Genetic transformation studies on *Coffea* sp.

A THESIS
submitted to the
UNIVERSITY OF MYSORE

For the award of degree of
DOCTOR OF PHILOSOPHY
in
BIOTECHNOLOGY

by
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December 2005

Dedicated to

My family and friends

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DECLARATION

I hereby declare that this thesis entitled "**GENETIC TRANSFORMATION STUDIES ON *COFFEA* Sp."** submitted to the **UNIVERSITY OF MYSORE**, for the award of the degree of **DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY**, is the result of research work carried out by me in the Department of Plant Cell Biotechnology, Central Food Technological Research Institute, Mysore, under the guidance of **Dr. G. A. Ravishankar** during the period August 2001-December 2005.

I further declare that the work embodied in this thesis is original and has not been submitted previously for the award of any degree, diploma or any other similar title.

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CERTIFICATE

This is to certify that the thesis entitled "**GENETIC TRANSFORMATION STUDIES ON *COFFEA* Sp."** submitted by **Mr. Vinod Kumar** to the University of Mysore for the award of the degree of **'Doctor of Philosophy' in Biotechnology**, is the result of work carried out by him in Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore under my guidance during the period August 2001-December 2005.

G. A. RAVISHANKAR

Place: Mysore (Research Supervisor)

Date:

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****

LIST OF ABBREVIATIONS

2,4-D	2, 4-Dichlorophenoxy acetic acid
2-iP	2-isopentinyl adenine
ABA	Abscisic acid
ACC	1-aminocyclopropyl carboxylic 0acid
$AgNO_3$	Silver nitrate
BA	Benzyl amino purine
BCIP/NBT	5-Bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium
bp	base pair
CaXMT	Xanthosine *N* methyl transferase
CaMXMT	Methyl xanthine *N* methyl transferase
CaDXMT	Dimethyl xanthine *N* methyl transferase
CCS	Coffea caffeine synthase
ccCDPK	*Coffea canephora* Calcium dependent protein kinase
CGA	Chlorogenic acid
CTS	Coffea theobromine synthase
DFMA	∝-DL-Difluromethylarginine
DFMO	∝-DL-Difluromethylornithine
DNA	Deoxy ribonucleic acid
DW	Dry weight
EC	Embryogenic callus
EDTA	Ethylene diamine tetra acetic acid
EGTA	Ethylene glycol-bis (2-aminoethylether) N, N, N,`N,` tetra acetic acid
Fig	Figure
FW	Fresh weight
GUS	*β*-glucuronidase
HFSE	High frequency somatic embryogenesis
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Kb	Kilo base
kDa	Kilo dalton
Kg	Kilo gram
KIN	Kinetin
l	litre
LB	Luria Bertani
LS	Linsmaier & Skoog

M	Molar
ml	Milli litre
mM	Milli moles
MS	Murashige and Skoog
NAA	α- Naphthalene acetic acid
NEC	Non embryogenic callus
NMT	*N* methyl transferase
OD	Optical density
PA	Polyamine
PCR	Polymerase chain reaction
PTGS	Post transcriptional gene silencing
Put	Putrescine
RT PCR	Reverse transcription PCR
SAM	*S* adenosyl methionine
SDS	Sodium dodecyl sulphate
Spd	Spermidine
Spm	Spermine
Tab	Table
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
TCS	Tea caffeine synthase
TDZ	Thidiazuron
TE	Tris-EDTA
TRIA	Triacontanol
Tris	Tris (hydroxy methyl) amino methane
U	Unit
X-Gluc	5-bromo 4-chloro indolyl-D-glucuronide
µg	Micro gram
µM	Micro moles
µm	Micro meter
%	Percentage
^{0}C	Degree centigrade

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CHAPTER - I
GENERAL INTRODUCTION & REVIEW OF LITERATURE

1.1. COFFEE CLASSIFICATION

Coffee is an important plantation crop belonging to the family *Rubiaceae,* subfamily *Cinchonoideae* and tribe *Coffeae* (Clifford *et al* 1989). The *Rubiaceae* members are largely tropical or subtropical with nearly 400 genera and 4800-5000 species. '*Quahweh*' is the Arabic word from which originated the name 'coffee'. The word was a poetic term used for wine, which is forbidden for strict muslims. Therefore, the name was transferred to coffee, and through its Turkish equivalent *quahweh* became *cafe* (French), *caffe* (Italian), *Kaffee* (German), *koffie* (Dutch) and coffee (English) and Latin *Coffea* for the botanical genus (Smith 1985). The stimulatory effects of roasted coffee beans were well known to the natives of Africa when the Arabs brought *Coffea arabica* seeds from Ethiopia to Yemen (Arabian Peninsula) during the 13th century, and established the first plantations (Monaco *et al* 1997).

Classification:

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Asteridae
Order	Rubiales
Family	Rubiaceae
Genus	*Coffea*

1.2. COMMERCIAL COFFEE

Commercial coffee is mainly confined to two species *C. arabica* (2n=44) and *C. canephora* (2n=22) (Ocampo and Manzanera 1991). These two species account for 75% and 25% of the market respectively (Berthouly and Etienne 1999). *C. arabica* is by far the most widely cultivated species. A few other species of coffee are consumed in areas producing them locally and these include *C. dewevrei*, *C. liberica* and *C. racemosa*

(Sreenath 2000). Small-scale cultivation of these species occurs in some African countries but the resultant beverages are generally of low quality and most of the beans are sold locally rather than exported (Mazzafera *et al* 1991). Caffeine free *Coffea arabica* have been recently discovered (Silvarolla *et al* 2004)

C. arabica is the only polyploid species so far described in this genus. It produces high quality coffee with comparatively low caffeine content, but is susceptible to diseases. The economic qualities of *C. arabica* together with its autogamous and perennial character has led to the development of homogeneous plantations all over the world (Carneiro 1999).

C. canephora Pierre ex Froehner cv. robusta, is a diploid (2n=22) species and contributes to 25% of the total coffee production (Berthouly and Etienne 1999), is the second most important species cultivated (Monaco *et al* 1997). *C. canephora* or robusta, as it is commonly called, accounts for 80% of African coffee production. However, robusta has also been cultivated in American and Asian countries (Carneiro 1999). *C. canephora* coffee was found to be a very good substitute for the *C. arabica* which was earlier devastated completely by the coffee leaf rust disease caused by *Hemileia vastatrix* (Waller 1987).

C. canephora produces coffee with higher caffeine content but grows more vigorously than *C. arabica* and *C. liberica*. The plant begins to yield earlier than *C. arabica* and the average yield is 4000-6000 Kg. per acre, while that of *C. arabica* is 2000-3000 Kg per acre (The Wealth of India 1950). *C. canephora* is widely used for the manufacture of instant coffee. Some of the major differences among *C. arabica* and *C. robusta* have been listed in Table 1.1

Table 1.1: Some differences between arabica (*Coffea arabica*) and robusta (*Coffea canephora*) coffee:

	Arabica	**Robusta**
Date species described	1753	1895
Chromosomes (2n)	44	22
Time from flower to ripe cherry	9 months	10-11months
Flowering	After rain	Irregular
Ripe cherries	Fall	Stay
Yield (Kg beans/hectare)	1500-3000	2300-4000
Root system	Deep	Shallow
Optimum temperature (yearly average)	24-30ºC	15-24ºC
Optimal rainfall	1500-2000 mm	2000-3000 mm
Growth optimum	1000-2000 m	0-700 m
Hemileia vastatrix (Leaf rust)	Susceptible	Resistant
Koleroga noxia (Black rot)	Susceptible	Resistant
Nematodes	Susceptible	Resistant
Tracheomycosis	Resistant	Susceptible
Coffee berry disease	Susceptible	Resistant
Caffeine content of beans	0.8-1.4% (Average 1.2%)	1.7-4.0% (Average 2%)
Shape of bean	Flat	Oval
Typical brew characteristics	Acidity	Bitterness

Adapted from International Coffee Organization, 2003, http:// www.ico.org).

1.3. COFFEE – CULTIVATION, PRODUCTION AND EXPORT

1.3.1. Global scenario

Global coffee production (Fig 1.1) has reached to 116 million bags (Anonymous 2005). Coffee has been harvested from an area of more than 104 million hectare (FAO 2003). It is grown in about 80 countries across the globe of which 51 are considered to be the major producers (Anonymous1996). In India as an agro based rural enterprise, coffee industry is a source of direct employment for about 4,00,000 people in the area of

cultivation apart from providing indirect employment to many in processing and trade sections. The world trade in coffee is an important contributor to the income of some fifty or more coffee producing countries. To a few countries coffee is the biggest earner; Burundi and Rwanda for example depend on coffee for 80% of foreign exchange income (Marshal 1985). The global coffee export data is presented in Fig 1.2.

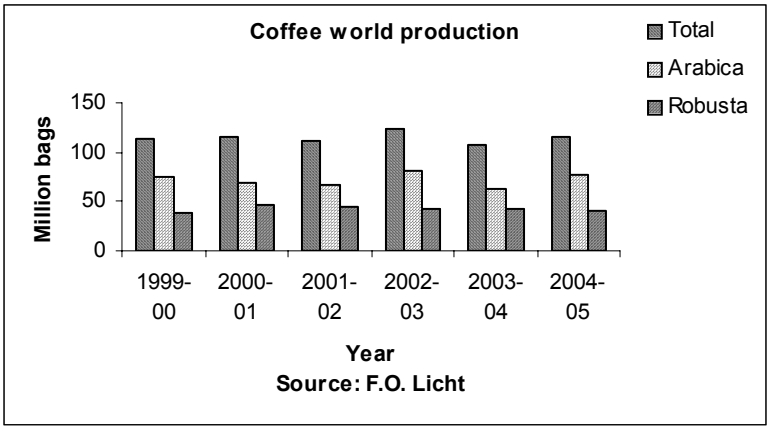


Fig 1.1 World coffee production (Adopted from Globe Scan, Indian Coffee, June 2005)

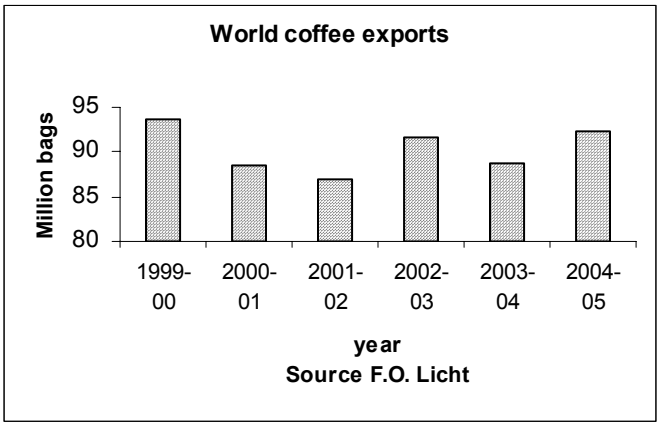


Fig 1.2. World coffee export (Adopted from Globe Scan, Indian Coffee, June 2005)

Indian scenario

Coffee is known to be introduced to India sometime during 1670's by a muslim pilgrim, Bababudan, who brought seven seeds from Yemen and raised seedlings on the hills near the town of Chikmagalur, Karnataka state (later named as Bababudan hills). Coffee was gradually planted in most of the neighbouring villages and later in the neighbouring states. Robusta coffee was introduced from Indo-China towards the end of the nineteenth century for planting in estates of lower elevation (Bheemaiah 1992). Commercial coffee

production in India began in around 1840 when the British established plantations throughout South India (John 1993).

Coffee production in India has reached to 275,000 tones from harvested area of 328,000 hectares in the year 2004 (FAO 2005) In India coffee is cultivated mainly in the southern states. India is a producer of both arabica and robusta varieties of coffee in proportion of 35:65. The total coffee production, area harvested and export from India has been presented in Fig 1.3, 1.4 and 1.5.

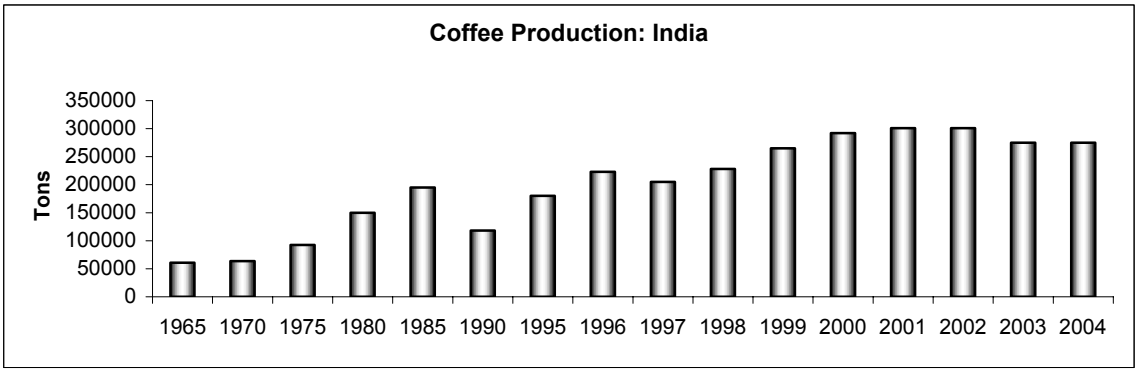


Fig 1.3 Total coffee production in India (Adopted from FAO 2005)

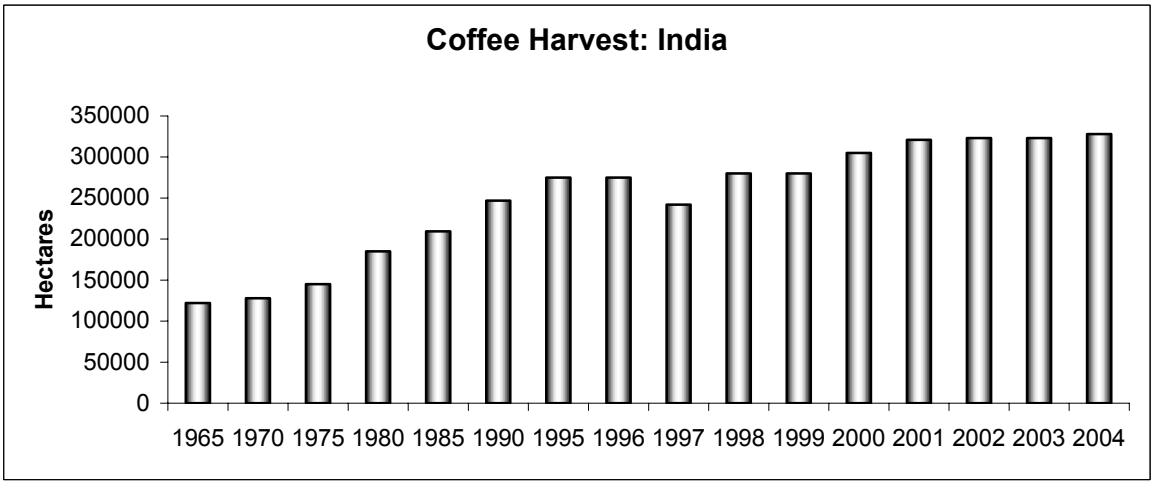


Fig 1.4 Total coffee annual harvest in India (Adopted from FAO 2005)

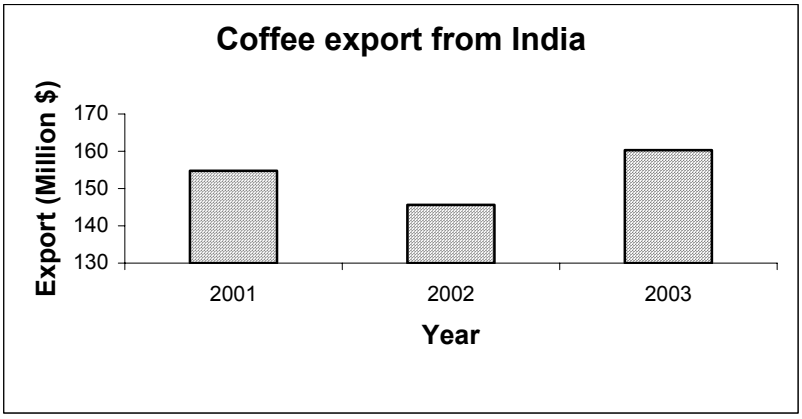


Fig 1.5 Total coffee export from India (Adopted from FAO trade yearbook 2003)

Today, in India, coffee occupies an important position among the export commodities particularly in the plantation sector. Production of coffee has risen from 18,000 tons during 1950s to 230,000 tons in 1998-99 (Anonymous 1996). The Indian coffee industry is also heading for the highest ever crop by the year 2000-2005 and estimated crop is 400,000 tons (Anonymous 2005).

1.4. COFFEE BEVERAGE

From a chemical point of view, a beverage can be defined as a liquid system with some nutritional factors (Petracco 2001). Coffee is an everyday part of our lives. Making coffee is both a ritual and a practical part of life. Unlike tea or cocoa, coffee lends itself readily to many different ways of making the infusion. All these methods share the basic principle which is to use hot water, to extract from the ground beans the natural essential oils, the caffeol, that give coffee its wonderful aroma and flavor. The resulting brew, or liquor, is a coffee infusion (www.ico.org). There are different ways of coffee preparation viz, Arab or Turkish coffee, the filter method, vacuum coffee, infusion methods, the plunger/cafetiere, the jug method, espresso and cappuccino, the moka-napoletana, the percolator, soluble or instant coffee, flavoured coffees, infusion methods, nepoletana coffee (Petracco 2001). The chemical composition of extracted coffee solids is presented below.

Table 1.2 Chemical compositions by mass of coffee solids (Petracco 2001)

Class	Percentage
Caffeine	8.25
Chlorogenic acid	18.5
Reducing sugars	1.45
Other carbohydrates	19.9
Peptides	6
Potassium	10
Other minerals	13.6
Acids	17.3
Trigonelline	5.15

1.5. COFFEE BIOTECHNOLOGY

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 have already been optimized successfully, including somatic embryogenesis and scale-up of somatic embryogenesis using bio-reactors, 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 (Reviewed by Carneiro 1999). 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 genetic transformation.

