroz-Figueroa et al., 2002). Histogenesis of indirect somatic embryogenesis of *C. arabica* has been described by Söndahl et al (1979a); they demonstrated that callus proliferation, in which some of the cells differentiate and develop into embryos, originated from spongy mesophyll cells. Nassuth et al. (1980) observed that all parenchymatic tissues between epidermis and vascular cambium were capable of being transformed into callus tissue, the cortex being the most active layer in this respect. After 14 days of culture, proembryos were observed in the zone of callus formation. Scanning electron microscopy observations (Söndahl et al., 1979b; Nakamura et al., 1992) revealed very young embryos with a mixture of rounded, elongated or curled cells of the explant.

While these studies have provided valuable information, they did not clearly demonstrate embryo development from one single cell to the final stages. Michaux Ferrière et al. (1989), and Menéndez-Yuffá and de García (1997) provided evidence for the hypothesis that somatic embryos in *Coffea* have unicellular origins. In both reports, the main stages of ontogenesis were not observed. Quiroz-Figueroa et al. (2002) demonstrated that the sequential events in embryo development arise through symmetric unicellular pathways. They concluded, on basis of their histological observations, that both direct and indirect somatic embryos of coffee formed on explanted leaf segments and callus, respectively, have a unicellular origin.

Physiological and biochemical advances

Coffee, as one of the most important major economic crops in the world, has been the subject of extensive research related to genetic improvement aimed at increasing yield. On the other hand, knowledge about the physiological and biochemical processes involved is scarce. The few studies carried out in this area can be resumed in three topics: the biosynthetic pathway of theobromine and caffeine, the changes during somatic embryogenesis, and the toxicity of aluminum on *in vitro* coffee cultures. Therefore, in order to enlarge our perception of basic scientific knowledge and to understand different mechanisms at the cellular and molecular level it is imperative that new research fields be opened up on these subjects.

Early studies related to the purine alkaloid (PA) production and metabolism in *in vitro* cultures of *Coffea* spp., including *C. arabica*, have been reported (Baumann and Frischknecht, 1988). Cell cultures of coffee were obtained which maintained the ability to produce caffeine and theobromine and to release these purine alkaloids into the medium (Keller et al., 1972; Waller et al., 1983). Besides PA, sterols, fatty acids, chlorogenic acids, and coffee aromatics were also produced (Townsley, 1974; Van de Voort and Townsley, 1975; Buckland and Townsley, 1975). Furuya et al. (1990) have demonstrated that a polyurethane foam cube was eventually capable of immobilizing more than 10 g (fresh weight) of coffee cells in long-term culture. Coffee cells in suspension culture were capable of biotransforming theobromine to caffeine (Furuya et al., 1991).

Based on recent knowledge of enzymology and molecular biology, it was proposed that caffeine is synthesized through multiple methylation of xanthine derivatives (Ashihara and Crozier, 2001). Enzymatic activities of N-methyltransferase for caffeine biosynthesis in coffee plants have been detected in cell-free extracts prepared from cultured cells (Baumann et al., 1983). N-methyltransferase proteins have also been purified (Mazzafera et al., 1994; Mösli Waldhauser et al., 1997; Moisyadi et al., 1998). Subsequently, cDNAs for 7-methylxanthine methyltransferase (MXMT or theobromine synthase) were successfully cloned from coffee plants (Ogawa et al., 2001; Mizuno et al., 2003).

Several studies have been reported concerning the modification of the embryogenic response in *Coffea* spp. Somatic embryogenesis of *C. arabica* was induced by the nitrogen source. The optimum nitrogen concentrations were between 3.75 and 15 mmol.L^{-1} nitrogen with a nitrate/ammonium molar ratio of 2:1 or 1:2 (Fuentes-Cerda et al., 2001). Also, the use of salicylic acid had a positive effect on cellular growth and somatic embryogenesis, causing a two-fold increase in both processes (Quiroz-Figueroa et al., 2001). The protein electrophoretic patterns during embryogenesis in *C. arabica* were determined and revealed qualitative and quantitative differences in size and charge (Menéndez-Yuffá et al., 1994). The two-dimensional analysis of embryogenic calli revealed seven characteristic polypeptides with a molecular weight of 23 to 35 kDa in a broad pI from acid to basic. Five of them were found in the neutral to acid pI. In the nonembryogenic calli, seven distinctive polypeptides were present in the range of 15 to 70 kDa. Four of the polypeptides were acidic, three of 70 kDa and one of 15 kDa. However, the identification and properties of these proteins remains to be determined in order to understand their role in somatic embryogenesis.

