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Developments in coffee biotechnology—in vitro plant propagation and crop improvement

Vinod Kumar · M. Madhava Naidu · G. A. Ravishankar

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Abstract Coffee is an important plantation crop grown in about 80 countries across the globe. In recent years, coffee attained lot of attention in the biotechnology research area. Since last three decades, there has been a steady flow of information on coffee biotechnology and now it is entering into the genomic era. Major milestones in coffee biotech research are successful in vitro manipulation and multiplication of coffee, development of gene transfer protocols and generation of transgenic coffee plants with specific traits. The isolation of genes involved in caffeine biosynthetic pathway has opened up new avenues for generating caffeine free transgenic coffee. With the initiation of international coffee genomics initiatives, the genomic research in coffee is expected to reach new dimensions. The IPR issues may play crucial role in sharing of benefits during international collaborations in near future. This review focuses on the basic and applied aspects of coffee biotechnology for newer potentials.

Keywords Biotechnology · Coffee · Somatic embryos · Tissue culture · Micropropagation · Caffeine pathway · Transgenic

Introduction

Presently, coffee is cultivated on 11.6 million ha and global production is 6.3 million tons. As an agro based rural enterprise this industry is a source of direct employment in the area of cultivation and provides employment in processing and trade sections. It is highly labour-intensive industry, employing an estimated 100 million people in over 60 developing countries. World average productivity is 505 kg/ha (Anonymous 2005).

Commercial coffee

From the commercial point of view, only two coffee species are cultivated extensively: *Coffea arabica* (Arabica) and *Coffea canephora* (Robusta). Some other species, such as *Coffea liberica*, *Coffea dewevrei* and *Coffea racemosa*, are only cultivated to satisfy local consumption. *Coffea arabica* is native to the highlands of

V. Kumar · G. A. Ravishankar (✉)
Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore, Karnataka 570020, India
e-mail: pcbt@cftri.res.in

M. M. Naidu
Plantation Products, Spices & Flavor Technology Department, Central Food Technological Research Institute, Mysore, Karnataka 570020, India

south-western Ethiopia and was brought from tropical Africa and introduced into the American continent in the first decade of the 18th century. It has contributed to the economic and cultural development of the countries where it has been cultivated. Arabica coffee, grown at altitudes of 1000–2000 m, is responsible for about 75% of commercial world coffee and for all the coffee production in Latin America (Carneiro 1997). This species is also produced in some African countries, such as Ethiopia and Kenya. The economic qualities of *C. arabica*, together with its autogamous and perennial character, have led to the development of homogeneous plantations all over the world.

Coffea canephora has a very wide geographic distribution, extending from the western to the central tropical and subtropical regions of the African continent, from Guinea and Liberia to Sudan and the Uganda forest, with a high concentration of types in the Democratic Republic of Congo (Carneiro 1997). *Coffea canephora* or *robusta*, as it is commonly called, grows at low altitudes (about 850 m), and accounts for 80% of African coffee production. However, robusta has also been cultivated in American and Asian countries.

Coffee breeding by conventional methods is a long process involving several different techniques, namely, selection from wild populations followed by hybridisation and progeny evaluation, back crossing and inter-specific crosses. Unfortunately, these traditional methods of improvement are slow and it takes more than 30 years to obtain a new cultivar using any of these methods and are also expensive, thus the resulting seed production and distribution is insufficient to satisfy the needs of coffee growers. Leaf rust caused by *Hemileia vastatrix* B.et.Br is a devastating disease of great economic significance for coffee production. Arabica coffee is very susceptible to leaf rust and this may be due to the narrow genetic base of the commercial populations, which are known to be derived from very few plants (Smith 1985). The tetraploidy of *C. arabica* versus the diploidy of all other species of Coffee prevents ready flow of genes between arabica and other species. Thus, improving arabica coffee with the specific objective of rust resistance without compromising on yield and productivity is a task of considerable dimension. Biotechnological approaches and transgenic research may be helpful in overcoming these bottlenecks and for supplementing conventional breeding programs.

The introduction of in vitro propagation techniques, with the potential to multiply genotypes of superior value at a faster rate, has proved to be a major advantage. Several methods of in vitro regeneration and propagation in coffee have already been optimised successfully, including somatic embryogenesis and scale-up of somatic embryogenesis using bioreactors, apical meristem and axillary bud culture, induction and development of adventitious buds, culture of zygotic embryos, anther/pollen culture, cell suspension culture and protoplast culture (Hatanaka et al. 1991; Neuenschwander and Baumann 1991; Sreenath et al. 1995; Madhava Naidu and Sreenath 1999; Giridhar et al. 2004a; b; c). The advances made to date in in vitro coffee techniques have allowed the manipulation of the coffee plant at cellular and molecular levels, making coffee a suitable crop for the application of biotechnological breeding programs, including genetic transformation.

Studies in coffee biotechnology have been pursued for the last three decades in various laboratories around the world and concentrated on the improvement of agronomic and processing qualities. In this communication, we intend to provide coffee researchers with information on advances made in crop improvement through biotechnology, which includes in vitro micropropagation, and genetic manipulation for desired traits.

In vitro multiplication

Conventionally, coffee is propagated from seed or by vegetative cuttings. Seed propagation is associated with inherent uncontrolled genetic variation in heterozygous cultivars, slow rates of multiplication of seed and short span of seed viability. Propagation of coffee by vegetative cuttings guarantees uniformity. Cuttings generate relatively low multiplication rates as they can only be obtained from orthotropic branches.