1.5.1. *In vitro* multiplication of coffee

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. This is insufficient to meet the demand. 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.

1.5.2. Micropropagation

Micropropagation to mass multiply the superior coffee genotypes using apical or axillary meristem culture (Reviewed by Carneiro 1999) has been reported. Techniques of *in vitro* multiplication of genotypes have been developed (Carneiro and Ribeiro 1989). Culture of micro cuttings in temporary immersion systems has been reported to increase the multiplication rate by 6-fold, in comparison with microcuttings multiplied on solid medium (Berthouly et al 1994, Teisson et al 1995).

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. In coffee, induction of adventitious buds was reported first time in the interspecific hybrid *Arabusta* a cross between arabica and robusta (Dublin 1980a, b). Carneiro (1990) reported induction of adventitious buds, for the first time, in *C. arabica* cultivars Caturra, Geisha and Catimor. Improved method for explant preparation for micropropagation of coffee through nodal culture has been reported (Ganesh and Sreenath 1999). Shoots of *C. arabica* and *C. canephora* reported to show increased growth when cultured on MS medium containing $AgNO_3$ (20 - 40 μM) and indole acetic acid (IAA) and BA (Giridhar *et al* 2003).

Success of newly formed plantlets is closely linked to the ability of the root system to adapt to the autotrophic conditions. Sondahl and Loh (1988) reported 60% rooting frequency in shoots treated with 10 mM IBA by using a two-layer medium consisting of basal B5 medium (Gamborg et al 1968) supplemented with IBA on top and basal medium with charcoal (2.5 gl^{-1}) on the bottom. Studies have been carried out with a view to improve the rate of rooting using different concentrations of MS mineral salts (Murashige

and Skoog, 1962), sucrose, activated charcoal, growth regulators, cytokinins, liquid and agar media (Reviewed by Carneiro 1999).

1.5.3. 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 and at a temperature of 28ºC. Successful somatic embryogenesis and plant regeneration from leaf explants of *C. arabica* was reported (Hermann and Haas 1975). Friable calluses, in the absence of auxin, were obtained by culturing endosperm tissue of *C. arabica* and *C. stenophylla* (Monaco et al 1977).

A number of protocols have been developed for various genotypes of *Coffea* (Reviewed by Carneiro 1999). High frequency somatic embryogenesis (HFSE) and low frequency somatic embryogenesis (LFSE) were established from leaf sections of *C. arabica* cv. Bourbon (Sondahl and Sharp 1977). HFSE was also reported in leaves of *C. canephora, C. congensis, C. dewevrei cv. Excelsa, C. arabica* cultivars Mundo Novo, Caturra, Laurina and C. *purpurascens* (Sondahl and Sharp 1977b). Dublin (1980a, 1981) described the induction of somatic embryogenesis in leaf segments of Arabusta. This technique of somatic embryogenesis was similar to LFSE reported by Sondahl and Sharp (1977).

Apart from this, two-step somatic embryogenesis process has been reported (Dublin 1980b, Pierson et al 1983, Neuenschwander and Baumann 1991). Effect of genotype on somatic embryogenesis in coffee was reported (Naidu *et al* 1999, Reviewed by Carneiro 1999). Picomolar concentrations of salicylates known to induce cellular growth and enhance somatic embryogenesis in *Coffea arabica* (Quiroz-Figueroa *et al* 2001) and histological studies on the developmental stages during embryogenesis have been

reported (Quiroz-Figueroa *et al* 2002). Santana *et al* (2004) reported various factors influencing embryogenesis in some recalcitrant genotypes of *Coffea canephora* under the influence of picloram as an auxin source.

Liquid cultures are known to be highly efficient in number of plant systems. Bioreactors play an important role in scaling up the production for commercialization of somatic embryogenesis based plant micro propagation. The induction of somatic embryogenesis of *C. arabica* in liquid medium using bioreactors was described (Zamarripa et al 1991, Zamarripa 1993). An estimated 15,000 embryos/l Erlenmeyer flask can be produced in 7 weeks of culture or 20,000 embryos/l in a bioreactor after 8 weeks of culture. Ducos et al (1993) and Noriega and Sondahl (1993) reported production of somatic embryogenesis in a commercial view. The maintenance of the embryogenic potential was reported to be maintained for 2 years in regeneration liquid medium (Van Boxtel and Berthouly 1996)**.** Although some authors (Zamarripa et al 1991, Ducos et al 1993, Noriega and Sondahl 1993) have reported obtaining 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 *Coffea* somatic embryo production (Reviewed by Etienne and Berthouly 2002). The effect of different cycle parameters on the quality aspects of somatic embryos was studied (Albarran *et al* 2005). Potentially, 40,000 embryos were reported to be produced from one gram of callus (Barry-Etienne et al 2002). The importance of this system lies in the simplification of handling and in the 10-fold reduction of labor costs. These results were reported for 9 different hybrids and about 15,000 plants were obtained (Barry-Etienne et al 2002).

Somatic embryogenesis was also obtained from integument (perisperm) tissues of coffee (Sreenath et al 1995). The role of ethylene in somatic embryogenesis from leaf discs of *Coffea canephora* was reported by Hatanaka et al (1995). Effect of genotype and growth regulators on somatic embryogenesis in coffee has been reported (Naidu and Sreenivasan 1999). Manuel de Feria *et al* (2003) reported effect of dissolved oxygen concentration on differentiation of somatic embryos of coffee. Triacontanol was reported to improve the embryogenesis in coffee (Giridhar *et al* 2004).

Table 1.3: Some of the reports on somatic embryogenesis of coffee

Variety	Explant	Cultural conditions	Period for embryog-enesis	Frequency of response (%)	Ref.
C. arabica	Leaf	LS + KN (0.1 mg l^{-1}) & 2,4-D (0.1 mg l^{-1})	2 months	-	Herman &Haas 1975
C. arabica & *C. canephora*	Internode	Two-step method	6 months	-	Raghura-mulu *et al* 1989
C. canephora	Leaves from *in vitro* plantlets	MS + NAA (0.1 mgl^{-1}) & BA (1 mg l^{-1})	5 months	93	Muniswamy & 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* 1991, 1995
C. canephora	Leaf	Two-step method	6-7 months	97	Van Boxtel & Berthouly 1996, Berthouly & Michaux-Ferriere 1996
C. canephora	Apical bud with hypocotyl	MS + BA (5 mg l^{-1})	5 months	-	Ganesh & Sreenath 1999
C. arabica	Cell suspension	10^{-12} & 10^{-10} M salicylic acid	1½ months	-	Quiroz-Figueroa *et al* 2001
C. canephora	Leaves	$AgNO_3$ 30-60 µM	2½ months	100%	Fuentes *et al* 2000
C. arabica & *C. canephora*	Leaves	Triacontanol	2 months	50-70% explant response	Giridhar *et al* 2004
C. canephora	Leaves	Picloram	-	-	Santana *et al* 2004

1.5.4. Induction and utilization of genetic variability

There are many ways of introducing variability in *in vitro* cultures and sometimes it is difficult to control these variations. Some techniques are well known, such as culture of

zygotic embryos, anther and pollen culture, cell suspension culture, *in vitro* selection and protoplast culture.

1.5.5. Culture of zygotic embryos

The culture of zygotic embryos allows the rescue of embryos resulting from interspecific crosses, which abort due to endosperm incompatibility. However, after F1 recovery, several cycles of back crossing and selfing are necessary to select the desirable gene combination and to eliminate deleterious genes. Collona (1972) for the first time demonstrated the zygotic embryo culture in four different coffee species namely, *C. canephora, C. dewevrei, C. excelsa* and *C. arnoldiana*. Sondahl and Loh (1988) reported the establishment of mature and immature embryo cultures of cultivars *Mundo Novo and Catuai* and the rescue of immature embryos resulted from *C. arabica* & *C. canephora* crosses. Raghuramulu *et al* (1989) demonstrated expansion of cotyledonary leaves, establishment of good rooting system and plantlet regeneration in zygotic embryo derived cultures. Hybrid plants have been reported from embryo culture of three interspecific crosses in coffee (Sreenath *et al* 1992).

1.5.6. Haploid production by androgenesis

The *in vitro* production of haploids is extremely valuable in plant breeding and genetics. This is useful in establishing homozygous lines of new varieties in a short period of time. This is particularly important for a highly heterozygous, long-generation tree species such as *Coffea*. Coffee anthers have about 2,000-40,000 microspores and each one of them may give a new combination during meiosis. The selected individuals could be crossed with commercial varieties to introduce plasticity for superior performance (Sondahl and Loh 1988)**.**

Sharp and co-workers developed the first coffee haploid plants (Sharp *et al* 1973). The authors reported the induction of callus from anthers of *C. arabica* cv. Mundo Novo *and* Bourbon Amarelo. Successful embryogenesis from isolated microspores was reported in *C. arabica* cv. Catuai and Catimor. (Carneiro 1990a, 1991, 1992, 1993, 1995). The regeneration of haploid plants by culturing *C. arabica* cv. Garnica anthers was reported by Ascanio and Arcia (1994). Carneiro (1995) referred to a repetitive or adventitious

embryogenesis in *C. arabica* cv. Catuai anthers. A protocol for the induction of androgenesis and plant regeneration from *C. arabica* cv. Caturra isolated microspores *in vitro* using colchicine pretreatment has been developed (Herrera *et al* 2002).

1.5.7. Protoplast culture

Protoplasts are highly useful in plant regeneration and somatic hybridization. Protoplasts are also used in direct transfer of foreign genes by electroporation or by permeabilization of the cell membrane by polyethylene glycol (Sondahl and Loh 1988). Protoplasts were prepared and regenerated from young leaves of hybrids of *C. arabica and C. canephora* (Orozco and Schieder 1982). Protoplasts were obtained from *C. arabica* cv. *Caturra* and plants regenerated *in vitro* (Rijo et al 1991). Plant regeneration after protoplast isolation from embryogenic callus suspensions of *C. arabica, C. canephora* and *Arabusta* was reported (Spiral and Petiard 1991, Acuna and de Pena 1991). The successful isolation and culture of coffee protoplasts from embryogenic calli and suspension cells has been reported (Mamatha and Sreenath 2000).

1.6. GENETIC TRANSFORMATION IN *COFFEA* SP

The success of transgenic research in Coffee has opened up new avenues for quality improvements. A number of transformation systems have been reported for arabica and robusta coffee, however only a few reports demonstrated the recovery of whole plant with stable integration of DNA. Rapid *in vitro* regeneration protocol is a basic need for the success of transgenic research in any plant species. A major hindrance for genetic transformations in coffee is slow rate of *in vitro* regeneration.

Transformation has been reported using the biolistic methods (Van Boxtel *et al*, 1995), DNA electroporation using protoplasts (Barton *et al* 1991) and various *Agrobacterium* systems (Ocampo and Manzanera 1991, Grezes 1993, Spiral *et al* 1993, Leroy *et al* 1997, Leroy *et al* 2000, Fernandez-Da Silva 2003). The first report of stable transformation of coffee plant used a protoplast system for electroporation (Barton *et al* 1991). Barton *et al* (1991) reported the recovery of *Coffea arabica* plantlets after selection of transformed protoplasts on kanamycin and regeneration. However the root system of the regenerated

transgenic plants was not well developed and plants capable of flowering were not produced.

Although many transformation systems have been investigated, most of the current work utilized the standard *Agrobacterium tumefaciens* mediated gene delivery system, despite the relatively low rate of transgenic plant production. Transgenic coffee plants containing the *cry1A(c)* gene were produced using both the *Agrobacterium rhizogenes* and *Agrobacterium tumefaciens* systems (Leory *et al* 1997, Leroy *et al* 2000). The *cry1A(c)* encodes for an insecticidal protein that is toxic to certain insects including the coffee minor, *Perileucoptera coffeela* (Oliveiro *et al*, 1998). Sugiyama and co workers (1995) reported *Agrobacterium rhizogenes* mediated transformation and regeneration plants from transgenic roots of *Coffea arabica* cv typica. However, All the plantlets reported to be with typical *rol* gene phenotype, short internodes and stunted growth. Spiral and co-workers (1999) found that the *npt* II kanamycin resistance was not an effective selection marker in *Coffea* somatic embryos. Transgenic selection with chlorosulfuron resistance was reported to be effective (Spiral *et al* 1999). In *Coffea* sp. the frequency of transgenic plants produced is considerably below that of many other transformation systems (Leroy *et al* 1997). In *Agrobacterium tumefaciens* system, 0.4% of the somatic embryos infected gave transgenic plants (Leroy *et al* 1997). However improvements in the selection systems have recently been reported. Leroy and co workers reported GUS gene expression after 10-12 months of selection under chlorosulfuron (Leroy *et al* 2000). Ocampo and Manzanera (1991) demonstrated that *C. arabica* tissues could be infected by wild strains of *Agrobacterium tumefaciens*. Hatanaka and co-workers (1999) reported stable transformation and regeneration of transgenic *Coffea canephora*. The authors adopted *Agrobacterium tumefaciens* strain EHA101 harbouring pIG121-Hm consisting of *β* glucournidase (*uid* A), hygromycin phosphotransferase (*hpt* II) marker gene for transformation. Grezes and co-workers (1993) reported *A. tumefaciens* mediated genetic transformation. Van Boxtel (1994) transformed coffee using biolistic gene delivery method. Studies on five selection markers i.e., chlorsulfuron, glufosinate, glyphosate, hygromycin and kanamycin indicated the potential of glufosinate for the detection of stably transformed coffee tissue (Van Boxtel 1994). Kanamycin resistant plantlets were obtained from coffee leaf segments through *Agrobacterium* mediated transformation

(Naidu et al 1998). De Pena (1995) reported optimization of conditions for direct DNA uptake using the poly ethylene glycol (PEG) method. Fernandez-Da Silva (2003) established an electroporation mediated genetic transformation method of coffee (*Coffea arabica* cv. Catimor) and incorporation of BAR gene for ammonium glufosinate resistance. Fernandez-Da Silva and Menendez (2003) reported optimization of gene delivery to *C. arabica* by electroporation mediated transformation.

1.7. COFFEE, CAFFEINE AND HEALTH

Caffeine has been the subject of extensive research for two reasons, its wide occurrence in nature and its long history of use. Investigators have identified over 60 plant species that contain caffeine, and history suggests that it may have been consumed, in one form or another, as far back as the Paleolithic period (Reviewed by Barone and Roberts 1996). In recent years, even more attention has been given to caffeine. The US Food and Drug Administration (FDA) focused regulatory attention on caffeine as part of its review of generally recognized as safe (GRAS) substances, which was initiated in the late 1960s. Caffeine again received close scrutiny in 1987 when the FDA proposed to establish a prior sanction regulation for caffeine (Reviewed by Barone and Roberts 1996). Coffee beans contain 0.8 - 2.8 % caffeine, depending on species and origin, and it contributes to 10 to 30% of the bitterness of the coffee beverage. There are only negligible losses of caffeine in the roasting process. Caffeine is mainly used in soft drinks, but also together with other active agents in remedies for headaches, cardiac insufficiency, migraine or the disorders of the respiratory center.

Caffeine is believed to be the most widely consumed and studied drug in history (Weinberg and Bealer 2001). Coffee has been known to possess free radical scavenging activity (Daglia *et al* 2004). However possible reported adverse effects include those to coronary heart disease (Kawachi *et al* 1994), fibrocystic breast disease (Garattini 1993, Curatolo and Robertson 1983), several types of cancer, (La Vecchia 1993), and reproductive health effects (Hinds *et al* 1996). Possible beneficial relations include reduced risk of suicide (Klatsky *et al* 1993) or liver cirrhosis (Klatsky and Armstrong 1992). No overall effect on mortality has been found (Klatsky *et al* 1993, Heyden *et al*

1978). Scientific information on the impact of coffee consumption on health has been summarized in Table 1.4.