In some tropical soils, heavy metal toxicity causes reduced growth and yield losses. Coffee plantations are mainly confined to acid soils, where several toxic forms of aluminum (Al) are present. Using as a model suspensions of *C. arabica* cells (Martínez-Estévez et al., 2001a), and in view of the suggestion that Al disrupts the metabolism of membrane phospholipids, the effect of $AlCl_3$ on different components of this pathway has been studied. When suspension cells were incubated with increasing concentrations of $AlCl_3$ (200-1000 μmol.L^{-1}), the protein phosphorylation pattern changed. The phosphorylated proteins with a molecular mass of 18, 31 and 53 kDa increased dramatically after *in vivo* treatment of cells with $AlCl_3$. When $AlCl_3$ was added to an *in vitro* phosphorylation reaction, no differences in phosphorylation were observed (Martínez-Estévez et al., 2001b). These results suggested that *in vivo* treatment of *Coffea* cells with $AlCl_3$ affects the activity of some protein kinases. The effect of Al on phosphoinositide-specific phospholipase C (PLC) and lipid kinase activities also was examined (Martínez-Estévez et al., 2003). Two main effects were seen when cells were treated with $AlCl_3$. In periods as short as 1 min, Al-exposed cells increased their activity of PLC. Over longer periods, PLC activity was inhibited by more than 50%. The activity of phosphatidylinositol 4-kinase, phosphatidylinositol phosphate 5-kinase and diacylglycerol kinase increased when cells were incubated in the presence of different concentrations of $AlCl_3$. These reports strongly support the hypothesis that Al disrupts the metabolism of membrane phospholipids regulating not only PLC but also other enzymes that have key roles in signal transduction pathways. Despite the research done in this field, we still need to understand the biochemical and molecular basis of Al-toxicity in coffee. This becomes particularly important for some countries like Brazil where acid soils are a major problem for coffee productivity.

Transformation of coffee

To establish a genetic transformation system a competent explant is required for the transformation process, together with an *in vitro* culture system that permits a high frequency of regeneration. Furthermore, a system for genic transfer is required that is simple, cheap, and reproducible. In coffee, there are efficient regeneration systems through somatic embryogenesis from several tissues and the genetic transformation of coffee plants has been achieved successfully by several research groups (Barton et al., 1991; Spiral et al., 1993; Sugiyama et al., 1995; van Boxtel et al., 1995; Hatanaka et al., 1999; Spiral et al., 1999; Leroy et al., 2000; Fernández-Da Silva and Menéndez-Yuffá, 2003; Ogita et al., 2004).

Several studies in coffee have indicated that some *in vitro* tissue cultures of coffee showed tolerance to high kanamycin concentrations (400 mg.L^{-1}) (Spiral et al., 1993; Giménez et al., 1996). In order to develop a selective growth system for genetically transformed coffee tissues, van Boxtel et al. (1997), studied the effects of the selective agents chlorsulfuron, glufosinate, glyphosate, hygromycin, and kanamycin on callus development from leaf explants and embryogenic suspension cultures. Chlorsulfuron and hygromycin caused strong inhibition and severe necrosis, whereas glyphosate and kanamycin showed variable inhibition. Glufosinate appeared to efficiently inhibit growth of both leaf callus and callus suspensions of all genotypes tested without inducing necrosis, indicating their potential for the detection of stably transformed coffee tissues.

Two kinds of transformation techniques have been tested: the indirect *Agrobacterium*-mediated transformation (which is the technique currently used to transfer genes in coffee plants) and direct transformation, through particle bombardment and electroporation of tissues.

Indirect gene transfer: Ocampo and Manzanera (1991), using wild-type *Agrobacterium* strains, observed the production of tumors on infected hypocotyls of *in vitro* germinated coffee

seeds. Spiral and Pétiard (1991) obtained preliminary results using protoplast co-culture with different *Agrobacterium* strains carrying neomycine phosphotransferase (NPTII) and β-glucuronidase (GUS) marker genes under control of the CaMV35S promoter. They observed transient expression by GUS histochemical assay on callus tissue derived from the treated protoplast.

Transformed plantlets of *Coffea canephora* were obtained by regeneration of somatic embryos infected with *Agrobacterium rhizogenes*. Integration of NPTII and GUS genes were demonstrated by polymerase chain reaction (PCR) and β-glucuronidase assay (Spiral et al., 1993). Sugiyama et al. (1995), after transforming *Coffea arabica* using the Ri plasmid of *Agrobacterium rhizogenes* strain IFO 14554, suggested that this kind of transformation is applicable to the production of plantlets with a phenotype of foreign genes.

The production of transgenic plants by cocultivating somatic embryos of *C. canephora* with *A. rhizogenes* (A4) and disarmed *A. tumefaciens* (LBA4404) was reported by Leroy et al., (1997). Hatanaka et al. (1999) reported the successful genetic transformation of *C. canephora* using *A. tumefaciens* EHA101 harboring pIG121-Hm from embryogenic calli. Somatic embryos were germinated and regenerated to small plantlets which were then transferred to medium containing both 100 $mg.L^{-1}$ hygromycin and 100 $mg.L^{-1}$ kanamycin for final selection of transgenic plants. The selected plantlets exhibited strong GUS activity in leaves and roots. Furthermore, the GUS and hygromycin phosphotransferase (HPT) genes were confirmed to be stably integrated into the genome of the coffee plants by PCR.