Multiplication by tissue culture techniques could provide a viable alternative to these traditional methods of coffee propagation. Tissue culture methods permit the production of relatively uniform plants on a massive scale in a shorter period, and with a narrower genetic base than those under the conventional methods. Various approaches that have been considered for in vitro multiplication of coffee species are somatic embryogenesis, meristem culture, axillary bud culture and development of adventitious buds.

Micropropagation

The coffee plant presents one apical meristem and each axil leaf has 4–5 dormant orthotropic buds and two plagiotropic buds. The plagiotropic buds only start development from the 10th and 11th node. For apical meristem culture and the culture of dormant buds, both orthotropic and Plagiotropic buds, give rise to plantlets, which can be used as initial explants for coffee micropropagation. Micro-cuttings or nodal culture comprise a tissue culture approach which entails culturing nodal stem segments carrying dormant auxiliary buds, and stimulating them to develop. Since this method involves the exploitation of buds already present on the parent stock plant, it provides means of clonal multiplication. Each single segment can produce 7–9 micro-cuttings every eighty days. Most of these work was carried out during the 1980s. These aspects have been covered extensively in an earlier review (Reviewed by Carneiro 1999).

Micropropagation to mass multiply the superior coffee genotypes using apical or axillary meristem culture have been reported by a number of groups during the 1980s (Reviewed by Carneiro 1999). However, the maximum rate of multiplication obtained was nine shoots per explant (Carneiro and Ribeiro 1989). Culture of micro-cuttings in temporary immersion systems has resulted in a 6-fold increase in the multiplication rate, in comparison with micro-cuttings multiplied on solid medium (Berthouly et al. 1994; Teisson et al. 1995). The field performance of embryo-regenerated plants have been reported and found to show normal response in terms of physiology and yield. In order to validate the propagation technology of *Coffea canephora* Pex Fr. var. Robusta via somatic embryogenesis in liquid medium, the clonal fidelity of regenerated trees has been assessed for the first time in large-scale field trials (Ducos et al. 2003). A total of 5067 trees originating from 5 to 7-month-old embryogenic cell suspension cultures were planted in the Philippines and in Thailand for comparing with control trees derived from in vitro axillary budding. For the observed morphological traits and the yield characteristics, no significant differences were seen between the somatic seedlings and the micro-cutting derived trees (Ducos et al. 2003). However, the occurrence of some phenotypic variants difficult to visualise or somaclonal variations at the DNA level cannot be excluded. However, the initial establishment of in vitro culture is a difficult task due to the presence of large amount of phenolics.

Adventitious shoot development is an alternative method of coffee micropropagation. Shoots originating in tissues located in areas other than leaf axil or shoot tips are subjected to one phase of dedifferentiation followed by differentiation and morphogenesis (Ganesh and Sreenath 1999, Reviewed by Carneiro 1999).

Somatic embryogenesis

Plant embryogenesis represents the most definitive stages of the plant life cycle, with the overall architectural pattern of the mature organism established during a relatively short interval. Endogenous and exogenously administered hormones and explant source play a crucial role in somatic embryogenesis in genotype specific manner. Embryo to embryo multiplication i.e., secondary embryogenesis process requires a fine balance in the reprogramming of cells towards re-differentiation and maturation. Somatic embryogenesis was well documented in coffee (Staritsky 1970; Sharp et al. 1973). The callus induction was more efficient in the absence of light. During the last 35 years, a number of protocols for somatic embryogenesis have been developed for various genotypes of Coffee (Reviewed by Carneiro 1999). High frequency of

somatic embryogenesis (HFSE) and low frequency of somatic embryogenesis (LFSE) were established from leaf sections of *C. arabica* cv. *Bourbon* (Söndahl and Sharp 1977). Dublin (1980a; b) described somatic embryogenesis in leaf segments of Arabusta. Somatic embryos and plantlets were obtained with different types of explants (Molina et al. 2002, Reviewed by Carneiro 1999). Even from coffee seed integument (perisperm) tissues of coffee, embryogenesis was successful (Sreenath et al. 1995).

The time required for embryogenesis in coffee reported by various groups range from 8 months to more than a year (Reviewed by Carneiro 1999). A number of attempts have been made to reduce the time needed for embryogenesis in coffee. Triacontanol, silver nitrate ($AgNO_3$), salicylic acid, thidiazuron and 2iP are the widely used growth regulators in coffee embryogenesis. Triacontanol incorporated at 4.55 and 11.38 μM in half strength MS basal medium containing 1.1 μM 6-benzylaminopurine (BA) and 2.28 μM indole-3-acetic acid (IAA) induced direct somatic embryos in both species of *C. arabica* and *C. canephora* (Giridhar et al. 2004a). Direct somatic embryogenesis was achieved from hypocotyl explants of in vitro regenerated plantlets of *C. arabica* and *C. canephora* on modified MS medium containing 10–70 μM $AgNO_3$ supplemented with 1.1 μM N^6 benzyladenine and 2.85 μM indole-3-acetic acid (Giridhar et al. 2004b). Somatic embryogenesis in just 2–4 months was the major breakthrough in the above reports.

Indirect modes of somatic embryogenesis i.e., callus initiation followed by embryogenesis is the major reason for long duration of time required for embryogenesis. Induction of embryos directly on leaf segments would obviously reduce the time needed for production of somatic embryos. The response of *C. arabica* and *C. canephora* genotypes with regard to direct somatic embryogenesis was reported by Giridhar et al. (2004c). Segments of cotyledonary leaf (CXR variety), first leaf and stalk of regenerated plantlets, produced cluster of somatic embryos directly from cut portions of explants on 2 mg l^{-1} thidiazuron (TDZ) containing medium. Sub-culturing of these embryo clusters reported to produce more secondary embryos on reduced TDZ (0.01–0.2 mg l^{-1}) containing medium and subsequently developed into plantlets followed by rooting on MS basal medium.