Table 1.4 Brief summary of published work on coffee consumption and health effects

Coffee consumption and health effects		**Ref**
Cancer	Weak positive correlation with ovarian cancer	Nehling and Debry 1996
	Did not reveal any association with breast cancer risk.	Folsome *et al* 1993, Tavani *et al* 1998, WHO/IARC 1991
	Did not reveal any association with renal cancer or cancers of urinary tract.	Nehling and Debry 1996, WHO/IARC 1991
	Weak positive correlation with bladder cancer.	WHO/IARC 1991, Nehling and Debry 1996,
	Did not reveal any association with esophageal and gastric cancers	WHO/IARC 1991, Nehling and Debry 1996,
	Weak positive correlation with esophageal and gastric cancers	Inoue *et al* 1998
	Weak positive correlation with pancreatic cancer in heavy coffee drinkers	WHO/IARC 1991, Nishi *et al* 1996
	Protection against colorectal cancer	WHO/IARC 1991, Nehling and Debry 1996, Giovannucci 1998
	Protection against carcinogens	Obana *et al* 1986
Mutagenic	Shown to be slightly mutagenic in *in vitro* systems (bacteria, fungi, mammalian cells)	Nagao *et al* 1986
	Did not reveal any association with mutagenicity in *in vivo* systems	Nehling and Debry 1994b
	Antimutagenic	Stadler *et al* 1994, Abraham 1991
Antioxidant	Free radical scavenging activity	Stadler *et al* 1994, 1995, Sanchez-Gonzalez *et al* 2005
Cardiovascular disease	Did not reveal any association with coronary heart disease	Myers 1992, Willett *et al* 1996, Sesso *et al* 1999
	Associated with myocardial infarction or coronary death.	Greenland 1993
	Associated with myocardial infarction in heavy coffee drinking women.	Palmer *et al* 1995

	Associated with increased norepinephrine resulting in transient high blood pressure	Debry 1994, Green *et al* 1996
	Associated with increase in serum cholesterol.	Thelle *et al* 1987
	Associated with higher plasma total homocysteine.	Nygard *et al* 1998, Oshaug *et al* 1998
	Increases the urinary excretion of calcium	Debry 1994
Bone health	Not associated with calcium loss under calcium-supplemented diet.	Heaney and Recker 1982
	Coffee with milk is sufficient to supplement the calcium loss	Barger-Lux and Heaney 1995
	Association of Coffee drinking with risk of osteoporosis is contradictory	Debry 1994, Heaney 1998
Reproductive and developmental studies	Most of the studies do not support the association between coffee consumption and congenital malformations.	Nehling and Debry 1994b, Brent 1998
	Associated with neurobehavioral disturbances in infants	Dumas *et al* 1982
	Sudden infant death associated with heavy maternal caffeine ingestion	Ford *et al* 1998
	Long term behavioral disturbances, increased locomotion, effects on learning abilities observed in rodents	Nehling and Debry 1994a
	Heavy Coffee consumption associated with low birth weight	Rondo *et al* 1996
	Fetal loss associated with caffeine intake before and during pregnancy	Infante-Rivard *et al* 1993
	Associated with spontaneous abortion in heavy coffee drinkers and possess high serum paraxanthine	Dlugosz *et al* 1996
	Associated with reduction in fertility	Wilcox *et al* 1988
	Associated with delayed conception	Stanton and Gray 1995
Beneficial health effects	Increase alertness, performance, vigilance tasks and reduce fatigue	Smith 1998
	Lower risk of suicide	Klatsky *et al* 1993
	Lower risk of suicide particularly in women	Kawachi *et al* 1996
	Fewer depressive symptoms among female but not male.	Mino *et al* 1990
	Protection against colon cancer	Giovannucci 1998
	Reduced risk of liver cirrhosis in induced by alcohol consumption	Klatsky and Armstrong 1992, 1993, Corrao *et al* 1994

	Reduces the level of serum γ-glutamyltransferase a marker of hepatobiliary disease	Nilssen and Forde 1994, Tanaka *et al* 1998
	Protection from radiotherapy induced late radiation injury	Stelzer *et al* 1994

1.7.1. Caffeine in drugs

Caffeine is present in many prescription and non-prescription (over-the-counter) drugs, including some taken for headache, pain relief, appetite control, staying awake, colds, asthma and fluid retention (www.ico.org). The caffeine content of drugs varies from 7mg to 200mg per tablet (www.ico.org). Twenty five percent of the total caffeine production goes into medicine (Fig 1.6) and 74 % is reported to be shared by the beverages (Heilmann 2001)

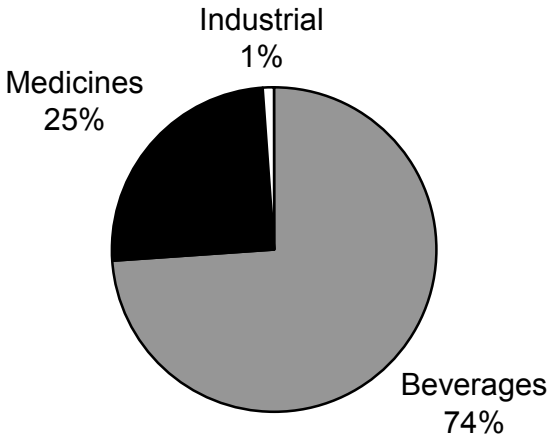


Fig 1.6: caffeine application worldwide

1.7.2. Levels of caffeine consumption

Caffeine is generally consumed in amounts less than 300mg per day, roughly equivalent to 3-4 cups of roast and ground coffee / 5 cups of instant coffee / 5 cups of tea / 6 servings of some colas or 10 tablets of some painkillers (*www.ico.org)*. Customary caffeine consumption has been classified as follows (Lecos1984): Low caffeine users:

less than 200mg per day; Moderate caffeine users: 200-400mg per day; High caffeine users: more than 400mg per day.

1.7.3. A summary of the physiological effects of caffeine

The physiological effect of caffeine has been dealt in detail in the following references (Anonymous 1987, www.ico.org, Tavani and Vecchia 2004). Soon after drinking a cup of coffee, or tea or cola, caffeine is distributed throughout the body. However, caffeine does not accumulate in the body, so its effects are short-lived and transitory. The caffeine half-life ranges from 0.7-1.2 h in rats, 3-5 h in monkeys, 2.5-4.5 h in humans and about 80 ± 23 h. in newborn infants (Reviewed by Nehling 1999). Effect of caffeine varies depending on a number of factors. Every individual reacts differently to caffeine. For example, caffeine may stay in the body of pregnant women for up to 3 times as long as is usual in adults, whereas smokers eliminate caffeine twice as quickly as non-smokers. Some of the effects of caffeine, such as those on the heart and blood vessels, are contradictory and have no net effect - others may only be noticeable when regular consumers suddenly cut out caffeine. The body can become habituated to caffeine so that regular users are less sensitive to the stimulant effects than others (Reviewed by Nehling 1999).

Of all the physiological effects of coffee, the best known is that it is a stimulant to the nervous system. One or two cups of coffee can make one feel more awake, alert and able to concentrate. However, in sensitive individuals, caffeine may delay the onset of sleep, decrease sleep time and even lower the subjective quality of the sleep. Caffeine has various effects on mood, ranging from pleasant stimulation and mood elevation to anxiety, nervousness and irritability, but these are transient and dose-related. Effects of caffeine have given rise to the development of several methods for removal of caffeine from beverages (Reviewed by Nehling 1999). Overall physiological effects of caffeine have been summarized in table 1.5

Table 1.5 Brief summary of physiological effects of caffeine

Reported physiological effects associated with caffeine		**Ref**
Positive effects	Potent antioxidant	Daglia *et al* 2004, Devasagayam *et al* 1996, Shi *et al* 1991
	Enhanced auditory vigilance and reaction time	Reviewed by Nehling 1999
	Caffeine is used in combination with ergotamine to treat vascular headaches	Gennaro 1985
	It is used in with analgesics and diuretics to relieve tension and fluid retention associated with menstruation.	Gennaro 1985
	Effective, as an analgesic adjunct in combination with aspirin or acetaminophen and aspirin to enhance pain relief,	FDC Reports. 1993
Negative or neutral effects	The caffeine withdrawal-induced behavioral changes lasts for few days	Griffiths & Mumford 1996
	Caffeine withdrawal translates into typical symptoms of headaches, weakness and drowsiness, impaired concentration, fatigue and work difficulty, depression, anxiety, irritability, increased muscle tension, occasionally tremor, and nausea and vomiting, as well as withdrawal feelings	Griffiths *et al* 1990, Hughes *et al* 1993, Nehlig & Debry 1994, Richardson *et al*, 1995, Silverman *et al* 1992, Strain & Griffiths 1995, Strain *et al* 1994, Nehlig *et al* 1992.
	Decreases in locomotor activity	Finn & Holtzman 1986, Holtzman *et al* 1983
	Operant behavior	Carney 1982, Carroll *et al* 1989, Mumford 1988
	Slow wave sleep	Sinton & Petitjean 1989
	Avoidance of a preferred flavor	Vitiello & Woods 1977
	Changes in cerebral blood flow.	Levy & Zylber-Katz 1983
	Caffeine was shown to be able to reinstate extinguished cocaine-taking behavior in rats.	Schenk *et al* 1996, Worley *et al* 1994
	The acute administration of caffeine does not lead to release of dopamine	Nehlig *et al* 1986, Nehlig *et al* 1984
	Caffeine leads to widespread cerebral metabolic increases	Nehlig *et al* 1986, Nehlig *et al* 1984

	Caffeine primarily acts on the extrapyramidal motor system leading to the release of dopamine in the caudate nucleus.	Okada *et al* 1996 &1997
	Caffeine fulfils some of the criteria for drug dependence	Nehlig 1992
	At higher concentrations, caffeine produces definite tachycardia and sensitive persons may experience other arrhythmias, such as premature ventricular contractions.	Gilman 1990
	Vascular Caffeine causes constriction of cerebral vasculature with an accompanying decrease in cerebral blood flow and in the oxygen tension in the brain.	Gilman 1990
	Caffeine stimulates voluntary skeletal muscle, possibly by inducing the release of acetylcholine, increasing the force of contraction and decreasing muscle fatigue	Gilman 1990
	Caffeine causes secretion of both pepsin and gastric acid from parietal cells.	Gilman 1990
	Caffeine increases renal blood flow and glomerular filtration rate and decreases proximal tubular reabsorption of sodium and water, resulting in a mild diuresis.	Feldman 1990
	Caffeine also inhibits uterine contractions, increases plasma and urinary catecholamine concentrations and transiently increases plasma glucose by stimulating glycogenolysis and lipolysis	Gennaro 1985
	Overdose symptoms: Abdominal or stomach pain, agitation, anxiety, excitement or restlessness, confusion or delirium, fast or irregular heartbeat, fever, frequent urination, head ache, increased sensitivity to touch or pain, irritability, muscle trembling or twitching, nausea and vomiting, swollen abdomen or vomiting in neonates	Harrisons 1998
	Ringing or other sounds in ear, seeing flashes of zigzag lights, seizures, trouble in sleeping, whole body tremors in neonates in acute overdoses	Harrisons 1998
	Caffeine has the property of reversing S and G2 checkpoint function during cell division and reducing DNA repair in irradiated cells.	Kaufmann *et al* 2003

1.7.4. Caffeine content in coffee

The amount of caffeine in a cup of coffee can vary greatly, depending on its origin or the composition of the blend, the method of brewing and the strength of the brew. Instant, or soluble, coffee generally contains less caffeine than roast and ground coffee, but may be consumed in greater volume. Robusta coffees have about twice as much caffeine as arabica.

Table 1.6 Caffeine range in different beverages

Beverages	**Caffeine Range** (mg per 150ml cup)
Roast and ground -drip method -percolator	 60-180 40-170
Instant coffee	30-120
Roasted and ground decaffeinated coffee	2-5
Instant decaffeinated coffee	2-8
Tea	8-91
Bagged tea	28-44
Instant tea	24-31
Regular cola soft drinks	15-24

(Adopted from Barone and Roberts 1996)

1.8. DISTRIBUTION OF CAFFEINE IN PLANTS

Worldwide, six caffeine-containing genera are used to prepare pleasant stimulants, namely *Coffea*, *Camellia*, *Theobroma*, *Cola*, *Ilex*, and *Paullinia*. Whereas the first three taxa have been well studied regarding the occurrence and within-the-plant distribution of purine alkaloids, *Coffea* (Charrier and Berthaud 1975) *Camellia* (Zheng *et al* 2002.), *Theobroma* (Hammerstone *et al* 1994), the remaining three, i.e., *Cola*, *Ilex*, and *Paullinia*, are poorly investigated in this respect, most likely because of their lesser economic importance and/or species richness. Caffeine has been found in 13 orders of the plant kingdom. Most caffeine containing plants are members of the dicotyledoneae, although *Scilla maritima* belongs to the monocotyledoneae (Reviewed by Ashihara and Suzuki 2004).

1.9. ROLE OF CAFFEINE IN PLANTS

Secondary metabolites are known to provide protection against physical and biotic factors such as pathogens and predators. Based on principles of evolution, a general strategy for optimal chemical defense against predation was postulated (Rhoades 1979)

Tissues with high dietary value (vegetative parts of plants) have high risk of predation (Rhoades 1979). Caffeine formation is strongly accelerated during early leaf emergence stage reaching up to 17mg/day/gram and breaks down during leaf senescence (Frischknecht *et al* 1986). These evidences demonstrated that tissues with a high rate of predation are preferably protected by a chemical defense system based on purine alkaloid may be established for a limited time (Frischknecht *et al* 1986). Caffeine is known to have a toxic effect on insects and fungi at concentrations found in coffee plants. Even at lower concentrations i.e., 0.3% caffeine killed tobacco hornworm (*Manduca sexta*) and the effect is mainly due to the inhibition of the phosphodiesterase activity and increases cyclic AMP (Nathanson 1984). In *Callosobruchus chinensis* (L) caffeine causes nearly 100% sterility at a concentration of 1.5% (Rizvi *et al* 1980). The fungitoxic effect was demonstrated in number of *Aspergillus* sp *Penicillium* sp (Buchanan *et al* 1981) and Saprolegniaceae (Prabhuji *et al* 1983)

There is strong evidence that caffeine released from leaves and beans may be allelopathic (Rizvi *et al* 1980, 1987, Waller *et al* 1986). Baumann *et al* (1995) has given a convincing explanation for caffeine as a deterrent compound in guarana fruits, which are used as food for some birds. They showed that the aril is rich in sugars and lacks caffeine related alkaloid. Caffeine did not release when the avian stomach conditions were artificially stimulated. Since the birds do not break the seeds during ingestion, it was suggested that a seed coat barrier prevented diffusion of caffeine. More recently Hollingsworth (2002) and co-workers clearly demonstrated that caffeine is a repellent for slugs and snails. Caffeine at the concentration 0.1% or more concentration found to be a lethal neurotoxin to these garden pests.

It was shown that seeds of selection of *C. arabica* from Ethiopia and Kenya with higher caffeine content were more resistant to *Colletotrichum coffeanum,* the causal agent of the coffee berry disease (Biratu *et al* 1996). Medeiros *et al* (1990) observed an increase of caffeine in infected leaves of coffee selections displaying resistance against the leaf rust *Hemeleia vastatrix.* Mazzafera (1991) observed in a field experiment for coffee yield selection that among 2500 plants, leaf-cutting ants preferred a few plants. Analysis showed that one noticeable difference in leaves of these plants was their lower caffeine content.

1.10. NATURAL LOW CAFFEINE *COFFEA* SP

A programme of genetic breeding was initiated in 1987 to obtain low caffeine lines at the Instituto Agronomico de Campinas, 3,000 coffee trees, representing 300 *C. arabica* accession from Ethiopia have been screened. Silverolla *et al* (2004) discovered three caffeine free plants obtained from Ethiopia and designated as AC1, AC2 and AC3. These plants found to possess theobromine but not caffeine indicating that the plants might be deficient in enzyme caffeine synthase. The results of radiolabel study demonstrated that the low caffeine trait of these plants is not because of rapid degradation, but due to deficiency of caffeine synthase activity.

1.11 BREEDING FOR CAFFEINE FREE COFFEE

Coffee breeding by conventional methods is a long process involving several different techniques, namely, selection from wild populations followed by hybridization 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.

The deleterious effects of caffeine are well documented and this led coffee industry to provide an artificially de-caffeinated coffee. Since solvent extraction is used for decaffeination, an alternative and health conscious approach would be to identify naturally occurring low caffeine lines (Srinivasan, 1996). This would be important for *C. canephora*, for which the caffeine content on a dry matter basis varies from 1.2% to 3.5% (Charrier and Berthaud 1975). Although genetic improvement using intraspecific diversity is an obvious approach, this approach seems inadequate to obtain caffeine-free coffee. Interspecific hybridization has been broadly developed in the genus *Coffea* (Carvalho *et al* 1965) and could lead to 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, 1991). Unfortunately, caffeine-free species are low yielding and give a beverage of poor tasting quality.

Although low caffeine material is currently available, for a variety of reasons, none is suitable for commercial exploitation (Mazzafera *et al* 1991). For example, seeds of *C.*

eugenioides contain 0.4% caffeine (Charrier and Berthaud 1975), but the form and low productivity of trees of this species preclude its use for commercial coffee production. Some wild coffees, e.g. the *Mascarocoffea C. vianneyi* from Madagascar, while virtually free from caffeine yield a bitter a beverage due to the presence of bitter diterpene glycosides such as mascaroside (Smith, 1985). Clifford *et al* (1991) showed that *C. kianjavatensis,* one taxon of the *Mascarocoffea*, contains about 0.55-0.81% caffeine in the beans which is traditionally viewed as caffeine-free. Economic exploitation of *Mascarocoffea* species is, however, impaired by poor agronomic characteristics and the bitter taste of the coffee caused by the presence of cafamarine (Charrier 1978). Attempts to transfer the caffeine-free trait from *Mascarocoffea* to *C. arabica* and *C. canephora* have been unsuccessful because of the infertility of the hybrid progeny (Charrier 1978).

The mean caffeine content of the descendants largely depends on the degree of heterozygosity of the parents. Therefore, the genetic variation of the caffeine content as determined in an entire collection of cultivated genotypes was found to be much higher in the allogamous *C. canephora* than in the autogamous *C. arabica*. (Charrier and Berthaud 1975).

1.12. DECAFFEINATED COFFEE:

Whatever method of decaffeination is used, the decaffeinated green coffee must contain less than 0.1 % caffeine (dry weight basis) to comply with EC regulations (www.ico.org). This corresponds to about 3mg caffeine in a cup of decaffeinated coffee. Decaffeinated coffee constitutes 17 per cent of coffee sales worldwide. In the United States the market for decaffeinated coffee, with 46 million American drinking decaffeinated coffee, represents 15 percent of total coffee consumption (www. ico.org). Exclusive decaffeinated drinkers represent 70 per cent of the decaffeinated market, the majority being women aged 35-55 who drink 80 per cent of their coffee in the morning, and who, according to the Swiss Water Decaffeinated Coffee Company, will prefer a chemical-free process and be willing to pay a premium price. A significant group, 30 per cent of decaffeinated coffee drinkers, also frequently drinks regular coffee. Other research indicates that decaffeinated coffee accounts for over 20 per cent of the US market, and 9 per cent of the UK market, with demand being greater in older age groups, with the over

60s in the US using 55 per cent of decaffeinated coffee. A recent survey (Anonymous 2004) indicated that the market for decaffeinated coffee is steady (www. ico.org).

In order to minimize flavor and aroma losses, the commercial decaffeination of coffee is at present carried out on the green coffee beans before roasting. (Lack and Seidlitz 1993). 'Decaffeinated Coffee' means in the EU countries maximum caffeine concentration of 0.1% related to the dry mass, in the US, it means less than 3% of the amount initially present in the beans.

Detailed information on different decaffeination process is reported by Heilmann (2001) Principally all the decaffeination process consists of five steps:

1. Swelling the raw beans with water in order to solubilize the caffeine potassium chlorogenate complex, and to make caffeine available for extraction.
2. Extracting the caffeine from the beans with the solvent.
3. Stream stripping to remove all solvent residues from the beans.
4. Regenerating the adsorbents.
5. Drying the decaffeinated coffee beans to their initial moisture content.