Leroy et al. (1999; 2000) reported the first transformation of coffee plants containing a *Bacillus thuringiensis* gene which had been integrated into the coffee genome. A synthetic gene encoding for a *B. thuringiensis* CryIAc endotoxin, which is active against the coffee leaf miner *Perileucoptera coffeela*, was introduced into three genotypes of the two cultivated species, *C. arabica* and *C. canephora*, conferring insect resistance. Somatic embryos were co-cultivated with the LBA4404 strain of *Agrobacterium tumefaciens* containing the *cry*1Ac gene. More than 100 transformed plants from independent transformation events were obtained for each coffee genotype. The integration and expression of the *cry*1AC gene was studied by western blotting analyses. Transgenic plants demonstrated effective resistance to leaf miner bioassays with the insects.

Direct gene transfer: The first genetic transformation of coffee cells reported was by protoplast electroporation. Barton et al. (1991) obtained plantlets of *Coffea arabica* genetically altered. They established suspension cultures to obtain protoplasts which were transformed with a kanamycin-resistance gene by an electroporation procedure. Embryos were formed from transformed cells and regenerated into plantlets. The regenerated embryos contained the inserted foreign DNA. However, the transformed coffee plantlets established feeble root systems and therefore did not develop into plants capable of flowering.

The application of the biobalistic system with coffee tissues was described for the first time by van Boxtel et al. (1995). The experiments were carried out on different tissues and revealed that suspension cultures and somatic embryos were less appropriate for transient expression studies of β-glucuronidase. Transient expression was most easily detectable and most frequently observed with bombarded leaves of microcuttings. Among four (CaMV-E35S, LTR, UBQ1, and EFα-A1) tested promoters controlling GUS expression, the best results with coffee were obtained using the EFα-A1 promoter of *A. thaliana*.

Fernández-Da Silva and Menéndez-Yuffá (2003) developed a method for coffee genetic transformation by electroporation. They evaluated different electroporation conditions on different plant tissues like embryogenic calli, leaf sections from *in vitro* plants, and somatic embryos at globular and torpedo stages obtained from cell suspensions. The optimal conditions for electroporation were one hour of enzymatic pretreatment of torpedo-shaped embryos, electroporation at 375 V and 900 μF. The secondary somatic embryos regenerated from electroporated torpedo-shaped somatic embryos were positive for *gus* expression, and also in the PCR analysis for the genes *gus* and *bar*.

Genetic Engineering: Genetic engineering of coffee has focused on seed production, ripening, insect control, and reduced caffeine. The decaffeination process is carried out by supercritical extraction that involves washing the beans with liquid carbon dioxide. The procedure is not only expensive but it also removes other compounds that give coffee its rich taste and aroma. Recently, an advance in transgenic plant research of coffee has been achieved: coffee plants (*Coffea arabica* and *Coffea canephora*) have been genetically modified to contain less caffeine (Ogita et al., 2003; 2004). The expression of the gene encoding theobromine synthase (CaMXMT1) was repressed by RNA interference

(RNAi). These reports provide evidence for the successful production of a low-caffeine phenotype in both 'Arabica' and 'Robusta' coffee species, with 100% decaffeination in embryogenic tissues and 70 % in plantlets, respectively. The 3'-untranslated region and coding region of CaMXMT1 cDNA were selected for design of double-stranded RNAi constructs. *Agrobacterium tumefaciens* EHA101 cells were transfected with these constructs and then used to transform embryogenic tissues of *C. arabica* and somatic embryos of *C. arabica* and *C. canephora*.

After 2-4 months of culture, most infected tissues turned brown and necrotic, however it was possible to regenerate hygromycin-resistant cells from these tissues. Seedlings were then cultured. Young leaves of one-year-old seedlings were collected 2-3 weeks after sprouting and their purine alkaloid content was measured. The wild type and transgenic lines that expressed green fluorescent protein (GFP) contained similar amounts of endogenous theobromine and caffeine (about 1 and 8.4 mg per g of fresh leaf tissue, respectively). In contrast, young leaves of transgenic lines, expressing RNAi showed a 40-70% reduction in theobromine content and a 50-70 % reduction in caffeine. At maturity, these transgenic plants should produce essentially normal coffee beans with low caffeine content. These decaffeinated coffee plants will, in theory, bring an end to the expensive, industrial decaffeination that results in a loss of flavour.

CONCLUSION

In the last few years, interest has grown in coffee as a model to study some very challenging aspects of modern biology, such as plant transformation and regeneration. Although coffee is the world's most heavily traded commodity apart from oil, research into some of the basic biological topics has indeed been neglected. The aim of this review was to point out the importance of developments in *in vitro* cell culture, somatic embryogenesis, plant regeneration and transformation in coffee, as well as call attention to some of the few biochemical and molecular studies that have been carried out.

Research on coffee harvesting, processing, pest and disease control, pathogen interaction, and abiotic stress is essential for the continued production of a high-quality coffee. Progress in our knowledge and understanding of the biochemical and molecular basis of the topics mentioned above can only come through continued research. This is the key to success in the quest for improving the yield and quality of this economically important crop, that would bring enormous benefits to both producers and consumers.

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