Shoots of arabica and robusta coffee showed increased growth when cultured on MS medium containing $AgNO_3$, IAA and BA (Giridhar et al. 2003). Incorporation of $AgNO_3$ at 10–40 μM concentration in the culture medium enhanced growth of both *C. arabica* and *C. canephora* coffee (Giridhar et al. 2003). According to a method reported by Giridhar et al. (2004b), it could be possible to get direct somatic embryogenesis in 2 months time. In general, 3–4 months time is required to get the yellow friable callus from leaf explants, which can be later, used for high frequency somatic embryogenesis (Van Boxtel and Berthouly 1996). Therefore, the observed effects of silver nitrate on somatic embryogenesis may further support the hypothesis that this compound acts as promoting agent for direct somatic embryogenesis and embryogenic callus formation which may be attributed to its ethylene regulation action during specific stages of *Coffea* embryogenesis.

Interestingly, picomolar concentrations of salicylates reported to induce cellular growth and enhance somatic embryogenesis in *C. arabica* tissue culture (Quiroz-Figueroa et al. 2001). Other than exogenous growth hormones ethylene (Hatanaka et al. 1995) and dissolved oxygen concentration (Manuel de Feria et al. 2003) known to play a crucial role in coffee somatic embryogenesis. Further work was carried out to assess the effect of different ethylene inhibitors such as cobalt chloride and salicylic acid. The results indicated that compared to other ethylene inhibitors, silver nitrate is highly efficient in eliciting secondary embryogenesis response from primary somatic embryos Table 1 contains a brief summary of reports on somatic embryogenesis from coffee.

Scale up of embryos in bioreactors

Liquid cultures are known to be highly efficient in eliciting embryogenesis response in a number of plant systems. Bioreactors play an important role in scaling up the production for commercialisation of somatic embryogenesis based plant

Table 1 Some of the reports on somatic embryogenesis of coffee

Genotype	Explant	Cultural conditions	Period for embryogenesis	Frequency of response (%)	Reference
C. arabica	Leaf	LS + KIN (0.1 mg l^{-1}) and 2,4-D (0.1 mg l^{-1})	2 months	–	Herman and Haas (1975)
C. arabica and *C. canephora*	Internode	Two-step method	6 months	–	Raghuramulu et al. (1987; 1989)
C. canephora	Leaves from in vitro plantlets	MS + NAA (0.1 mg l^{-1}) and BA (1 mg l^{-1})	5 months	93	Muniswamy and Sreenath (1995)
C. arabica	Leaf	MS + BA (1 mg l^{-1})	5 months	76	Yasuda et al. (1995)
C. canephora	Leaf	MS + 2-iP (1 mg l^{-1})	1½ months	100	Yasuda et al. (1995)
C. arabica	Callus	MS + 2-iP (1 mg l^{-1}) + asparagine 10 mM	1 month	100	Nishibata et al. (1995)
C. canephora	Leaf	MS + 2-iP (1 mg l^{-1})	2 months	100	Hatanaka et al. (1995)
C. canephora	Leaf	Two-step procedure	6–7 months	97	Van Boxtel and Berthouly (1996), Berthouly and Michaux-Ferriére (1996)
C. canephora	Apical bud with hypocotyl	MS + BAP (5 mg l^{-1})	5 months	12	Ganesh and Sreenath (1999)
C. arabica	Cell suspension	10^{-12} and 10^{-10} M Salicylic acid	1½ months	Positive effect on number and quality of embryos	Quiroz-Figueroa et al. (2001)
C. canephora	Leaves	$AgNO_3$	2½ months	100	Fuentes et al. (2000)
C. arabica and *C. canephora*	Leaves	Triacontanol	2 months	60–100% embryogenesis and secondary embryogenesis	Giridhar et al. (2004a)
C. arabica and *C. canephora*	Leaves, hypocotyl	BA 1.1 µM + IAA 2.85 µM $AgNO_3$ 30–60 µM	2 months	50–70% explant response	Giridhar et al. (2004b)
C. arabica and *C. canephora*	Leaves, hypocotyl	MS medium, 2% sucrose, 2.27–11.35 µM TDZ	2 months	40–60% response	Giridhar et al. (2004c)

micro propagation. The induction of somatic embryogenesis of *C. arabica* in liquid medium using bioreactors was described (Zamarripa et al. 1991; Zamarripa 1993). Although some reports (Zamarripa et al. 1991; Ducos et al. 1993; Noriega and Söndahl 1993) suggested somatic embryogenesis for commercial uses, the feasibility of scale-up of somatic embryogenesis was described for the first time by Zamarripa (1993) in *C. canephora* and *Arabusta*. Extensive studies have been made in the use of conventional (Reviewed by Carneiro 1999) and temporary immersion system for Coffee somatic embryo production (reviewed by Etienne and Berthouly 2002; Albarran et al. 2005). However, till today the major hindrance in scale up is synchronisation of embryogenesis and conversion of plantlets.

Temporary immersion systems

Temporary immersion systems for coffee micropropagation have been described and grouped into four categories according to operation: tilting and rocker machines; complete immersion of plant material and renewal of the nutrient medium; partial immersion and a liquid nutrient renewal mechanism; complete immersion by pneumatic driven transfer of liquid medium and without nutrient medium renewal (Etienne et al. 2002). Using a culture system based on temporary immersion in liquid medium, 40,000 embryos were obtained per gram of callus. The importance of this system lies in the simplification of handling and 10-fold reduction of labour costs. These results were reported for nine different hybrids and reported production of about 15,000 plants (Etienne et al. 1999; 2002).