The different decaffeination procedures can be classified in to following major groups:

1.12.1. Solvent Decaffeination:

Decaffeination with chemical solvents (which are approved under the rules of all food legislations) such as methylene chloride or ethyl acetate was used in decaffeination process. Due to its relatively low investment and operating costs and the high coffee quality, more than 50% of the worldwide capacity is based on the solvent decaffeination. More than 30 solvents have been tested in the past 50 years, but dichloromethane and ethyl acetate predominates representing about 98% of all solvent processes.

Another invention relates to a process where in extraction of caffeine and substances that are potentially detrimental to health from green coffee is carried out by the use of sufficient acid to remove chlorogenic compound from coffee (Heilmann 2001). In this organic solvent is mixed with an acid (such as acetic, formic or citric acid up to 25% and water upto 15%). In this decaffeinated process a mild coffee is produced in which caffeine, wax and chlorogenic acid compounds are removed simultaneously.

Zeller and Saleeb (1999) reported improved process for decaffeination using 2,4-dihydroxycinnamic acid. Another decaffeination method for green and roasted coffee extracts has been developed (Kaleda *et al* 1986). Caffeine containing extract solution is mixed with caffeic acid crystals in the presence of water. The caffeine and caffeic acid form an insoluble complex. This complex can be separated by filtration or centrifugation.

1.12.2.Water decaffeination:

Berry and Walters (1943) used green coffee extract with equilibrium quantities of non-caffeine soluble solids and removal of caffeine from the extract with dichloromethane in liquid- liquid extraction (Heilmann 2001). The application of the preloaded activated carbon for the caffeine adsorption instead of solvent extraction was the basis for the 'Swiss water process' (Fischer and Kummer 1979). This is much more expensive than the solvent decaffeination method, but is used in 22% of decaffeination plants (Heilmann 2001).

Originally fresh water is used for decaffeination of the swollen beans. After removal of the caffeine the solution is concentrated and re-adsorbed on the pre-dried decaffeinated beans from which it had been obtained, thus replacing non-caffeine solids otherwise lost.

1.12.3. Supercritical CO_2 decaffeination:

There has been a growing disquiet among consumers about the use of synthetic mainly chlorinated chemical solvents in the food industry. This has lead to the development of an alternative processes using solvents of natural origin (Lack and Seidlitz, 1993). One of those new technologies was the application of supercritical CO_2 for the extraction of caffeine from green coffee beans. The CO_2 decaffeination is used for about 19% of the worldwide capacity (Heilmann 2001).

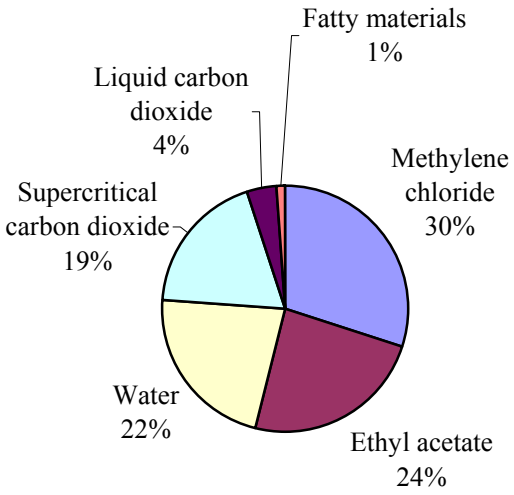


Fig: 1.7 Percentage distributions of decaffeination methods (Heilmann 2001)

1.12.4. Economic aspects

Due to an increase in consumption of decaffeinated coffee in the 1970s and 1980s there have been installed decaffeination plants of very large capacities, mainly from roast coffee companies. The installation followed the main areas in which decaffeinated coffee is consumed, namely central Europe and United States. Approximately 50% of the worldwide capacities are located in Germany and France (Heilmann 2001).

Whereas the consumption of decaffeinated coffee in the Western countries is declining, this is compensated for by an increasing demand in Eastern Europe. In total, the worldwide-decaffeinated coffee represented, in 1998 a volume of 382000t with sales value of US$5 billion.

Since the early 1970's, sales of decaffeinated coffee have increased markedly because of a growing belief that the ingestion of large amounts of caffeine can cause adverse effects on health (Mazzaferra *et al* 1991). Hence caffeine-free coffee is demanded in the world market. However, such product is poor in quality, and other methods to produce caffeine-free coffee are being intensively investigated (Ogita *et al* 2002).

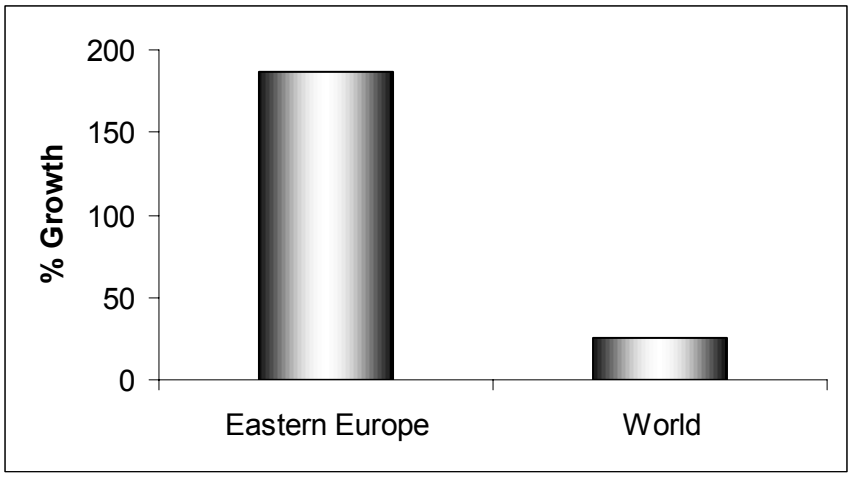


Fig: 1.8 Percentage growth of decaffeinated coffee sales in world and Eastern Europe during 1994-1998 (Heilmann 2001)

1.13. CAFFEINE BIOSYNTHESIS

1.13.1. Caffeine Biosynthetic Pathway and *N* methyltransferases

The two SAM-mediated methylations of the 7-methylxanthine to theobromine and caffeine respectively were the first-known steps of the purine alkaloid synthesis (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 catalyzed the *N*-3- and *N*-1- methylations (Baumann *et al* 1983). *C. arabica* cell free extracts of callus cultures revealed that the active biosynthesis of caffeine was exhibited from 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 SAM dependent 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 Km values obtained for

theobromine and 7-methylxanthine were 0.196 and 0.496 respectively. Gillies *et al* (1995) improved the 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, 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 second *N*-methyltransferase activity was always considerably higher than the 3rd one. However, they frequently paralleled each other (Waldhausser *et al* 1997b), as they were components of one entity (Waldhausser *et al* 1997b). 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. 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 plants. Subsequently, this fragment was used as a probe, and four independent clones were isolated from a cDNA library derived from coffee young leaves. One of them was found to encode a protein possessing 7-methylxanthine methyltransferase activities and was designated as *CaMXMT* upon expression in *Escherichia coli*. 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 - 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. Km values were estimated to be 50 and 12 μM for 7-methylxanthine and *S*-adenosyl-L-methionine, respectively and this was found to target the cytoplasm (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 (RACE) 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 characterization and in vitro reconstitution of the enzymes have been carried out. The cDNAs were named as *CaMXMT2* and *CaDXMT1* respectively. *CaMXMT2* catalyzed the formation of theobromine from 7-methylxanthine with a Km of 251 μM, and CaDXMT1 catalyzed the formation of caffeine from theobromine with a Km of 1,222 μM (Uefuji *et al* 2003). These reports suggest that, in coffee plants, caffeine is synthesized through three independent methylation steps from xanthosine (Ogawa *et al* 2001, Uefuji *et al* 2003). The main biosynthesis route utilizes the purine nucleotides through the steps (AMP and/or GMP) → IMP → XMP → xanthosine → 7-methylxanthosine → 7-methylxanthine → theobromine → caffeine pathway.

Sub cellular compartmentation of purine alkaloids has not yet been verified. However, it is in keeping with many other secondary metabolites that caffeine and other purine alkaloids are stored in vacuoles (Wink 1997). The mechanisms for uptake and sequestration of caffeine in vacuoles are yet to be investigated. Active/passive transport by channels and/or transporters and membrane/vesicle fusion, may be involved in these processes. Binding of caffeine to polyphenols such as catechins (tea) and chlorogenic acids (coffee) may allow the accumulation of these compounds in vacuoles against a concentration gradient. Compartmentation of enzymes of tea caffeine biosynthesis and the SAM cycle has been examined biochemically. It was found that caffeine synthase, which is a key enzyme catalyzing the final two steps of caffeine biosynthesis, is located in chloroplasts (Reviewed by Ashihara and Suzuki 2004).

Examination of the subcellular localization of coffee theobromine synthase (*CaMXMT*) using the fusion protein of *CaMXMT* and green fluorescent protein found it present predominantly in the cytoplasm of onion epidermal cells (Ogawa *et al* 2001). The PSORT program running with the deduced amino acid sequence also predicted a high possibility of cytosolic localization for CaMXMT. Based on these results, Ogawa *et al* (2001) suggested that caffeine biosynthesis occurs in the cytoplasm (cytosol) of cells in buds and young leaves. The observed discrepancy in the localization of the caffeine

biosynthetic pathway according to the different techniques is not yet resolved. The proposed pathway of the final stage of caffeine biosynthesis in coffee is illustrated in Figure 1.9

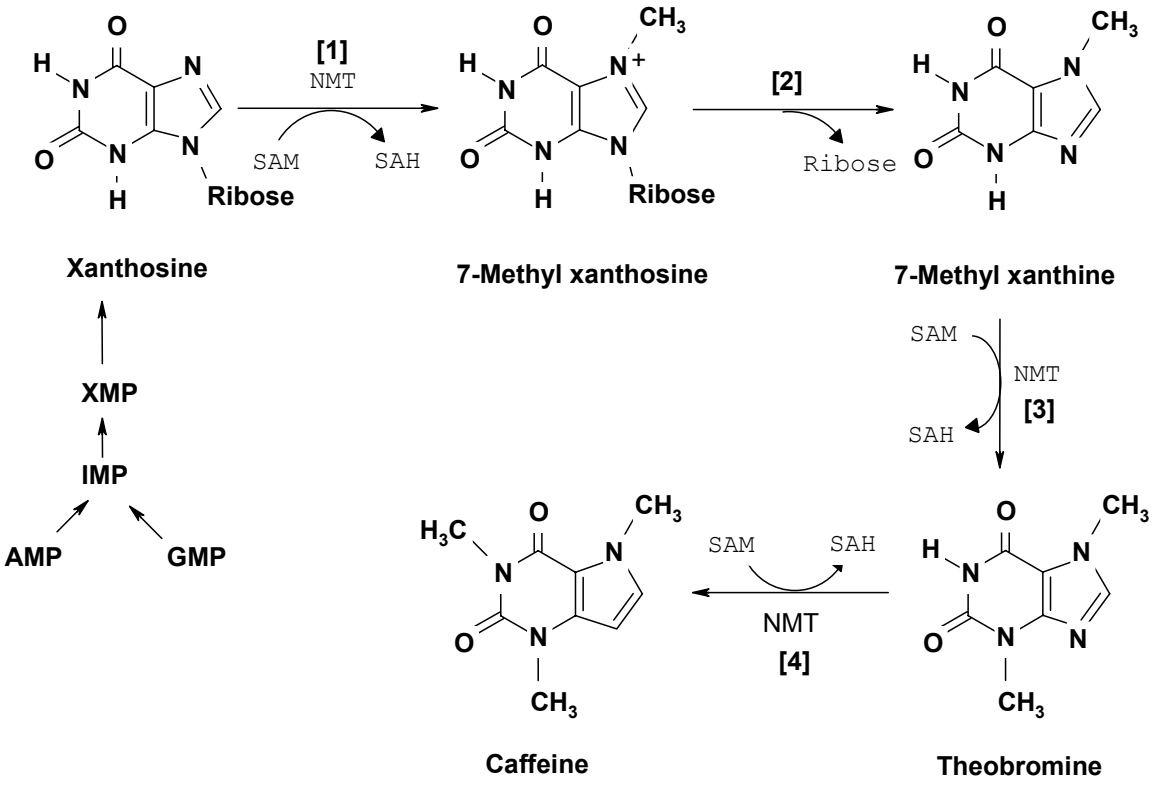


Fig 1.9. The major route of caffeine biosynthetic pathway in coffee plants.

[1] 7-methylxanthosine synthase (xanthosine *N* methyltransferase, EC 2.1.1.-), [2] N-methylxanthine nucleosidase (EC3.1.3.-), [3] theobromine synthase (7-methylxanthine *N* methyltransferase, EC 2.1.1.-) and / or caffeine synthase (7-methylxanthine and theobromine *N*-methyltransferase, EC 2.1.1.-), [4] caffeine synthase (7-methylxanthine and theobromine *N*-methyltransferase, EC 2.1.1.-). EC numbers have not yet been given for all enzymes of this pathway (Ashihara and Suzuki 2004).

1.13.2. Promoter for *N* methyltransferase gene

Using gene-walking technique, the promoter for *N*-methyl transferase enzyme has been cloned (Satyanarayana *et al* 2004, 2005). The analysis of sequence revealed the successful 5' walking to the extent of 745bp from the known 5' end of the theobromine

synthase gene. The 745 bp fragment spanning the 5´ upstream region and the first exon of theobromine synthase was used in making promoter::GUS construct pPCTS745 in the binary vector pCAMBIA1381 and introduced into tobacco through *Agrobacterium tumefaciens* mediated transformation. GUS activity was detected in transgenic calli transformed with the promoter::GUS fusion construct pPCTS774, confirming the ability of the isolated promoter to drive gene expression (Satyanarayana *et al* 2004, Satyanarayana *et al* 2005).

Recently, naturally decaffeinated arabica coffee plants have been reported, where the low caffeine content observed was not due to enhanced degradation of caffeine, but more likely due to possible mutation in caffeine synthase gene (Silvarolla *et al* 2004). The regulation for this naturally decaffeinated coffee might be promoter controlled, instead of a mutation in the coding region of the caffeine synthase gene as suggested. 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 (RdDM) (Mette *et al* 2000).

Recently 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 should be possible to produce coffee beans that are intrinsically deficient in caffeine (Ogita *et al* 2003).

1.13.3. Caffeine degradation in coffee plants

Kalberer (1965) reported the biodegradation of caffeine to xanthine. Xanthine is degraded further by the conventional purine catabolism pathway to CO_2 and NH_3 via uric acid, allantoin and allantoic acid in leaves of *C. arabica* (Crozier *et al* 1995, Kalberer 1965). Biodegradation of caffeine and formation of theophylline and theobromine from caffeine was noticed in mature *Coffea arabica* fruits (Suzuki and Waller 1984 a, b). Theophylline and theobromine have been identified as the first degradation products in immature and mature *C. arabica* fruits (Mazzafera 1990, Mazzafera *et al* 1991) and in leaves (Crozier *et al* 1995). For (8-^{14}C) theophylline, the

biodegradation products were 3-methylxanthine, allantoin, allantoic acid, urea and an unknown compound (Suzuki and Waller, 1984a,b, Crozier *et al* 1995). In addition to being broken down to CO_2, via the purine catabolism pathway, xanthine was metabolized to 7-methylxanthine (Crozier *et al* 1995).

1.13.4. Caffeine degradation in microbial systems:

Several bacterial isolates from different soils cultivated with coffee showed high ability to degrade this alkaloid (Yamaoka-Yano and Mazzafera, 1997). Microbial degradation of caffeine offers a biological alternative for using enzymes for decaffeination. The microbial degradation of caffeine has been reported in fungi (Schwimmer *et al* 1971, Hakil *et al* 1999), yeast (Sauer *et al* 1982) and bacteria (Komeda and Yamada, 1993). In a study conducted by Yamaoka-Yano and Mazzafera (1999) using C^{14}-labelled caffeine has indicated that *Pseudomonas putida* demethylates caffeine sequentially by removing methyl groups. Hakil *et al* (1998) demonstrated the degradation and product analysis of caffeine and related dimethylxanthines by filamentous fungi. Recently Sarath Babu and co-workers (2005) reported the degradation of caffeine by *Pseudomonas alcaligenes* CFR 1708. Figure 1.10 illustrates degradation pathway of caffeine in *P. putida.*

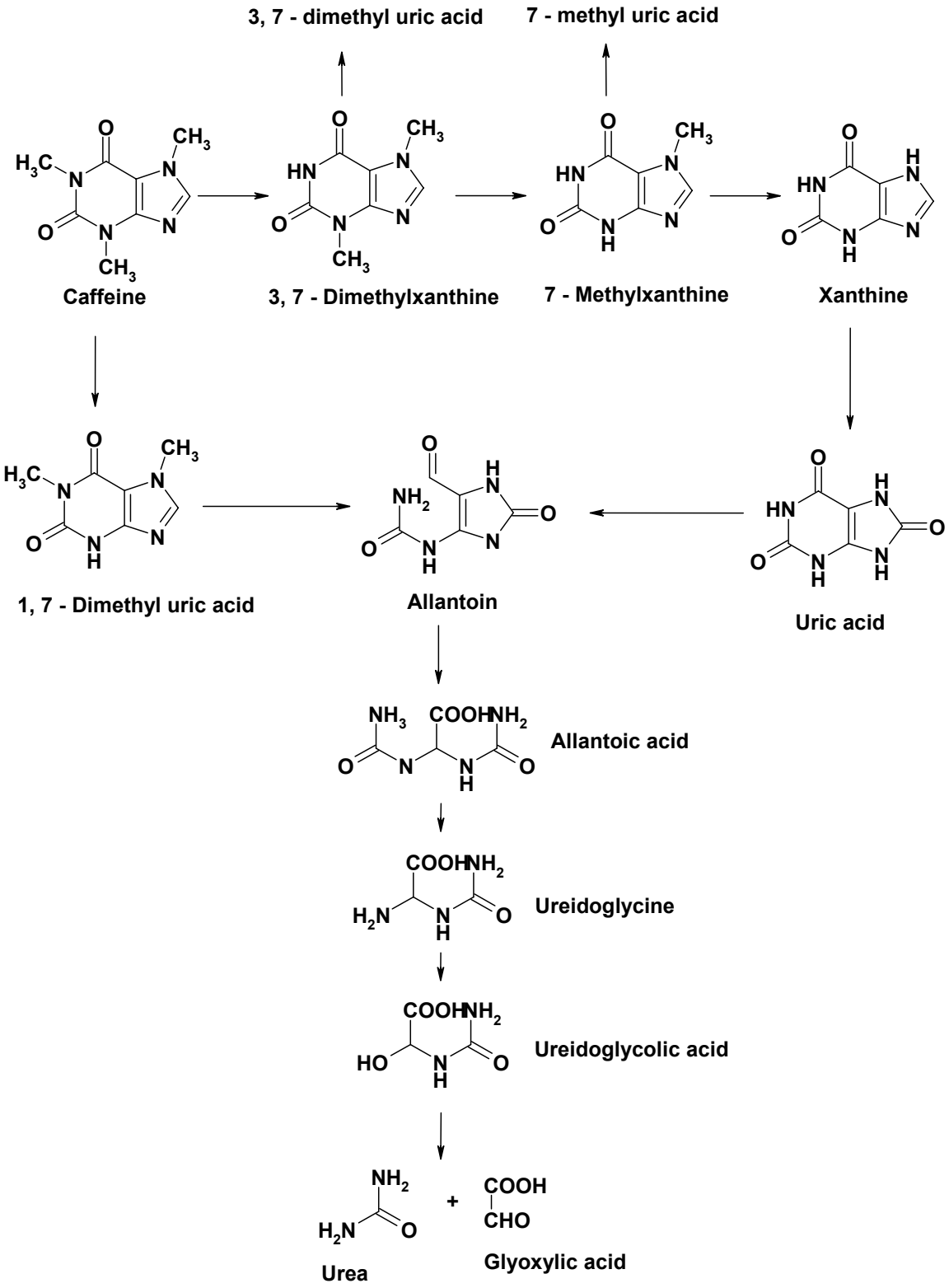


Fig. 1.10a Pathways of caffeine degradation by *Pseudomonas putida*. (Yamaoka-Yano and Mazzafera 1999)

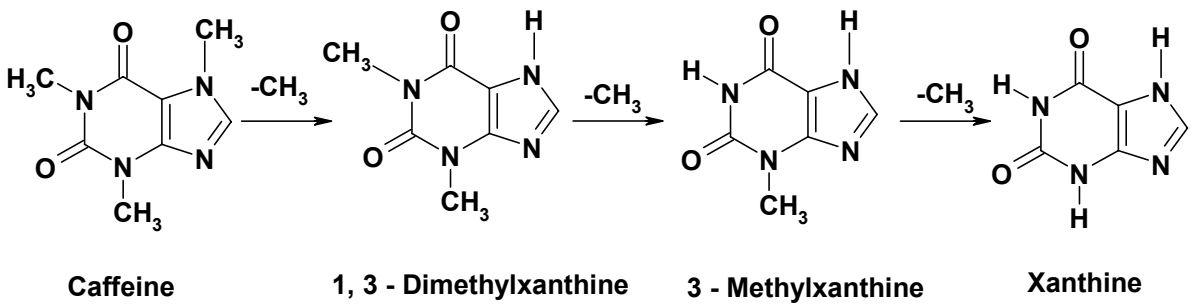


Fig. 1.10b Pathways of caffeine degradation by *Rhizopus delemar* (Tagliari *et al* 2003)

1.14. GENE SILENCING IN PLANTS

Silencing of endogenous genes or transgenes can occur by transcriptional gene silencing (TGS) or posttranscriptional gene silencing (PTGS). TGS can result from the impairment of transcription initiation through methylation or chromatin condensation or could drive from the mechanisms by which transposed copies of the mobile eliments and T-DNA insertions are tamed. PTGS results from the degradation of mRNA when aberent sense, antisense or double stranded forms of RNA are produced (Fagard and Vaucheret 2000). Three papers published in 1990 (Napoli *et al* 1990, Smith *et al* 1990, Van der Krol *et al* 1990) demonstrated that introduction of transcribed sense transgenes could down-regulate the expression of homologous endogenous genes, a phenomenon called co-suppression (Napoli *et al* 1990). Post-transcriptional gene silencing also known as RNA silencing in plants, is an RNA degradation process through sequence-specific nucleotide interactions induced by double-stranded RNA. Over the last several years much progress has been made in unraveling the mechanism of RNA silencing, a process leading to the degradation of homologous mRNAs, which is also termed RNA interference in animals, posttranscriptional gene silencing (PTGS) in plants, and quelling in fungi (Reviewed by Yu and Kumar 2003). This has opened up new avenues for down regulation of genes encoding undesirable traits with special reference to processing characteristics and anti-nutritional traits.

The RNA silencing is triggered by the presence of endogenous or exogenously introduced double-stranded RNA (dsRNA), which is further cleaved into small RNAs to become functional in a number of epigenetic gene-silencing processes (Zamore *et al*

2000, Eckardt 2002). In plants, RNA silencing, as an efficient part of gene silencing, but also plays important roles in the regulation of endogenous gene expression (Voinnet 2002). The signals of intracellular RNA silencing can be transmitted systemically from cell to cell over a long distance through the phloem. Short interfering RNAs (siRNAs), aberrant RNAs, and dsRNAs are the suggested candidates for such mobile silencing signals, although the mechanism of their involvement in the process is not clear so far (Palauqui *et al* 1997, Voinnet *et al* 1998)

The mechanisms of silencing have been discussed extensively in the recent review article (Yu and Kumar 2003). The mechanism of RNA silencing induced by dsRNA can be simplistically summarized as having two major steps, viz., initiation and effector steps (Cerutti 2003). The initiation step involves the cleavage of the triggering dsRNA into siRNAs of 21–26 nucleotides with 2-nucleotide 3` overhangs, which correspond to both sense and antisense strands of a target gene (Hamilton and Baulcombe 1999, Voinnet 2002). In the effector step, the siRNAs are recruited into a multiprotein complex referred to as the RNA-induced silencing complex (RISC), in which the degradation of target mRNAs occurs with the siRNA as a guide (Hammond *et al* 2000, Zamore *et al* 2000). Each RISC appears to have a single siRNA, an RNase and an mRNA homology-recognition and binding domain. The Dicer protein is involved in generating siRNA. The processing of a long dsRNA into siRNA is mediated by an RNase-III-like dsRNA-specific ribonuclease, designated Dicer, initially in *Drosophila* (Bernstein *et al* 2001). The members of the Dicer protein family may be functionally conserved in fungi, plants and animals (Tijsterman *et al* 2002).

Grafting experiments revealed that PTG silenced plants produce a sequence specific systemic silencing signal that propagates long distance from cell to cell and triggers PTGS in non silenced graft connected tissues of the plant (Palauqui *et al* 1997, Voinnet and Baulcombe 1997)

1.14.1. Application of RNA silencing in plants

With the completion of *Arabidopsis* and rice genome sequencing and the expanding crop sequence databases, the practical use of RNA silencing to reduce gene expression in a sequence-specific manner promises to be an essential and routine reverse genetics

approach in plant functional genomics. Technologically reliable and high-throughput methods of RNA silencing are being developed by the recent progress on the understanding of the core RNA silencing mechanism. Particularly, the discovery of dsRNA as an inducer of RNA silencing has provided a scheme of dsRNA-mediated interference to direct gene-specific silencing that is more efficient than antisense suppression or co-suppression by over expression of target genes (Fire *et al* 1998, Kennerdell and Carthew 1998, Waterhouse *et al* 1998, Sanchez-Alvarado and Newmark 1999). The dsRNA-mediated silencing was first demonstrated in plants by the simultaneous expression of antisense and sense gene fragments targeted against both an RNA virus and a nuclear transgene (Waterhouse *et al* 1998). The methodology of the specific and heritable genetic interference by dsRNAs in *Arabidopsis* was further established in the investigation of several genes involved in floral development (Chuang and Meyerowitz 2000). In this respect, transformation vectors capable of dsRNA formation were constructed by linking the gene-specific sequences in both sense and antisense orientation under the control of a strong viral promoter. These dsRNA-expressing constructs, when delivered into *Arabidopsis* with *Agrobacterium*-mediated transformation, created a heritable phenotypic series in the transformants, which corresponded to mutant alleles of different strengths. Thus, the dsRNA interference can generate transformants showing both reduction and loss of function. It is reported that, inclusion of an intron as a spacer between the sense and antisense arm of a dsRNA construct greatly increases the silencing effect (Wesley *et al* 2001).

1.14.2. Examples of gene silencing

Due to the paucity of mutations in biochemical pathways in plants, an alternative approach to classical genetics was tested by expressing antisense RNA in plant cells. A series of plasmids was constructed with the bacterial gene for chloramphenicol acetyltransferase linked in either the sense or antisense orientation to several different plant gene promoters. Transcription of antisense RNA was found to effectively block the expression of target genes in plants too (Ecker and Davis 1986). Some of the examples of gene silencing in plants have been summarized in Table 1.7.

Table 1.7 Brief summary of examples of gene silencing in plants

Description	Reference
Inhibition of gene expression in plant cells by expression of antisense RNA.	Ecker and Davis 1986
Silencing of polygalacturonase glycanhydrolase by antisesse RNA in tomato to decrease cell wall degradation and fruit softening.	Sheehy *et al* 1988.
Antisense RNA has been shown to reduce expression of polygalacturonase gene in stably transformed tomato.	Smith *et al* 1988
Compared the effects of strong and weak promoters that drive sense chalcone synthase (Chs) transgenes and discovered that a strong transgene promoter is required for high-frequency cosuppression of Chs genes for the production of the full range of co-suppression phenotypes.	Que *et al* 1997
Constitutive expression of an anti-sense chalcone synthase gene in transgenic petunia and tobacco plants results in an altered flower pigmentation due to a reduction in levels of target mRNA.	Van der Krol *et al* 1988
Demonstrated RNA-mediated RNA degradation and chalcone synthase a silencing in petunia	Metzlaff *et al* 1997
Antisense RNA technique has been applied to know the function of genes such as gene involved in the conversion of 1-amino-cyclopropane-1-carboxylic acid to ethylene by the ethylene-forming enzyme (ACC-oxidase).	Hamilton *et al* 1990
Antisense RNA to study gene function. The study was focused on inhibition of carotenoid biosynthesis in transgenic tomatoes.	Bird *et al* 1991
An antisense dihydroflavonol reductase (DFR) gene-construct made using the cDNA for DFR from *Antirrhinum majus* and introduced into *Lotus corniculatus* and effectively down-regulated tannin biosynthesis in the hairy roots.	Carron *et al* 1994.
An antisense potato polyphenol oxidase (PPO) cDNA was	Thipyapong *et al*

introduced into tomato (*Lycopersicon esculentum* L.) to assess the impact of PPO expression on resistance to *Pseudomonas syringae* pv. *tomato*. Antisense PPO expression dramatically increased susceptibility to *P. syringae.*	2004
Polyphenol oxidase (PPO) activity of Russet Burbank potato was inhibited by sense and antisense PPO RNAs expressed from a tomato PPO cDNA. Expression of tomato PPO RNA in sense orientation led to the greatest decrease in PPO activity and enzymatic browning due to co-suppression.	Coetzer *et al* 2001
An antisense coproporphyrinogen oxidase cDNA was introduced to tobacco (*Nicotiana tabacum*) and transformants showed reduced coproporphyrinogen oxidase activity and accumulation of photosensitive coproporphyrin (ogen).	Kruse *et al* 1995
Lotus corniculatus L. were transformed with an antisense chalcone synthase (CHS) gene construct made using a stress induced CHS17 cDNA from *Phaseolus vulgaris.* After elicitation with glutathione, the level of tannin accumulation was found to increase in a number of antisense root cultures.	Colliver *et al* 1997
Antisense inhibition of sorbitol synthesis resulted in up-regulation of starch synthesis without altering CO_2 assimilation in apple leaves.	Cheng *et al* 2005
Double antisense plants for ascorbate peroxidase and catalase were created. They were found to be less sensitive to oxidative stress than single antisense plants lacking ascorbate peroxidase.	Rizhsky *et al* 2002
Described in detail the potential of RNA-induced gene silencing approaches for investigating plant gene function in a high-throughput, genome-wide manner.	Waterhouse and Helliwell 2003
Silencing of caffeine biosynthesis in coffee by RNAi using the coding and untranslated regions (UTR) of *N* methyltransferase.	Ogita *et al* 2004
Agrobacterium rhizogenes mediated transformation of broccoli	Henzy *et al* 1999

(*Brassica oleracea L. var. italica*) with an antisense 1-aminocyclopropane-1-carboxylic acid oxidase gene.	
Reported the silencing of codeinone reductase (COR) in the opium poppy, *Papaver somniferum*, through RNAi and the transgenic plants produced high level of nonnarcotic alkaloid reticuline.	Allen *et al* 2004
Attempts were made to increase tomato fruit nutritional value by suppressing an endogenous photomorphogenesis regulatory gene, *DET1*, using fruit-specific promoter combined with RNA interference (RNAi) technology. Both carotenoid and flavonoid contents were increased significantly and demonstrated use of organ-specific gene silencing to improve the nutritional value of plant-derived products.	Devuluri *et al* 2005
Demonstrated that transforming tomato leaf curl virus-infected plants with the homologous replicase gene constructs that produce RNAs capable of duplex formation confers gene silencing and results in recovery of infected plants and proposed that the antisense suppression in the virus-infected plants provides a threshold level of dsRNA needed to induce gene silencing leading to the virus suppression.	Praveen *et al* 2005

RNA interference is a gene-silencing technology that is being used successfully to investigate gene function in several organisms. Waterhouse and Helliwell (2003) described in detail the potential of RNA-induced gene silencing approaches for investigating plant gene function in a high-throughput, genome-wide manner. Although genome sequence data are available for model dicotyledon (*Arabidopsis*) and monocotyledon (rice) plant species, the functions of many proteins encoded by these genomes are unknown. Finding the function of these proteins is a major challenge for plant biology. Loss of a gene's expression can give insight into its function. RNA-induced gene silencing (RNA interference) is a good way to remove gene function, because it is driven by RNA hybridization, so genes can be targeted specifically and directly, as can gene families; it can be used in a wide variety of plant species and it reduces gene

expression to varying degrees. RNAi can be induced in plants by double-stranded RNA (dsRNA), by self-complementary 'hairpin' RNA (hpRNA) or by viral RNA. A key trigger of RNA-induced gene silencing is the production of dsRNA, which leads to the production of small interfering RNAs (siRNAs) of ~21 nucleotides that are used to target of degradation RNA with complementary sequence. RNAi can be delivered by transient methods (such as particle bombardment, *Agrobacterium* infiltration or viral infection) or by stable ones (such as the introduction of hpRNA or amplicon transgenes) Reviewed by Waterhouse and Helliwell (2003).

1.15. 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. 5. 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.

Table 1.8. Selected patents in the area of Coffee biotechnology

Title	Inventors	Patent Number
Induction and selection of somaclonal variation in coffee	Sondahl MR, Romig W R, Bragin A.	US5436395
Swiss water process for decaffeination	Fischer A, Kummer P	EP 008398
Process of decaffeinating coffee.	Berry NE, Walters RH	US 2309092
Process of decaffeinating coffee.	Kaleda WW, Saleeb FZ, Zeller BL	US 4467634
Induction and selection of somaclonal variation in coffee	Romig W R, Bragin A, Sondahl MR.	EP0606759
Constitutive and inducible promoters from coffee plants	Herbert S, Gaitan AL.	US 545686

Purified proteins, recombinant DNA sequences and processes for controlling the ripening of coffee plants	Stiles JI, Moisyadi I, Neupane KR	*US2003084487*
Coffee plant with reduced alpha-D-galactosidase activity	Marraccini P, Rogers J, Deshaya.	WO03032713
Caffeine Synthase polypeptide of coffee plant and the gene encoding said polypeptide	Koizumi N, Kusano T, Sano H.	EP1197558 A3
Leaf specific gene promoter of coffee	Marraccini P, Rogers J.	EP1256629
A process for the selection of transgenic cells in the meristematic region of cotton, coffee, cocoa, banana or grape	Lima AFJ, Rech FEL.	WO0056904
Process for decaffeinating green coffee	Hermsen M, Sirtl W.	EP0316694 A3 B1
Method for decaffeinating coffee materials including a reverse osmosis permeate recycle	Katz SN, Prasad R, Spence JE, Vogel GJ.	*CA1329595*
Process for decaffeinating green coffee beans	Ben-Nasr H, Coenen FWH.	*US5089280.*
A process for decaffeinating green coffee.	Kaper L.	*EP0248482B1*
Method for decaffeinating coffee with a supercritical fluid	Katz SN.	*CA1304978*
Method for the decaffeination of roasted coffee extracts and the products thereof	Gottesman M D, Kramer KC, Musto JA, Meinhold JF.	*CA1293148*
Production of transformant of plant of *Coffea* and transformant coffee plant	Sano H, Kusano T.	*JP2000245485*
Stably transformed coffee plant cells and plantlets	Adams TL, Zarowitz MA.	*US5334529*
Theobromine synthase polypeptide of coffee plant and the gene encoding said polypeptide	Sano H, Kusano T, Koizumi N.	*US2004154055*
Isolated purified xanthosine N-7-methyl transferase from coffee plant, coding nucleic acid thereof, coffee plant transformed cells, caffeine free transformed coffee plants and coffee beans, and methods for inhibiting production of caffeine by coffee plant cells	Stiles JI, Moisyadi I, Neupane KR.	*EA3835*
Xanthosine methylase and use thereof	Uefuji H, Sano H,	*WO03080833*