Problems associated with large-scale micropropagation

Commercialisation of large-scale somatic embryogenesis is still in infancy. The main reasons are difficulty in maintaining aseptic conditions for long duration of in vitro culture. Apart from that, maintaining synchrony in induction of embryogenesis and maturation stages is difficult in coffee. Other problems include, embryo to plant conversion rate is highly variable among different genotypes and explants.

However, direct somatic embryogenesis has several advantages over propagation by micro-cuttings and in vitro nodal culture. It does not have the problems of phenolic accumulation in the medium, and the contamination rates are generally very low (Muniswamy and Sreenath. 1996). Furthermore, the genetic variability is relatively low and the method permits the development of plantlets in larger numbers than is possible with the micro-cuttings method. For example, one leaf segment can produce hundreds of plantlets in a single culture cycle, whereas the micro-cutting method requires several sub-culturing cycles in order to get a comparable number of plantlets.

Induction and utilisation of genetic variability

There are many ways to produce variability in vitro and sometimes it is difficult to control these variations. Some of the well-known techniques include culture of zygotic embryos, anther and pollen culture, cell suspension culture, in vitro selection and protoplast culture. The culture of zygotic embryos allows the rescue of embryos resulting from inter-specific crosses, which abort due to endosperm incompatibility. The first contribution for this field was reported by Collona (1972) for different diploid species, namely, *C. canephora*, *C. dewevrei*, *C. excelsa* and *C. arnoldiana*. Immature embryos of San Ramon (*C. arabica*) cv. S73 were rescued and plant regeneration was achieved at high frequency (Raghuramulu et al. 1989). Sreenath et al. (1992) reported embryo culture for three inter-specific crosses in coffee. Coffee anthers have about 2,000–40,000 microspores and each one may give a new combination during meiosis. The first attempt to obtain coffee haploid plants was made by Sharp et al. (1973). These authors reported the induction of calli from anthers of *C. arabica* cultivars *Mundo Novo* and *Bourbon Amarelo*. Successful embryogenesis in *C. arabica* cv. *Catuai* and in different progenies of *Catimor* was reported after the culturing of isolated microspores and

anthers in liquid and solid media (Carneiro 1993). Söndahl et al. (1980) for the first time reported protoplast isolation and callus proliferation from friable embryogenic callus of *C. arabica.* Protoplasts were regenerated from young leaves of hybrids of *C. arabica* and *C. canephora*. (Orozco and Schieder 1982). The successful isolation and culture of coffee protoplasts from embryogenic calli and suspension cells has been reported by Mamatha and Sreenath (2000). More detailed information on zygotic embryo culture, haploid culture, androgenesis and protoplast culture is available in an earlier review (Carneiro 1995).

Cryopreservation of coffee germplasm

Conservation of genetic resources of Coffee is difficult because coffee seeds loose viability during conventional seed storage. Most of the coffee germplasm are maintained in field collections, which are expensive to maintain and susceptible to natural disasters or disease. Storing Coffee seeds in genebanks would reduce costs and provide a safer conservation strategy for these important genetic resources. Though not completely tolerant of drying, embryos from most *Coffea* sp. studied survived water contents as low as or less than 0.20 g water/g dry mass (Eira et al. 2002). This relatively high tolerance of desiccation suggests that seeds and embryos can be cryopreserved if both water content and cooling and heating rates are optimised. Consistent with this hypothesis, no loss of viability was observed when *Coffea arabica* seeds were dried to 0.20 g/g (in equilibrium with 75% RH) and cooled in liquid nitrogen. Successfully cryopreserved *Coffea arabica* seeds showed minimal viability loss after 1-year storage in liquid nitrogen (Eira et al. 2002). Some of the critical factors in cryopreservation of coffee includes optimisation of moisture content (Normah and Vengadasalam 1992; Dussert et al. 1998), cooling rate (Eira et al. 2002; Dussert et al. 1998), pre-cooling parameters (Dussert et al. 1997), tolerance to cooling (Eira et al. 1999) and physiological changes (Dussert et al. 2003) during cooling. Mycock et al. (1995) reported detailed study on cryopreservation of different genus including coffee. Embryos of *Coffea* sp. has been successfully conserved by cryopreservation techniques (Mari et al. 1993; Abdelnour et al. 1993; Florin et al. 1993).

Caffeine in coffee

Caffeine is one of the most widely used psychoactive substances in the world, its estimated global consumption being 120,000 tones per year. The caffeine content of green coffee bean varies among the species (Table 2). A typical cup of regular coffee contains 70–140 mg of caffeine depending on preparation, blend and cup size (Roger and Richardson 1993).

Chemically, caffeine is a purine derivative xanthine with methyl groups attached at position 2, 3, and 7. Biosynthesis and degradation of caffeine both processes occur more rapidly in immature than mature fruit (Keller et al. 1972). Caffeine and chlorogenic acid (CGA) is known to form complex in the plant tissues. When comparing the caffeine (Campa et al. 2005a) and CGA (Campa et al. 2005b) contents in green beans of 21 Coffee species or taxa, variations in these parameters showed similar trends, with an increase in CGA content always being accompanied by an increase in caffeine content. Nevertheless, CGAs were always more abundant than caffeine, thus indicating that all the CGA was not trapped as caffeine–chlorogenate complex in the seed (Campa et al. 2005b).