	Koizumi N, Shinmyo A.	
Caffeine synthetase and its use	Kamifuji H, Sano H, Koizumi N, Niina A.	*JP2003304879*
Joint application of caffeine biosynthetic system genome	Yokei K, Hiroshi S, Nozomu Y.	*CN1495261*
Method of constructing caffeine less coffee plant by genetic recombination	Ogita S, Sano H, Koizumi N, Shinmyo A.	*EP1541016*
Constitutive and inducible promoters from coffee plants	Aldwinckle HS, Gaitan AL.	*20030163837*
Theobromine synthase polypeptide of coffee plant and the gene encoding said polypeptide	Sano H. Kusano T, Koizumi N.	*20040154055*
Purified proteins, recombinant DNA sequences and processes for controlling the ripening of coffee plants	Stiles JI, Moisyadi I, Neupane KR.	*20030084487*
Coffee plant with reduced alpha-D-galactosidase activity	Marraccini P, Edmond D, Alain FP, Rogers WJ.	*0199943*
Purified proteins, recombinant DNA sequences and processes for producing *caffeine*-free beverages	Stiles JI, Moisyadi I, Neupane KR.	*6348641*
Method for producing the transformants of *coffee* plants and transgenic *coffee* plants	Sano H, Kusano T.	*6392125*
Method and product for decaffeinating an aqueous solution using molecularly imprinted polymers	Leone, AM	*20020031580*
Method and system for effecting withdrawal from *caffeine* dependency	White HB.	*5760095*
Method and product for decaffeinating an aqueous solution using molecularly imprinted polymers	Leone AM	*20020012727*
Fortified *coffee* drink	Atkinson JR Deis DA, Marchio AL.	*6,207,203*
Decaffeinating microorganism and process of bio-decaffeination of caffeine containing solutions	Thakur MS, Sarath BABU VR, Karanth NG, Varadaraj MC.	*20040191333*

Composite utilization of a group of genes in biosynthetic pathway of caffeine	Uefuji H, Sano H, Koizumi N, Shinmyo A.	*20040128709*
Coffee substitute	Zhao IG.	*6,171,635*
Coffee mannanase	Marraccini P, Rogers J, Pridmore R D, Gysler C	*20030131380*
Coffee compositions with enhanced flavor characteristics and method of making	Hardesty, DC, Young, JD.	*20020155210*
Caffeine detector	Catania D, Ignelzi S, Baugh S.	*6,461,873*
An improved culture medium for regeneration of transgenic secondary embryos of *Coffea Canephora.* Pex. Fr.	Vinod Kumar, Sathyanarayana KV, Indu EP, Giridhar P, Chandrashekar A Ravishankar GA.	*397 DEL 03* Indian Patent

Biotechnology has come a long way to play its role in the well being of human. The progress in the transgenic research led to the development of genetic manipulation strategies for specific traits. The compiled information provides an idea about the latest developments in Coffee biotechnology area. Several workers have reported somatic embryogenesis, formation of embryogenic and non-embryogenic callus in coffee. However, information is lacking on what are the factors, which determine embryogenic nature in coffee. Development of rapid regeneration protocols in coffee is a key factor for optimizing genetic transformation. So far it was not possible to transform coffee and regenerate plants devoid of *rol* gene phenotype by *A. rhizogenes* mediated transformation. Several physical and chemical factors may influence the transformation efficiency by *A. rhizogenes* in plants. Information on influence of sonication, calcium ions, cellulase and pectinase enzyme on *A. rhizogenes* mediated transformation frequency is completely lacking. The need for naturally decaffeinated coffee can be answered in a better way by molecular breeding of coffee. Alternative gene transfer methods such as electroporation seem to be a good approach to analyze promoters to drive the expression

of reporter genes in coffee. With this background a series of experiments were designed for plant regeneration through somatic embryogenesis and genetic transformation of coffee with the following objectives. The results of these studies form the substance of this thesis.

1.16. Objectives of the present study

1. Development of somatic embryogenesis protocols in *Coffea* sp.
2. Optimization of *A. rhizogenes* mediated genetic transformation system using a model system.
3. Development of genetic transformation system for *Coffea* sp.
4. Expression of desired antisense genes for *N*-methyl transferases to block caffeine production with a view to improve processing characteristics and value addition.
5. Development of electroporation mediated gene delivery systems in *Coffea* somatic embryos and endosperm tissues.

CHAPTER II
MATERIALS AND METHODS

2.1. Establishment of *in vitro* cultures

2.1.1. Source of material and *in vivo* germination

Certified ash coated seeds of *C. canephora* cv S-274, cv CxR and *C. arabica* cv Hemavathy were obtained from Central Coffee Research Institute, Coffee Research Station, Chikmagalur District, Karnataka, India.

2.1.2. Sterilization of coffee seeds

The seeds were washed with water; hard testa and silver skin were removed manually. The seeds were washed with 1% Bavistin (Carbendazim, 50% w/w, BASF India LTD Thane) for 30 min; 3 minutes in 70% alcohol followed by copious water. Subsequently the seeds were sterilized in 0.2 % (w/v) mercuric chloride (Hi-media, Mumbai, India) solution for 10 min and washed five times in sterile distilled water. The seeds were soaked in sterile distilled water and incubated on a shaker at 90 rpm (Jeo Tech SI-600R, Korea) for one day.

2.1.3. Media and culture conditions for seed germination

The sterilized seeds were blot dried and inoculated (2-4 seeds/bottle) on to ¼ MS basal medium and vitamins (Murashige and Skoog, 1962) with 2% sucrose, 40 mgl^{-1} cysteine HCl. The medium was gelled with 0.8% (w/v) tissue culture grade agar (Hi-media, Mumbai, India) in 200ml glass jars, containing 40ml of the medium. The medium contained polyvinyl pyrrolidone (PVP) 0.5%, activated charcoal 0.5%, cefotaxime 500 mgl^{-1} (Alkem Laboratories Ltd., Mumbai, India), bavistin 100 mgl^{-1} in different combinations to prevent browning and contamination. The jars were closed with polypropylene caps. The pH was adjusted to 5.6 using a pH meter (Cyber Scan 510, Oakton, USA) prior to autoclaving at 121ºC, 1.2 kg cm^{-2} pressure for 20min. The cultures were maintained at 25±2 ^{o}C in the dark for 120 days.

2.1.4. Callus induction

C. canephora cv S-274 was used in initial experiments. The cotyledonary leaf (5x5mm square explants were cut from leaf blade with a scalpel, excluding the basal and apical

portions, mid vein and margins) and hypocotyl explants (10mm length) were placed on callus induction medium (Van Boxtel and Berthouly 1996) containing ½ MS salts and B5 vitamins (Gamborg *et al* 1968), isopentenyladenine (2-iP) 2 mgl^{-1}, 2,4-dichloroacetic acid (2,4-D) 0.5 mgl^{-1} and Indole 3-butyric acid (IBA) 0.5 mgl^{-1}, another medium containing 2-iP 2 $mgl^{-1}$and 2,4-D 1 mgl^{-1}, control medium did not contain any hormones. All hormones were obtained from Sigma (USA). The cultures were incubated at 25±2ºC in the dark for the first 45 days and later sub-cultured onto a medium (Van Boxtel and Berthouly 1996) containing ½ MS salts and B5 vitamins, 2,4-D 1 mgl^{-1} and benzyl amino purine (BA) 4 mgl^{-1} another medium containing 2,4-D 1mgl^{-1} and BA 1 mgl^{-1} for callus multiplication. The cultures were incubated at 25±2ºC in dark. Callus was sub-cultured on 45th day. The callus was cultured in this medium for two and a half months.

2.1.5. Somatic embryogenesis

The callus was sub-cultured on medium comprising ½ MS salts and B5 vitamins IAA 0.5 mgl^{-1} and BA 0.25 mgl^{-1} another medium containing BA alone 2mgl^{-1} to induce somatic embryos. The callus was cultured for 45 days. The cultures were incubated at 25±2ºC with a 16 h photoperiod under the light intensity of 25µmol m^{-2} s^{-1}. Embryogenesis was monitored using a Leica (Wild M3Z) stereo microscope. The results were expressed in terms of percentage response for non-embryogenic callus, embryogenic callus and somatic embryogenesis. The percentage response was calculated based on the total number of callus clumps inoculated and the number of callus clumps producing somatic embryos. The embryogenic callus and non-embryogenic callus was distinguished depending on their ability to produce somatic embryos in subsequent culture and since there was clear morphological difference in embryogenic and non-embryogenic callus, the percentage responses were calculate on the basis of morphological appearance of the callus clumps. The non-embryogenic callus was spongy dark brown/black and the embryogenic callus was creamish yellow and friable in appearance.

2.2. Influence of polyamines on secondary embryogenesis

2.2.1. Exogenous incorporation of polyamine, polyamine inhibitors and silver nitrate

Mature torpedo shaped embryos were used for secondary embryogenesis. Medium used for secondary embryogenesis comprised of half strength MS salts, B5 vitamins, IAA 0.5 mgl^{-1}, BA 0.25 mgl^{-1} alone or along with either of $AgNO_3$ 40 µM, putrescine 50 mM and DFMA 1mM +DFMO 1mM. Control medium comprised of IAA 0.5 mgl^{-1}, BA 0.25 mgl^{-1}. Media pH was adjusted to 5.8 and autoclaved at 121^0C, 1.2 kg cm^{-2} pressure for 15min.

For studies on caffeine levels under polyamine treatments, callus cultures were used. Embryogenic callus lines were derived according to the procedure mentioned in section-2.1.4. The callus was grown for 60 days in medium comprising MS salts and vitamins, 1 mgl^{-1} 2,4-D, 4 mgl^{-1} BA and either of putrescine 50mM, spermidine 50mM, spermine 50mM, DFMA 1mM, DFMO 1mM, DFMA+DFMO 1mM each, $AgNO_3$ 50µM, DFMA 1mM +DFMO 1mM + $AgNO_3$ 50µM. Putrescine, spermine, spermidine obtained from Sigma (USA) DFMO and DFMA was procured from Marrion Merrel Research foundation (Cincinatti, Ohio) $AgNO_3$ was obtained from (Qualigens, India). These were added to the media after filter sterilization using a 0.22 µM disposable filter to obtain the desired concentration range. All the chemicals were of analytical grade and the solvents of High Performance Liquid Chromatography grade (Qualigens, India).

Five torpedo shaped somatic embryo explants in ten replicates were inoculated per treatment for secondary embryogenesis response. Approximately five 50 mg callus clumps each were inoculated in individual petri plates for embryogenic response. The petri plates were closed with parafilm. Cultures were incubated at 25±2 ^{0}C in dark for 45 days for each treatment.

2.2.2. Extraction and estimation of endogenous polyamines

The extraction of endogenous polyamines (PAs) was carried out by acid hydrolysis of perchloric acid. PAs were analyzed according to Flores and Galston (1982). The tissues were ground in 5% cold perchloric acid at a ratio of about 100mg/ml perchloric acid. Samples were incubated for 1hr in ice bath and centrifuged at 10,000rpm (Hettich D-78532, Germany) for 20 min. and the supernatant containing the free polyamines were

benzoylated. To 0.5 ml PCA extract 1ml of 2 N NaOH and 10 µl benzoyl chloride was added, vortexed for 20 seconds, incubated for 20 min at room temperature. Saturated NaCl (2 ml) was added to the mixture. The benzoylated polyamines were extracted in 2 ml diethyl ether, centrifuged, the ether phase was collected and evaporated to dryness and re-dissolved in 100 µl methanol. The standards were treated in the same way and subjected to HPLC analysis.

Each sample replicated thrice for HPLC and average values were expressed in µg g^{-1} FW of the tissue. The mobile system contained 64% methanol at a flow rate of 1 $mlmin^{-1}$. The benzoylated extracts were eluted at room temperature through a Bondapak C-18 reverse phase column and detected at 254 nm. Parameters were controlled by a Shimadzu LC 10–AS liquid chromatograph equipped with a dual pump and a UV spectrophotometric detector (Model SPD-10 A). The recorder Shimadzu C –R7A chromatopac was set at a chart speed of 2.5cm/ min. Injection volume was 10 µL, injected with Rheodyne 7125 injector. Peak identification was achieved by comparing with the retention time of standards (SIGMA, USA) and confirmed by spiking the samples with standards. HPLC analysis was carried out for each treatment in triplicates.

2.2.3. Estimation of caffeine

Caffeine extraction from the samples was carried out using 80% ethyl alcohol. Estimation of caffeine was carried out by high performance liquid chromatography on a Bondapak C18 column (5µ x 25cm) with 50mM sodium acetate (pH 5.0), methanol, tetrahydrofuran in the ratio 91:8:1 (mobile phase). HPLC analysis was performed as explained in section-2.2.2. Detection was carried out at 270nm

2.3. TDZ induced direct embryogenesis in *c. arabica* and *c. canephora*

C. arabica L. variety Hemavathy and *C. canephora* P ex Fr. variety 274 and CXR variety was used in direct embryogenesis experiments using *in vivo* and *in vitro* coffee plants. From *in vivo* coffee seedlings, the cotyledons and hypocotyls were used as explants. Similarly leaf and stalk portion of *in vitro* regenerated plantlets were used as explants. The *in vitro* plantlets were obtained through inducing embryogenic callus, subsequently somatic embryos and plantlets by using reported protocol (Van Boxtel and Berthouly

1996). Surface sterilization for *in vivo* explants was performed as explained in section 2.1.2. Ten mm squared explants were cut from leaf blade with a scalpel, excluding the basal and apical portions, mid vein and margins. Hypocotyl and stalk explants were cut into ten mm segments and collected in petridishes containing 0.025% cystein HCl. The explants were cultured in 100mmD X 20mmH disposable petridishes containing approximately 25 ml of the medium. Leaf explants were cultured with their adaxial side in contact with the medium. The medium compositions used for the study is given below.

Medium A comprises MS basal medium (Murashige and Skoog 1962), 2% sucrose (w/v) supplemented with 2.27-11.35 µM thidiazuron (TDZ, i.e. 1-phenyl-3- (1,2,3-thiadiazol-5-yl) urea). Medium B comprises MS basal, 3% sucrose (w/v), 2.27-11.35 µM TDZ, medium C is MS basal, 10% (v/v) coconut water (CW), 2% sucrose + 2.27-11.35µM TDZ and medium D contains 2% sucrose+ 10% CW. Controls were maintained on medium E comprising MS basal and 2% sucrose. MS salts full or half strength concentration along with B5 vitamins, 2–3% (w/v) sucrose, supplemented with TDZ 2.27-11.35µM and CW10% (v/v), and were also used in the experiments. The clumps of the leaf-derived primary embryogenic nodules were further cultured on medium comprising half strength MS salts and B5 vitamins supplemented with 0.91µM TDZ.

The maturation of the somatic embryos was carried out in embryo development medium containing half strength MS salts, 10 mgl^{-1} thiamine HCl, 3.2 mgl^{-1} pyridoxine HCl, 2.85µM indole-3-acetic acid (IAA), 2% (w/v) sucrose and cultured for 60 days. The regenerated plantlets were rooted on half strength MS basal medium containing 2% (w/v) sucrose.

2.4. Somatic embryogenesis under the influence of silver nitrate

The experiments conducted with leaf explants of *C. arabica* variety Hemavathy and *C. canephora* cv 274 of approximately 2year old *in vitro* plants. *In vitro* plants were raised as explained by Van Boxtel and Berthouly (1996). The embryos of different growth stage - round globular embryos and green tubular stage embryos were used for subsequent rapid multiplication of primary embryos into secondary embryos. The medium used for embryo multiplication in this study contains half strength MS micro and macro salts

(Murashige and Skoog 1962) thiamine HCl 10 mgl^{-1}, inositol 100 mgl^{-1}, pyridoxine 5 mgl^{-1}, niacin 0.5 mgl^{-1}, glycine 0.5 mgl^{-1} and sucrose 2% (w/v) along with growth regulators BA 1.1μM, IAA 2.85μM. A stock solution of silver nitrate (1mg ml^{-1}) was prepared, autoclaved and incorporated into the autoclaved medium at the required concentrations (10-70μM). The pH was adjusted at 5.7±0.2. The medium was gelled with 0.7% agar (Himedia, Mumbai) and autoclaved for 20 minutes at 121^0C.

2.5. Involvement of calcium in somatic embryogenesis

2.5.1. Callus induction, somatic embryogenesis and secondary embryogenesis

The seeds were germinated *in vitro* as explained in section 2.1.3. Two successive media were used for the induction of callus from cotyledonary leaves and hypocotyls of germinated seedlings. The protocols were explained in sections 2.1.1 to 2.1.4. The callus was sub-cultured every 25 days for multiplication of callus. Stock solutions of MS salts devoid of calcium were prepared for this experiment. The callus multiplication medium contained either 0.1mM EGTA or 0-50 mM calcium chloride. Somatic embryos were produced in the same medium after 60 days of culture. Prolonged culture for another 45 days in the same medium gives rise to mature tubular and torpedo stage embryos. Mature torpedo stage embryos were used for secondary embryogenesis. Medium used for secondary embryogenesis comprised of half strength MS salts, B5 vitamins, IAA 0.5 mgl^{-1}, BA 0.25 mgl^{-1} and $AgNO_3$ 40 μM. Calcium was incorporate in the range of 0.1 to 50mM concentration in the secondary embryogenesis medium. The Ca^{2+} channel blocker verapamil HCl (Sigma, USA) was dissolved in 2% (v/v) DMSO. Verapamil was added to the medium in a concentration range of 0 to 1mM. Embryogenesis medium containing DMSO served as control. Media pH was adjusted to 5.7 sterilized by autoclaving.

Approximately five, 50 mg callus clumps were inoculated in individual petri plates for embryogenic response under each treatment. The experiments were carried out in triplicates and the mean values of response for primary embryogenesis and secondary embryogenesis was expressed in terms of percentage response. Each treatment given was in triplicates. Embryo induction and development was monitored by microscopy. The petri plates were closed with parafilm. Cultures were incubated at 25±2 ^{0}C for 45 days for each treatment. The percentage explant response for embryogenesis and secondary

embryogenesis as well as number of embryos and secondary embryos were counted after 45 days of culture for each treatment.