Caffeine biosynthetic pathway

In coffee plants caffeine is synthesised from xanthosine via 7-methyl xanthosine, 7-methyl xanthine, and theobromine. *S*-adenosyl methionine (SAM) is the actual source of the methyl groups (Reviewed by Ashihara and Suzuki 2004). The caffeine is degraded relatively slowly and involves demethylation steps to yield theobromine and theophylline. Theophylline is catabolised to xanthine via 3-methyl xanthine. However, it is unclear whether 3-methyl xanthine and/or 7-methyl xanthine are intermediates in the conversion of theobromine to xanthine (Waller and Suzuki

Table 2 Geographical origin and caffeine content in different *Coffea* sp. (Campa et al. 2005a)

Species and taxa	Geographical origin	Bean Caffeine content % dry mass basis (dmb)
C. brevipes	Cameroon	2.36–2.96
C. canephora	Cote-d'Ivoire	1.51–3.33
C. congensis	Congo Democratic Republic	1.08–1.83
C. eugenioides	Kenya	0.44–0.60
C. heterocalyx	Cameroon	0.86–0.99
C. humblotiana	Comores	0.00–0.01
C. humilis	Cote-d'Ivoire	1.67–2.27
C. kapakata	Angola	1.04–1.39
C. liberica dewevrei	Central African Republic	0.81–1.10
C. liberica Koto	Cameroon	0.91–1.70
C. liberica liberica	Cote-d'Ivoire	1.12–1.39
C. pseudozanguebariae	Kenya	0.00–0.00
C. racemosa	Tanzania	0.86–1.25
C. salvatrix	Tanzania	0.01–0.06
C. pocsii	Tanzania	1.04–1.71
C. stenophylla	Cote-d'Ivoire	2.05–2.43
Coffea sp. *Bakossi*	Cameroon	0.00–0.03
Coffea sp. *Congo*	Congo Democratic Republic	2.11–2.37
Coffea sp. *Ngongo 2*	Congo Democratic Republic	1.90–2.32
Coffea sp. *Moloundou*	Congo Democratic Republic	0.52–0.61
Coffea sp. *Nkoumbala*	Cameroon	1.89–2.89

1989). Simulation of caffeine biosynthesis in suspension—cultured coffee cells and the in situ existence of 7-methylxanthosine was reported (Schulthess and Baumann 1995).

The two SAM-mediated methylations of the 7-methylxanthine to theobromine and caffeine, respectively, were the first-known steps of the purine alkaloid synthesis (Fig. 1.) (Roberts and Waller 1979; Baumann et al. 1983; Waller et al. 1983). In a study conducted by Looser et al. (1974), theobromine and 7-methylxanthine were identified as precursors of caffeine. The results of mixed substrate experiments have indicated that separate enzymes catalysed the *N*-3- and *N*-1-methylations (Baumann et al. 1983). *Coffea arabica* cell free extracts made from callus cultures in which active biosynthesis of caffeine was occurring exhibited *N*-methyltransferase enzyme activity (Waller et al. 1983). The cell suspensions showed high activity with transfer of methyl groups from *S*-adenosyl-L-methionine to 7-methylxanthine and to theobromine producing theobromine and caffeine, respectively. The same methyltransferase activities have later been detected in cell suspension (Baumann et al. 1983). Mazzafera et al. (1994) reported the purification of theobromine 1-*N*-methyltransferase (STM), the enzyme responsible for the methylation of theobromine leading to caffeine formation in coffee. STM was purified from developing endosperms of immature fruits of *C. arabica*. STM is a bifunctional enzyme since it also methylated 7-methylxanthine, the immediate precursor of theobromine in the caffeine biosynthetic pathway. The K_m values obtained for theobromine and 7-methylxanthine were 0.196 and 0.496, respectively. Gillies et al. (1995) reported improved method of purification of *N*-methyltransferases from coffee endosperm. Incorporation of 20% (v/v) glycerol in buffers during anion-exchange chromatography resulted in 54–78% yield of *N*-methyltransferase activity and a 10–20 fold purification (Gillies et al. 1995). Analysis of a gel filtration purified preparation containing all three *N*-methyltransferase activities revealed the presence of three bands at 49, 43 and 40 kDa (Waldhausser et al. 1997a). Maximum relative and absolute second and third *N*-methyltransferase activities coincide with leaf emergence (Waldhausser et al. 1997b). The cDNAs for 7-methylxanthine methyltransferase (MXMT or theobromine synthase) (CaMXMT, CTS1 and CTS2) were successfully cloned from coffee plants (Ogawa et al. 2001; Mizuno et al. 2003a, b), although CTS1 and CaMXMT were later found to be identical.