2.5.2. Extraction of soluble proteins

Soluble proteins were extracted from endosperm, pericarp of mature fruit embryogenic callus tissue, non-embryogenic callus tissue, somatic embryos and plantlet. Tissues were homogenized using a pestle and mortar at 4^0C and suspended in the extraction buffer containing 2.5 mM EDTA, 20 mM Tris [tris(hydroxymethyl)aminomethane]-HCl, pH 7.2, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The extracts were spun at 12,000*g* for 30 min at 4°C in a refrigerated centrifuge (Hettick, model D-78532 Germany). The supernatant containing the soluble proteins was used for immunodetection. Protein concentrations were determined according to the method of (Bradford 1976) using bovine serum albumin as the standard.

2.5.3. Fractionation of proteins by SDS PAGE:

Protein extracts were mixed with Laemmli's sample buffer (Laemmli, 1970), boiled for 5 min, and resolved on a 12% (w/v) SDS polyacrylamide gel (Sambrook *et al* 1989).

2.5.4. Immunodetection (Western Blot) of ccCDPK using polyclonal anti-soybean CDPK

Protein extracts were resolved by SDS-PAGE and transferred to nitrocellulose membrane (Towbin *et al* 1979) using Sci-Plus Electrobloting unit (model V20-SDB unit UK). The blot was treated with blocking buffer (1X phosphate-buffered saline, pH 7.4, 0.05% [v/v] Tween 20) containing 2% (w/v) gelatin for 1 h. This was followed by incubation in TBST buffer (20mM Tris, 0.9%NaCl, 0.1% Tween 20, pH adjusted to 7.4 with HCl) containing polyclonal antibodies directed against the CaM-like domain of soybean CDPK (Bachmann *et al* 1996) (kind gift from Prof. Alice Harmon, Department of Botany, University of Florida, Gainesville, USA) at a dilution of 1:5000 for 3 h. Excess antibodies were removed by washing the blot with three changes of TBST buffer for 1 h with constant rotation at 30rpm at 28^0C (Hybridization Oven, Shell Lab, 1004-2E). The blot was further incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase

that was diluted to 1:1,000 in TBST buffer. The unbound secondary antibodies were removed by washing with TBST. ccCDPK was visualized by incubation in 5-Bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium (BCIP-NBT) substrate (Ready to use solution obtained from Bangalore genei, Bangalore, India).

2.5.5. Immunofluorescence

Samples were fixed in formaldehyde: acetic acid: 70% (v/v) ethanol (5:5:90). Immunolocalization was performed as explained by Anil *et al* (2000) in transverse sections of somatic embryos of coffee. Polyclonal antibodies directed against the CaM-like domain of soybean CDPK (Bachmann *et al* 1996) was used as primary antibody. The sections were observed under Olympus microscope equipped with fluorescence filters (Olympus, BX40. Emission wavelength 450-480, Excitation wavelength 510-550).

2.5.6. Scanning Electron Microscopy

The samples were processed for scanning electron microscopy (SEM) according to Larry *et al* (1994). The samples were fixed in 2% glutaraldehyde in 0.2 M phosphate buffer (pH 6.8) for 6 hrs, dried in alcohol series upto 100%, sputter coated with gold and examined in a LEO Scanning Electron Microscope 435 VP (Leo Electron Microscopy Ltd. Cambridge UK).

2.5.7. Autophosphorylation assay

Soluble proteins of embryogenic, non-embryogenic cultures were extracted in 0.1M potassium phosphate buffer, dialyzed against 0.1M potassium phosphate buffer at 4^0C and assayed for ccCDPK autophosphorylation activity. The reaction was carried out in a total reaction volume of 30 µl, containing 30 µg protein, 10mM $MgCl_2$ 0.1mM $CaCl_2$, ^{32}P -ATP 1µl (specific activity 3000 Ci /mmol, concentration 1 mCi/ml) with no exogenously added substrate and incubated for 20 min at 37^0C. All the buffers and reagents ware made in calcium free milliQ water (Millipore India Ltd, Bangalore). The reaction was terminated by addition of Laemmli's sample buffer (Laemmli 1970), boiled for 2 min, and resolved in 12% SDS-PAGE. Subsequently the gel was rinsed in wash solution containing 20% TCA and 0.2% sodium pyrophosphate to wash free unbound ^{32}P -ATP.

The gel was exposed (Fuji Film BAS cassette 2025) and documented using a phosphor-imaging system (Fugifilm BAS 1800).

2.5.8. Substrate phosphorylation assay

Protein kinase activity was determined by measuring the incorporation of ^{32}P from (γ-^{32}P) ATP into the in vitro substrate histone III-S. The reaction was carried out in a total reaction volume of 30 µl, containing 30 µg protein, in the presence or absence of 0.1mM $CaCl_2$, 10mM $MgCl_2$, ^{32}P -ATP 1µl (specific activity 3000 Ci /mmol, concentration 1 mCi/ml), 150 µg histine III S substrate and incubated for 20 min at 37^0C. The reaction was terminated by addition of Laemmli's sample buffer, boiled for 2 min, and resolved in 12% SDS-PAGE. Subsequently the gel was rinsed in wash solution containing 20% TCA and 0.2% sodium pyrophosphate to wash free inbound ^{32}P -ATP. The gel was exposed (Fuji Film BAS cassette 2025) and documented using a phosphor-imaging system (Fugifilm BAS 1800).

2.6. Optimization of *A. rhizogenes* mediated genetic transformation in *Nicotiana tabacum*: assessment of physical and chemical treatments.

2.6.1. Plant material and bacterial strain

Seeds of tobacco (*Nicotiana tabacum var.* Anand 115) were surface sterilized with 0.1% mercuric chloride (Hi-media Mumbai, India) cultured on MS basal media (Murashige and Skoog 1962) without any phytohormone for *in-vitro* germination. Approximately 2x2cm square leaf discs from 20 to 25 day old plant were used for infecting with *Agrobacterium rhizogenes*. *A. rhizogenes* strain A4 (Kind gift from Dr. Juan B Perez, Instituto Canaro Investiganes Agrswas, Spain) was grown on LB medium and used for all the treatment.

2.6.2. General protocol for co-cultivation and induction of hairy roots

The leaf explants of Tobacco were co-cultivated with *Agrobacterium rhizogenes* for infection to induce hairy roots. *Agrobacterium* cultures were maintained by sub culturing onto a 100×15mm culture tubes containing 25 ml Luria Bertani (LB) slant medium and grown in LB broth for 48 hr in the dark at 28^oC. The leaf segments were subjected to different physical and chemical treatments prior to *A. rhizogenes* co-cultivation. The

combination of treatments has been summarized in table 2.1. Wounded segments from the treatment groups were immersed in the bacterial broth culture of $OD_{600\ nm}$ (~18 mg/ml) and swirled for 15-30 min. The explants were blotted to remove excess of bacterial inoculum. LB liquid medium without bacteria was applied to the explants as a control.

A minimum of 20 explants were used for each experiment. All the explants were cultured on sterilized petriplates comprising semi solid MS (Murashige and Skoog 1962) medium without phyto-hormones, however supplemented with sucrose 30 gl^{-1} and myo-inositol 100 mgl^{-1} (Hi-media Mumbai, India) The medium was gelled with 0.8% (w/v) agar (Hi-media Mumbai, India). The pH was adjusted to 5.8 ± 0.2. The medium was sterilized by autoclaving.

Table 2.1. Summary of treatments subjected to leaf segments of *Nicotiana tabacum* prior to co-cultivation with *A. rhizogenes*

Treatment	Sequence of treatments prior to co-cultivation or during co-cultivation
A	Manual wounding
B	Ultra-sonication
C1	Ultra-sonication + Pectinase [a] 0.1%
C2	Ultra-sonication + Pectinase 0.5%
C3	Ultra-sonication + Pectinase 1%
D1	Ultra-sonication + Cellulase [a] 0.1%
D2	Ultra-sonication + Cellulase 0.5%
D3	Ultra-sonication + Cellulase 1%
E1	Ultra-sonication + Pecinase and Cellulase [a] 0.1%
E2	Ultra-sonication + Pecinase and Cellulase 0.5%
E3	Ultra-sonication + Pecinase and Cellulase 1%
F1	Ultra-sonication + acetosyringone [b] 50µM
F2	Ultra-sonication + acetosyringone 100µM
F3	Ultra-sonication + acetosyringone 150µM
G1	Ultra-sonication + $CaCl_2$ [b] 5mM
G2	Ultra-sonication + $CaCl_2$ 10mM
G3	Ultra-sonication + $CaCl_2$ 20mM

[a] Prior to co-cultivation with *A. rhizogenes*.
[b] During co-cultivation with *A. rhizogenes*

2.6.3. Manual wounding of leaf disc explants

The leaf discs were kept in disposable sterile petriplate, pricked manually with metal needle (~10 wounds/cm^2) (Table 2.1, Treatment A), dipped in *Agrobacterium rhizogenes* culture and incubated in a shaker at 70 rpm for 30 minutes in dark. The explants were blot dried using sterile filter paper and inoculated on co-cultivation medium as described above.

2.6.4. Ultra sonication of leaf segments

Sonication assisted *Agrobacterium tumefaciens* mediated transformation (Trick & Finer 1997) was modified and adopted for transformation experiments. The leaf segments were taken in a 50 ml polypropylene tube (Tarson, India) and ultrasonicated (Bandelin Sonoplus ultrasonicator, Germany) at 50MHz for 10-100 sec at 80% amplitude (Table 2.1, Treatment B). The duration and frequency of sonication was standardized by series of initial experiments. The sonicated explants dipped in *Agrobacterium rhizogenes* culture incubated in a shaker at 70 rpm for 30 minutes in dark. The explants were blot dried using sterile filter paper and inoculated on co-cultivation medium.

2.6.5. Macerozyme treatment of sonicated leaf segments

The explants were sonicated as described above. Macerating enzymes pectinase (P 9932, Sigma chemical Co, USA) 0.1 to 1% v/v (Table 2.1, Treatment C1, C2, C3) and cellulase (Onozuka R-10 Yakult Pharmaceutical Industry Co., Ltd, Japan) 0.1 to 1% w/v (Table 2.1, Treatment D1, D2, D3), was freshly prepared and dispensed in cell wall degrading enzyme solution (KH_2PO_4 27.2 gml^{-1}, KI 0.16gml^{-1}, $CuSO_4.5H_2O$ 0.025gml^{-1}, KNO_3 0.101gml^{-1}, $MgSO_4.7H_2O$ 0.246 mgl^{-1}, Mannitol 9% (Hi-media Mumbai, India), 2-(N-Morphplino)-ethane-sulphonic acid (Sigma USA)-KOH [P^H 5.8] 3mM). The leaf discs were sonicated and treated with either cellulase and pectinase alone or in combination (Table 2.1) at concentration of 0.1-1% each or in combination for partial cell wall degradation for 1 hour in a shaker at 70rpm under dark. The explants were washed thoroughly to remove traces of cellulase and pectinase enzyme. The explants after sonication and macerating enzyme treatments were dipped in *Agrobacterium rhizogenes*

culture incubated in a shaker at 70 rpm for 30 minutes in dark. The explants were blot dried using sterile filter paper and inoculated on co-cultivation medium.

2.6.6. Treatment with acetosyringone

Acetosyringone (Sigma USA) 50 -150 μM was incorporated in the co-cultivation medium (Table 2.1, Treatment F1, F2, F3). This was filter sterilized using 0.22 μm disposable sterile syringe filter (Sartorius) added to sterilized, cooled co-cultivation medium. The sonicated explants were dipped in *A. rhizogenes* culture and shaken at 70 rpm for 30 minutes in the dark. The explants were blot dried using sterile filter paper and inoculated on co-cultivation medium.

2.6.7. Calcium treatment

Five to 20mM $CaCl_2$ (Hi-media Mumbai, India) was incorporated in co-cultivation medium (Table 2.1, Treatment G1, G2, G3). The sonicated explants dipped in *Agrobacterium rhizogenes* culture incubated in a shaker at 120 rpm for 30 minutes in dark. The explants were blot dried using sterile filter paper and inoculated on co-cultivation medium.

2.6.8. Elimination of *A. rhizogenes* from hairy roots

The cultures were incubated in the dark for 24 hrs, they were then transferred to fresh MS medium containing cefotaxime 500 mgl^{-1} (Alkem Laboratories Ltd., Mumbai, India) augmentin 300 mgl^{-1} (Glaxo Smith Kline, Mumbai, India). Hairy roots were made bacteria free by transferring to fresh medium for every 15 days containing the antibiotics mentioned above. The roots were checked for *Agrobacterium* contamination by culturing hairy root samples on LB medium.

2.6.9. Detection of Ri T-DNA integration

The Polymerase Chain Reaction (PCR) was used to detect the Ri T-DNA integration in hairy roots. The bacteria-free roots grown in MS basal medium were removed, dried on sterile filter paper and quickly frozen in liquid N_2. Thereafter, genomic DNA from putative transformed and normal roots was extracted using Gen Elute DNA extraction kit

(Sigma, USA). PCR was performed to detect the *rol* A gene using a set of *rol* A specific primer pair (Sigma, USA). A 308 bp *rol* A gene fragment was amplified with the following primer sets. Forward- 5`-AGAATGGAATTAGCCGGACTA-3` and reverse- 5`-GTATTAATCCCGTAGGTTTGTTT-3`. The PCR mixture (25 µl) contained 50 ng of DNA prepared from normal and hairy roots respectively as the template, 1X PCR buffer, 25 pmoles of each primer, 2.5 mM of dNTPs and 1 unit of Taq DNA polymerase (MBI Fermentas, Lithuania). PCR for *rol* A was carried out with initial denaturation at 94^{o} C for 5 min, 35 cycles of 1 min denaturation at 94^{o} C, 1 min annealing at 55^{o} C and 1 min extension at 72^{o} C and a final extension of 72^{o} C for 10 min using a thermal cycler (MWG Biotech, Germany). PCR was performed using *vir* C genes to detect the presence of contaminating *Agrobacterium* in the hairy roots. The PCR conditions were same as those used for amplifying the *rol* A gene. The primers (Sigma, usa) for *vir* C were, 5` ATCATTTGTAGCGACT-3` and 5`-AGCTCAAACCTGCTTC-3`. The PCR products obtained were run on 1% agarose gel, stained with ethidium bromide, observed and documented using a transilluminator equipped with a gel documentation system (Herolab GMBH, Germany).

2.6.10. Analysis of results

The results were expressed in percentage transformation frequency.

$$\% \text{ Transformation frequency} = \frac{\text{Number of explants inducing hairy roots}}{\text{Total number of explants infected with } A.rhizogenes} \times 100$$

Fifty to 60 leaf explants were inoculated with *A. rhizogenes* for each treatment in each experiment and 25 explants were cultured as positive and negative control using live and killed *A. rhizogenes* respectively for infection. All the experiments were carried out in triplicate and the results were expressed as mean $\pm$ SD.

2.7. *A. rhizogenes* mediated genetic transformation studies on *Coffea canephora*

2.7.1. Plant materials

Details were provided in p. 52, Section 2.1.1

2.7.2. Sensitivity tests for selection of transformed tissue

The embryos were wounded and inoculated into secondary embryogenesis medium containing different levels hygromycin (2.5, 5, 10, 20, 30, 40 and 50 mgl^{-1}). Filter sterilized hygromycin was added to the sterilized medium. The cultures were maintained under dark for a period of 2 months. The minimum concentration of hygromycin required for complete inhibition of regeneration response was determined and overall data was recorded as percentage regeneration response. The experiment was carried out in triplicates and data was represented in terms of mean and standard deviation.

2.7.3. *Agrobacterium* culture and transgene expression cassette

Agrobacterium rhizogenes agropine type wild strain A4 (Obtained from Dr. Juan B Perez, Instittuto Canaro Investiganes Agrswas, Spain) and binary vector pCAMBIA 1301 (Obtained from Center for the Application of Molecular Biology to the International Agriculture, Canberra, Australia) was used in the experiments.

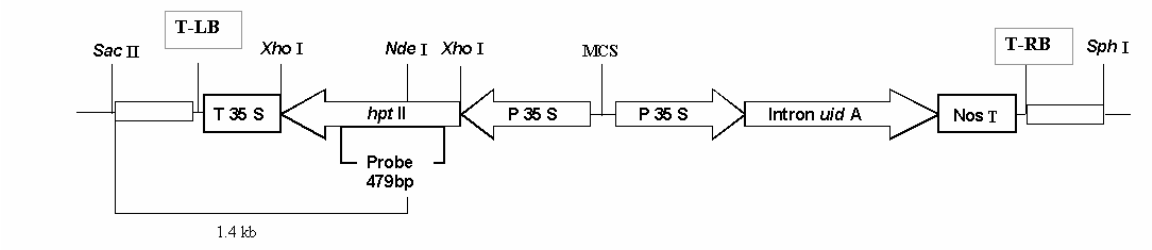


Fig 2.1: T-DNA region of pCAMBIA 1301.

The vector pCAMBIA 1301 contains the selectable marker gene hygromycin phosphotransferase (*hpt* II) under the control of the CaMV 35S promoter and CaMV 35S terminator; β-glucuronidase (*uid* A) gene with a catalase intron under the control of CaMV 35S promoter and NOS terminator (Fig 2.1).

2.7.4. Maintenance of binary vector

The binary vectors were maintained in *E. coli* strain DH 5α. The vectors were introduced in *E. coli* competent cells by $CaCl_2$ mediated transformation as explained below.

Materials

Luria-Bertani broth (LB)	Gram per liter
Bacto-tryptone	10.0
Bacto-Yeast extract	5.0
Sodium chloride	10.0

Adjusted the pH to 7.0 with 2N NaOH and the total volume was made to 1 liter with deionized water.

SOB medium	Gram per liter
Bacto-tryptone	20.0 g
Bacto-Yeast extract	5.0 g
Sodium chloride	0.6 g
Potassium chloride	0.19 g
Magnesium sulphate	10.0 mM (added from 1.0 M stock)
Magnesium chloride	10.0 mM (added from 1.0 M stock)

First four components were autoclaved and sterilized magnesium salt solutions were added separately and then mixed to constitute the SOB medium.