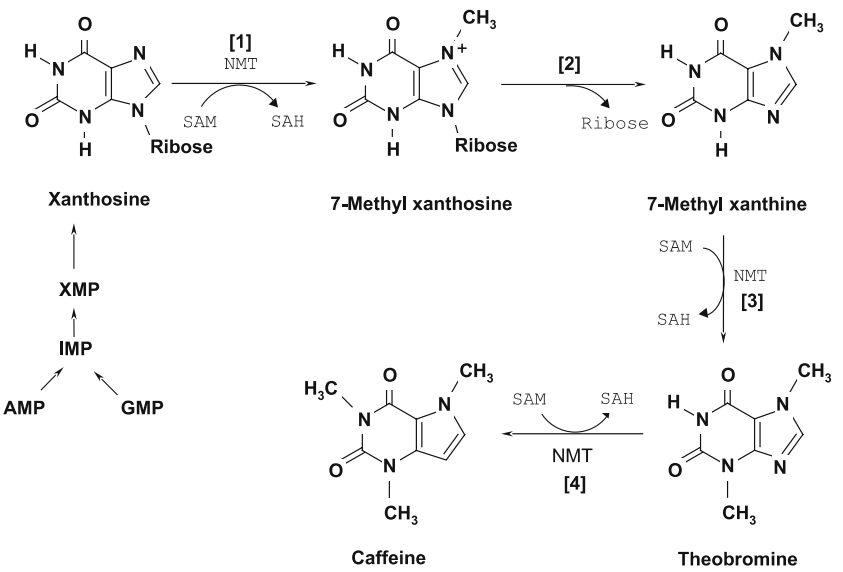

Fig. 1 The major route of caffeine biosynthetic pathway in coffee plants. [1] 7-Methylxanthosine synthase (xanthosine *N*-methyltransferase), [2] *N*-methylxanthine nucleosidase, [3] theobromine synthase (7-methylxanthine *N*-methyltransferase) and/or caffeine synthase (7-methylxanthine and theobromine *N*-methyltransferase), [4] caffeine synthase (7-methylxanthine and theobromine *N*-methyltransferase) (Ashihara and Suzuki 2004)

Using primers designed on the basis of conserved amino acid regions of tea caffeine synthase and *Arabidopsis* hypothetical proteins, a particular DNA fragment was amplified from an mRNA population of coffee. Subsequently, this fragment was used as a probe, and four independent clones were isolated from a cDNA library derived from coffee young leaves. Upon expression in *Escherichia coli*, one of them was found to encode a protein possessing 7-methylxanthine methyltransferase activity and was designated as CaMXMT. It consists of 378 amino acids with a relative molecular mass of 42.7 kDa and shows similarity to tea caffeine synthase (35.8%) and salicylic acid methyltransferase (34.1%). The bacterially expressed protein exhibited an optimal pH for activity ranging between 7 and 9 and methylated almost exclusively 7-methylxanthine with low activity toward paraxanthine, indicating a strict substrate specificity regarding the 3-*N* position of the purine ring. K_m values were estimated to be 50 and 12 μM for 7-methylxanthine and *S*-adenosyl-L-methionine, respectively (Ogawa et al. 2001). Mizuno et al. (2003b) reported isolation of a bifunctional coffee caffeine synthase (CCS1) clone from coffee endosperm by reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends technique using previously reported sequence information for theobromine synthases (CTSs). The predicted amino acid sequences of CCS1 are more than 80% identical to CTSs. CCS1 has dual methylation activity (Mizuno et al. 2003b). Genes encoding 7-methylxanthine methyltransferase (MXMT) and 3,7-dimethylxanthine methyltransferase (DXMT) were isolated from immature fruits of *C. arabica*. Functional characterisation and in vitro reconstitution of the enzymes have been carried out. The cDNAs were named as CaMXMT2 and CaDXMT1, respectively. CaMXMT2 catalysed the formation of theobromine from 7-methylxanthine with a K_m of 251 μM, and CaDXMT1 catalysed the formation of caffeine from theobromine with a K_m of 1,222 μM (Uefuji et al. 2003). The results suggest that, in coffee plants, caffeine is synthesised through three independent methylation steps from xanthosine (Ogawa et al. 2001; Uefuji et al. 2003).

The main biosynthesis route utilises the purine nucleotides through the steps (AMP and/or GMP) → IMP → XMP → xanthosine → 7-methyl xanthosine → 7-methylxanthine → theobromine → caffeine. More detailed information on caffeine biosynthesis, the provider pathways, SAM cycle and other aspects of purine alkaloids have been discussed by Ashihara and Suzuki (Reviewed by Ashihara and Suzuki 2004). The proposed pathway of the final stage of caffeine biosynthesis in coffee is illustrated in Fig. 1.

Using gene-walking technique, the promoter for one of the *N*-methyl transferase gene has been cloned (Satyanarayana et al. 2005). The analysis

of sequence revealed the successful 5′ walking in the unknown region to the extent of 745bp from the known 5′ end of the theobromine synthase gene. The ability of the isolated promoter to drive reporter gene expression was confirmed using a model system tobacco (Satyanarayana et al. 2004; 2005).

The cloning of the promoter for the gene involved in caffeine biosynthetic pathway opened up the possibility of studying the molecular mechanisms that regulate the production of caffeine. The promoter sequence could be used for specific down regulation of individual member of NMT gene family through transcriptional gene silencing by RNA directed DNA methylation (Mette et al. 2000).

Breeding for caffeine free Coffee

The deleterious effects of caffeine on the central nervous system are well documented and this led coffee industry to provide an artificially de-caffeinated coffee. The health conscious consumers will welcome the developments towards naturally decaffeinated coffee. Although genetic improvement using intra-specific diversity is an obvious approach, this approach seems inadequate to obtain caffeine-free coffee. Caffeine is lacking in most Malagasy species and in the East-African species *C. pseudozanguebariae* (Charrier and Berthaud 1975; Clifford et al. 1989). Unfortunately, caffeine-free species are low yielding and give a beverage of poor tasting quality. *Psilanthus* species indigenous to India were thought to be caffeine free.

However, Santa Ram et al. (2005) reported very low caffeine content (0.05–0.07%dw) in *P. bengalensis.* A tetraploid *Ligenioides*, derived from the cross *C. liberica* × *C. eugenioides* found to possess reduced caffeine (1.29–1.39% dw) content. The seeds of *Racemusta* hybrids (*C. racemosa* × *C. canephora*) contain 0.82–1.43% caffeine, which was found to be much lower than that of *C. canephora* (~3%) (Santa Ram et al. 2005)

Silvarolla et al. (2004) have been able to discover a naturally decaffeinated *C. arabica* plant from Ethiopia, a species normally recognised for the high quality of its beans. They have studied 3,000 coffee trees representing 300 *C. arabica* accessions from Ethiopia. The study revealed that, three of these accessions are completely free of caffeine and speculated to be due to mutation in the caffeine synthase gene (Silvarolla et al. 2004). These developments clearly opened up new avenues towards breeding for low or zero caffeine coffee.

Decaffeination

The first decafffeination process was developed in Germany during 1900. Swiss water process was introduced to the American market in New York in 1979, and French water decaffeinated coffee was introduced in 1992. The use of solvent super critical CO_2 for decaffeination of coffee, commercialised by HAH-GF in Germany, is based on the 1970 patent of Kurt Zosel, at Max Planck Institute, Germany. Patents have been the major sources of public information. There have been numerous process developments in decaffeination technology, the most important being solvent extraction of caffeine from pre-wetted beans. There are two basic methods for producing decaffeinated coffee using solvent, direct solvent extraction of the beans and water extraction of the beans followed by solvent extraction of the caffeine from the water extract. More detailed information is available in a review article by (Ramalakshmi and Raghavan 1999).

The Coffex Company developed the water process using water for decaffeination in 1938 in Switzerland. However, commercialisation of the process was slow; only in the late 1970s, was able to realise the commercial production, with an initial capacity of 4000 tons per year. Caffeine is transferred from steamed green beans to an intermediate from that it is recovered by thermal desorption (Hinrichsmeyer and Chammenga (1985). Some of the absorbents used are activated charcoal, molecular sieves, silica gels, bentonites, and organic ion-exchange resins. Caffeine containing green coffee extract can be decaffeinated by contact with caffeic acid, which forms an insoluble caffeine/caffeic acid, complex and can be separated (Kopsch et al. 1989).

The adverse effects of caffeine have increased the market for decaffeinated coffee to about 10% of the coffee consumption worldwide (www.ncausa.org), despite the loss of key flavour compounds in the industrial decaffeinating process.

Genetic transformation of *Coffea* sp.

The success of transgenic research in Coffee has opened up new avenues for quality improvements. The early reports on coffee genetic transformation referred to co-cultivation of protoplasts with different strains of *Agrobacterium tumefaciens*. Barton et al. (1991) reported electroporation mediated gene delivery for the first time in protoplasts and plantlets were recovered from electroporated embryos using kanamycin as a selective marker. But now it is well known that kanamycin selection is not very efficient as hygromycin for the selection of transgenic coffee.

Ocampo and Manzanera (1991) demonstrated that *C. arabica* tissues could be infected by wild strains of *Agrobacterium tumefaciens*. Transgenic plants of *C. canephora* and *C. arabica* were obtained using genetic transformation mediated by *A. rhizogenes* (Spiral et al. 1993; Sugiyama et al. (1995). Transient expression of *uid* A gene was demonstrated by GUS histochemical assay on callus tissues derived from transformed protoplasts (Spiral et al. 1999).

Transgenic work on coffee was continued with different approaches. Some of them include, *A. tumefaciens* mediated genetic transformation (Grèzes et al. 1993; Madhava Naidu et al. 1998), biolistic gene delivery (van Boxtel 1994, 1995), PEG mediated direct DNA uptake (De Peña 1995), electroporation mediated gene delivery (Fernandez and Menendez 2003). We have been working on development of suitable transformation system in coffee for several years. *Agrobacterium rhizogenes* strain A4 harbouring plasmid pCAMBIA 1301 with an intron *uid* A reporter and hygromycin phosphotransferase (*hpt* II) marker gene was used for sonication assisted transformation of *C. canephora* (Kumar et al. 2003; 2004; 2006). PCR and Southern blot analysis confirmed the independent, transgenic nature of the analysed plants and indicated single and multiple locus integrations (Kumar et al. 2006). The study clearly demonstrated that *Agrobacterium rhizogenes* harbouring binary vectors could be used for generating transgenic coffee without hairy root phenotype and the T DNA from the Ri plasmid and the T DNA of the binary vector integrates independently in transgenic plants (Kumar et al. 2006). However, only upto 3% transformation efficiency has been achieved.

In a recent report by Canche-Moo et al. (2006), *Agrobacterium*-mediated plant transformation protocol was evaluated as a fast method to obtain genetically modified *C. canephora* plantlets. Leaf explants were used as source material for *A. tumefaciens*-mediated transformation involving a vacuum infiltration protocol, followed by a step of somatic embryogenesis induction and a final selection of the transformed plants. *Agrobacterium tumefaciens* strain C58CI containing the binary vector pER10W-35SRed was used. For the first time, transformation efficiency of 33% was achieved in coffee. The authors claim to generate transgenic embryos in just two months (Canche-Moo et al. 2006). Stable transformation of *C. canephora* was obtained by particle bombardment of embryogenic tissue (Ribas et al. 2005). The somatic embryos and embryogenic tissue were bombarded with tungsten particles (M-25) carrying the plasmid pCAMBIA3301 (containing the *bar* and *uid* A genes) using a high-pressure helium microprojectile device. The transgenic plants were successfully transferred to green house (Ribas et al. 2005).

Transgenic coffee for down regulation of caffeine biosynthesis

Ogita et al. (2003; 2004) have reported the simultaneous down-regulation of three distinct methylation steps of the caffeine biosynthetic pathway by RNAi. Specific sequences in the 3′ untranslated region (UTR) of CaMXMT1 messenger RNA were selected for construction of RNAi short and long fragments. The caffeine content of the transgenic plants reduced by up to 70%, indicating that it is possible to produce coffee beans that are intrinsically deficient in caffeine (Ogita et al. 2003). In a study reported by

Kumar et al. (2004), *Agrobacterium tumefaciens* mediated genetic transformation was achieved in *C. canephora* for silencing of *N*-methyl transferase involved in caffeine biosynthesis (Kumar et al. 2004).

Transgenic coffee for resistance against leaf miner

With regard to molecular breeding for agronomic qualities, transgenic coffea have been developed for resistance to leaf miner pest. The possible use of *Bacillus thuringiensis* in coffee biotechnology and the production of genetically modified coffee plants expressing the *Bacillus thuringiensis cry*1Ac gene for resistance to leaf miner was reported (Oliveiro et al. 1998; Leroy et al. 2000; Dufour et al. 2000). Perthuis et al. (2005) reported a field trial of transgenic clones of *C. canephora* transformed for resistance to the *Lepidopteran* coffee leaf miner *Leucoptera coffeella* was installed in French Guiana. Fifty-eight transformed clones produced by transformation of the *C. canephora* were planted. They were harboring the pEF1α constitutive promoter of *Arabidopsis thaliana* controlling either the *Bacillus thuringiensis* native gene for the *cry1Ac* insecticidal protein (eight clones) or a synthetic *cry1Ac* gene (53 clones). Over a 4-year period after plantation, a majority of the independent transformed clones harboring the synthetic gene displayed resistance against *L. coffeella* when compared to controls.

GM coffee

So far, no GM coffee has hit the market. However, GM coffee with desired traits would come to the market. Since first commercialisation in 1996, GM crop areas have grown at rapid rates, making this one of the most rapidly adopted technologies in agriculture. Worldwide, strict regulatory frameworks are in place in many countries to ensure that all candidate GM crops are evaluated according to the latest standards of scientific knowledge for impacts on human health, animal health, and the environment before authorisations for marketing are granted. The safety assessment is extremely rigorous and varies slightly from country to country (Jaffe 2004). Assessments of the GM crops currently on the market have uncovered no adverse health or environmental effects. Also, there have been no validated adverse findings during their consumption over the last 8 years (UK GM Science Review Panel 2003; 2004). Labelling the product is very essential to give freedom to the consumer on their preference towards GM or non-GM coffee. However, there is no doubt that a systematic scientific evaluation and safety assessments of GM coffee is required before releasing it to the market. This has to be followed for every new trait, within the context of a sound international broadly harmonised legislative framework.

IPR Issues

Since coffee biotechnology has tremendous application in the pre-harvest and post harvest application, protection of intellectual property rights has gained importance. Selected patents in the area of coffee biotechnology has been compiled and presented in Table 3. These studies have bearing in the future applications of the technology for both basic and applied research. The search for value addition and improvement is bound to continue in view of the fact that coffee could continue to be an important cash crop of the world. With the initiation of international coffee genomics initiatives, it is expected that, a large number of scientific data in terms of DNA sequence, clones, gene constructs, protocols are expected to come into picture. In this case it is essential to draw a framework on sharing and distribution of materials coming under the IPR issue.

Near future of coffee biotechnology

Coffee biotechnology sector is going to take quantum leap in near future. International coffee genomics initiatives have been started with the intention of venturing into cutting edge research

Table 3 Selected patents filed or granted in the area of Coffee biotechnology

	Number
Patents on decaffeination of coffee	EP0316694 A3 B1, CA1329595, 2002031580, 20020012727, 20040191333, US5089280, EP0248482B1, CA1304978, CA1293148
Patents on genes involved in caffeine pathway and its regulation	EP1197558 A3, US2004154055, JP2003304879, WO03080833, CN1495261, 20040154055
Patents on transgenic coffee	EA3835, EP1541016, 6392125, 397Del03-Indian Patent, JP2000245485, US5334529,
Other patents on coffee biotechnology	0199943, 6348641, 5760095, 6207203, EP1256629, 20030131380, 20020155210, 6461873, US 545686, US2003084487, EP0337541 A3, WO03032713

in the area of coffee biotechnology. Major objectives would be to obtain a large number of EST and genomic sequence data for functional genomic studies. The major focus would be on development of pest and disease resistant coffee, improved processing characteristics, more work on transgenics and development of marker less transformation protocols.

Conclusion

Biotechnology offers new ideas and techniques that can be applied in agriculture. It uses the conceptual framework and the technical approaches of molecular biology to develop commercial processes and products. Plant breeding has been benefited by the introduction of genetic engineering techniques, based on the knowledge of gene structure and function. Advances in cellular, developmental and molecular genetics, combined with traditional breeding, can target and achieve improvements in specific agronomic, processing and quality traits. The high performance already achieved in in vitro coffee plant regeneration systems constitutes an important breakthrough in various in vitro manipulation and regeneration processes.

Genetic transformation methods, such as direct DNA uptake, gene bombardment and mediation by *Agrobacterium* sp., vacuum infiltration have been shown to be reliable techniques for coffee. These facts allow coffee breeders to advance with projects aimed at developing plants resistant to the pests and diseases of coffee, plants resistant to herbicides and to other specific characteristics, particularly cold resistance, regulation of chlorogenic acid synthesis and plants which overproduce the amino acid contents involved in coffee flavour (cysteine and methionine). The improvements made in gene silencing techniques made revolutionary advancements in molecular breeding programmes. The best example is down regulation of caffeine pathway and this would be a model study for future developments towards molecular breeding for improved processing characteristics of coffee. The public concerns over marker genes in GM coffee would probably drive the researchers to think in those directions of development of marker

free transgenic plants of coffee harbouring silencing constructs containing coffee gene fragments driven by the coffee promoters.

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