SOC (per 100 ml) medium

To 1.0 ml of SOB added 7 µl of filter-sterilized glucose solution (50%w/v)

0.1 M $CaCl_2$ stock solution

Dissolved 1.47 g of $CaCl_2$ in 100 ml of deionized water. The solution was filter sterilized and stored as 20 ml aliquots at -20°C.

2.7.5. Kanamycin stock solution

Kanamycin sulphate (Sigma USA) was dissolved in water, filter-sterilized and stored at -20^{o}C. The stock solution was of 10mg ml^{-1}. Kanamycin was used at a working concentration of 100 μg ml^{-1}.

2.7.6. Preparation of competent cells using $CaCl_2$

Single colony of *E. coli* DH5α was picked from a plate, freshly grown for 16-20 h at 37°C and transferred to 50 ml LB broth in a 250 ml conical flask. Incubated the culture at 37°C with vigorous shaking. Determined the OD_{600} of the culture periodically to monitor cell growth. The cells were aseptically transferred to sterile polypropylene tubes when OD_{600} reached to 0.45-0.50. The culture was cooled by storing the tube on ice for 10 minutes. The cells were recovered by centrifugation at 4000 rpm for 10 minutes at 4°C. The media was decanted from the cell pellet. The tubes were kept in an inverted position for 1 minute to allow the last traces of media to drain away. Each pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ and stored on ice. The cells were recovered by centrifugation at 4000 rpm for 10 minutes at 4°C. The pellet was resuspended in 2.0 ml of ice-cold 0.1 M $CaCl_2$ and stored the cells at 4°C overnight.

2.7.7. Transformation of competent *E. coli* cells

The competent cells (200 μl) were transferred to sterile microcentrifuge tube. DNA (~50 ηg) was added to each tube, mixed the contents of the tube by swirling gently and stored the tubes on ice for 30 minutes. The following control samples were included: (a) competent cells that receive standard supercoiled plasmid DNA and (b) competent cells that receive no plasmid DNA. The tubes were transferred to water bath set at 42°C and incubated the tubes for 90 seconds. Rapidly the tubes were transferred to ice and allowed the cells to chill for 1-2 minutes. Added 800 μl of SOC medium to each tube and incubated the cultures for 45 minutes at 37°C in a shaker incubator set at 150 rpm.

2.7.8. Selection of transformants

Plated 100 µl of transformation mix onto LB agar plates containing 100 µg ml^{-1} kanamycin and incubate the plates at 37°C overnight for the colonies to grow.

2.7.9. Analysis of transformants

The selected colonies were checked for the presence of transferred plasmid vector by agarose gel electrophoresis and PCR for *npt* II gene.

2.7.9.1. Plasmid isolation (Sambrook *et al* 1989):

Materials

Solution I:

Glucose	50 mM
Tris HCl	25 mM (pH 8.0)
EDTA	10 mM (pH 8.0)

Lysis solution, Solution II:

NaOH	0.2 M (prepared freshly from 10 M NaOH)
SDS	1.0%

Prepared freshly before use

Solution III (for 100 ml):

5 M Potassium acetate	60 ml
Glacial acetic acid	11.5ml
Distilled water	28.5 ml

Single colonies were picked from kanamycin LB plates and inoculated in 20 ml of LB broth. The cultures were grown overnight at 37^0C temperature, the cells were transferred to microcentrifuge tubes and harvested by centrifugation at 5000 rpm for 10 min. 200 µl of solution I was added to the pellet in tube and mixed well. Added 200 µl of cell lysis solution (solution II) mixed completely by repeated gentle inversion of the tube and incubated on ice for 5 min. 150 µl of solution III was added, mixed well and placed on

ice for 5 min. The tubes were centrifuged at 10,000 rpm for 15 min at 4^{O}C, the supernatant was transferred to a fresh tube and equal volume of equilibrated phenol-chloroform (1:1) was added and vortexed thoroughly, centrifuged at 4 ^{O}C with 10,000 rpm for 10 min. The upper aqueous phase was transferred to a fresh tube and equal volume of chloroform was added. The tubes were centrifuged at 12,000 rpm at 4^{O}C for 10 min to remove traces of phenol. The upper aqueous phase was transferred to a fresh tube and distilled ethanol was added to fill the microcentrifuge tube. The tubes were kept in freezer overnight for plasmid precipitation. The tubes were centrifuged at 12,000 rpm at 4 ^{O}C for 30 min. Pellets were washed with 70 % ethanol and centrifuged at 12,000 rpm at 4 ^{O}C for 10 min. The pellet was dissolved in 20µl nuclease free water or TE (10mM tris, 0.1 mM EDTA, pH 7.8) buffer and stored at -80 ^{O}C (Heraeus, Kendro Lab Products, Germany). The isolated plasmids were observed by agarose gel electrophoresis.

2.7.9.2. Agarose gel electrophoresis

Materials

Agarose (Electrophoretic grade SRL, India)

50X TAE buffer (1000ml)

Tris base	242.0 mgl^{-1}
Glacial acetic acid	57.1 mgl^{-1}
0.5 M EDTA (pH 8.0)	100 ml

DNA loading dye (6X)

Xylene cyanol	0.25%
Bromophenol blue	0.25%
Glycerol	30%

Stored at 4 ^{O}C

The gel casting boat was sealed with adhesive tape and placed the comb for making the wells. Agarose was used at 0.8% (w/v) level and melted in 1X TAE. Agarose was allowed to cool to about 50^{O}C and poured into the sealed gel casting boat. The gel was allowed to set for 30 min. The comb and adhesive tapes were removed and the gel was placed in the electrophoresis tank (Bangalore genei, India). Electrophoresis was carried out at 80V using a power pack (Consort Power Pack- E861, Belgium). The tank was filled with 1X TAE buffer to the electrode chamber to cover the gel to a depth of about 1

mm. The samples were loaded by mixing 10 µl aliquot with 2 µl of loading dye. The samples were run at 50 volts till the loading dye reaches ¾th of the gel. The gel was removed from the tank and placed in ethidium bromide (5µg ml^{-1}) solution for 15 min. subsequently destained with distilled water to remove unbound ethidium bromide and examined the gel on a UV transilluminator equipped with a documentation system (HeroLab, GMBH Germany).

2.7.10. Mobilization of binary vectors to *Agrobacterium rhizogenes*

Binary vectors were mobilized to *Agrobacterium* strains by freeze-thaw method of An et al (1988). *Agrobacterium* sp was grown in 5 ml of LB medium overnight at 28^{O}C in a shaker. Added 2 ml of the overnight culture to 50 ml LB medium in a 250 ml conical flask and shaken vigorously (200 rpm) at 28^{O}C until the culture grew to an OD of 0.5-1 at 600 nm. The culture was chilled on ice and the cell suspension was centrifuged at 3000 rpm for 5 min at 4 ^{O}C. The supernatant was discarded and resuspended the cells in 1 ml of 20 mM $CaCl_2$ solution (ice-cold). This was dispensed 0.1 ml aliquot into prechilled microcentrifuge tubes. About 1µg of plasmid DNA was added to the cells. The cells were frozen in liquid nitrogen for 2 min. The cells were thawed by incubating the tubes in 37^{O}C water bath for 5 min. Added 1 ml of LB medium to the tube and incubated at 28 ^{O}C for 2-4 hours with gentle shaking. This period allowed the bacteria to express the antibiotic resistance genes. The tubes were centrifuged for 5 min at 5,000 rpm., discarded the supernatant and resuspended the cells in 100 µl of LB medium. The cells were spread on an LB agar plate containing 100 mgl^{-1} kanamycin. The plates were incubated at 28 ^{O}C. The transformed colonies appeared in 2-3 days.

2.7.11. Isolation of binary vectors from *Agrobacterium rhizogenes*

Inoculated 5ml culture and the cells were incubated at 28^{0}C for 24hrs. The cells were harvested by centrifugation at 12,000rpm for 10 min in 1.5 ml microcentrifuge tube. The bacterial pellet was resuspended in 100µl cell suspension solution containing 50mM glucose, 25mM Tris, 10mM EDTA pH 8. Subsequently 20µl lysozyme (20mg/ml stock) (Sigma USA) was added, mixed well and incubated at 37^{0}C for 15 min. Freshly prepared 200 µl of cell lysis solution (0.2 M NaOH and 1.0% SDS) was added and mixed

completely by repeated inversion. Equilibrated phenol 50 µl was added with 2 volumes of cell lysis solution and mixed thoroughly. Added 200 µl of neutralization solution (3M sodium acetate pH 5.2), mixed completely by repeated inversion of the tube. The tubes were centrifuged at 12,000rpm for 5 min, transferred the upper aqueous phase to a fresh microcentrifuge tube, added 2.5 volumes of 95% ethanol and placed on ice for 10 min. Centrifuged at 12,000rpm for 15 min to spin down the DNA/RNA pellet and washed the pellet in 70% ethanol for further purification. Centrifuged at 12,000rpm for 5 min and resuspended the pellet in 50 µl TE buffer (10mM tris, 0.1 mM EDTA, pH 7.8)

2.7.12. PCR for the detection of binary vector in *A. rhizogenes*

Plasmid DNA was isolated from control and transformed *Agrobacterium* using the above protocol. PCR was performed using primers designed for hygromycin phosphotransferase (*hpt* II) gene. The primer sequences (*hpt* II) were as follows, forward: 5' CGGAAGTGCTTGACATTGG 3' and reverse: 5' AGAAGAAGATGTTGGCGA 3' The PCR mixture (25 µl) contained 50 ng of DNA prepared from *Agrobacterium rhizogenes*, control untransformed *Agrobacterium rhizogenes,* 1X PCR buffer, 2.5 mM of dNTPs and 1 unit of *Taq* DNA polymerase (MBI Fermentas, Lithuania), 25 pmoles of each primer (Genosys, Sigma USA). PCR for *hpt* II gene was performed at initial denaturation at 94^{o} C for 5 min followed by 35 cycles of 1 min denaturation at 94^{o} C, 1 min annealing at 55^{o} C and 1 min extension at 72^{o} C with a final extension of 72^{o} C for 10 min. PCR for *rol* A was carried out as described above with annealing at 55^{o} C for 1 min. The thermal cycler used was Primus 25 PCR system (MWG, AG Biotech, Germany). The PCR products obtained were separated on 1% agarose gel, stained with ethidium bromide and observed under UV light and documented (Hero-Lab Gmbh. Germany).

2.7.13. Preparation of *Agrobacterium rhizogenes* for coffee transformation

Wild *A. rhizogenes* and *A. rhizogenes* with pCAMBIA 1301 plasmid were used for transformation experiments. Single colonies were inoculated in 10 ml of LB broth. Kanamycin was used at a concentration of 100 mgl^{-1} in LB medium for *A. rhizogenes* harboring the binary vector pCAMBIA 1301. One ml of the overnight grown bacterial cultures was inoculated in 50 ml of LB broth. The bacterial cultures were grown for one day in LB broth till the optical density (OD) reaches one at 600nm. The cultures were centrifuged at 3000rpm in 1.5ml microcentrifuge tubes to harvest the culture, resuspended in one ml of the MS liquid medium containing acetosyringone 100µM (Sigma, USA), and shaken for half an hour at 100rpm at 28^0C.

2.7.14. Sonication assisted transformation and regeneration

Sonication-assisted *Agrobacterium* mediated transformation (Santarem *et al* 1998) was adopted for transformation. The somatic embryos and *Agrobacterium rhizogenes* were suspended in infection medium comprising half strength MS salts and B5 vitamins (Gamborg *et al* 1968), 2% sucrose and 200µM acetosyringone in 30ml Oakridge tubes (Tarson, India) and ultrasonicated (Bandelin Sonoplus ultrasonicator, Germany) for 30 sec at 80% amplitude and then incubated in a shaker at 120 rpm for 2 hrs. The duration and frequency of sonication was standardized during a series of preliminary experiments. The embryos were blot dried using sterile filter papers and co-cultivated on half strength MS salts and B5 vitamins supplemented with 2% sucrose, 100µM acetosyringone, 0.8% agar for solidification, pH 5.6 for 2 days.

Subsequently, the embryos were washed with sterile water thrice, followed by 500 mgl^{-1} cefotaxime (Alkem Laboratories Ltd., Mumbai, India) in sterile water. The embryos were blot dried and cultured on secondary embryogenic selection media. Selection and regeneration medium consisted of half strength MS salts and B5 vitamins, 0.45 mgl^{-1} IAA, 0.25 mgl^{-1} BA, 2% sucrose. Thidiazuron 0.5 mgl^{-1}, $AgNO_3$ 60 µm, 2ip 1 mgl^{-1} or triacontanol 5µM was used as adjuvant. Hygromycin (Duchefa, The Netherlands) 3-20 mgl^{-1} was used for selection and cefotaxime was used along with potassium clavulanate (Glaxo Smith Kline, Mumbai, India) 100 mgl^{-1} for killing *A. rhizogenes*. Every two

months, the surviving embryos were transferred to higher hygromycin concentrations from 3 mgl^{-1} to 5 mgl^{-1}, 10 mgl^{-1} and up to 20 mgl^{-1}.

2.7.15. GUS assay

GUS assay was performed by immersing somatic embryos for 12 hrs at 37^0C in a GUS assay buffer containing 100mM sodium phosphate (pH-7), 20mM EDTA, 0.1% triton X-100, 1mM potassium ferrocyanide, 1mM potassium ferricyanide, 20% methanol, and 1mM X-Gluc (5-bromo 4-chloro indolyl-D-glucuronide cyclo-hexamonium salt, Sigma, USA) (Jefferson *et al* 1987). Methanol was added to the reaction mixture to suppress endogenous GUS like activity (Kosugi *et al* 1990). The tubes were incubated at 37^OC in water bath for 16 hours. Dark blue color was developed in transformed callus.

2.7.16. Isolation of genomic DNA from *Coffea* sp

Genomic DNA was isolated from control and transgenic plants using the Gen Elute Plant Genomic DNA isolation kit (Sigma, USA).

2.7.17. Polymerase chain reaction (PCR) for transgenic *Coffea* sp

PCR was performed using primers designed for hygromycin phosphotransferase (*hpt* II) gene. The primer sequences (*hpt* II) were as follows, forward: 5' CGGAAGTGCTTGACATTGG 3' and reverse: 5' AGAAGAAGATGTTGGCGA 3' Detection of Ri T-DNA integrations were performed by PCR for the *rol* A gene from the putative transformants which were positive for the *hpt* II gene. The primer sequences for *rol* A gene were, forward- 5' AGAATGGAATTAGCCGGACTA 3' reverse 5' GTATTAATCCCGTAGGTTTGTTT 3'. The PCR mixture (25 µl) contained 50 ng of DNA prepared from *Agrobacterium rhizogenes*, control untransformed plantlets and transformed plantlets as the template, 1X PCR buffer, 2.5 mM of dNTPs and 1 unit of *Taq* DNA polymerase (MBI Fermentas, Lithuania), 25 pmoles of each primer (Genosys, Sigma USA). PCR for *hpt* II gene was performed at initial denaturation at 94^o C for 5 min followed by 35 cycles of 1 min denaturation at 94^o C, 1 min annealing at 50^o C and 1 min extension at 72^o C with a final extension of 72^o C for 10 min. PCR for *rol* A was carried out as described above with annealing at 55^o C for 1 min. The thermal cycler used

was Primus 25 PCR system (MWG, AG Biotech, Germany). The PCR products obtained were separated on 1% agarose gel, stained with ethidium bromide and observed under UV light and documented (Hero-Lab Gmbh. Germany).

2.7.18. Southern hybridization

The isolated genomic DNA (approx. 40µg quantity) from putative transformants was digested with N*de* I and S*ac* II enzymes, for Southern blot analysis. The digested DNA was electrophoresed on 0.8% (w/v) agarose gel, transferred (Sambrook *et al* 1989) to Biobond plus nylon membrane (Sigma, USA) and hybridised with the 479-bp *hpt* II gene probe. The probe for *hpt* II gene was prepared using a psoralen biotin labelling kit (Ambion, USA). Hybridisation signals were detected using a Biodetect Kit (Ambion, USA). The detailed protocol is presented below.

2.7.18.1. Restriction digestion of pCAMBIA 1301 vector and *Coffea* sp genomic DNA

Materials

1. Restriction enzymes: N*de* I and S*ac* II (MBI Fermentas, Lithuania)
2. 10 X restriction enzyme buffer (MBI Fermentas, Lithuania)
3. BSA, acetylated, 1mg ml-1 (MBI Fermentas, Lithuania)
4. Nuclease-free deionized water (Bangalore Genei, India).

The following were added in a micro centrifuge tube in the order stated:

Nuclease-free water	13.8 µl
Restriction enzyme 10X buffer	2.0 µl
BSA, acetylated (1mg ml^{-1})	0.2 µl
DNA	3.0 µl
N*de* I or S*ac* II	1.0 µl (10 units)
Final volume	20.0 µl

Since the enzymes are not compatible the digestion was carried out separately. Initially the DNA was digested with N*de* I, precipitated, resuspended in water and re-digested

with S*ac* II. For digestion, the samples were incubated (after brief centrifugation) at 37^0 C for 8 h and the reaction was stopped by heating them at 65^0C for 2 min. An aliquot of the digested products were fractionated and observed on agarose gel (0.8%).

2.7.18.2. Transfer of DNA to nylon membrane and hybridization

Materials

1. Target DNA
2. Primers for probe preparation (Genosys Sigma, USA)
3. Nylon membrane (Sigma, USA)
4. 20XSSC:

NaCl	3M
Sodium citrate pH 7.2.	0.3M

5. Hybridization buffer:

SSC	5X
N-lauroylsarcosine	0.1% (w/v)
SDS	0.02% (w/v)

6. Post-hybridization washing buffer I:

SSC	2X
SDS	0.1%

7. Post-hybridization washing buffer II :

SSC	0.1X
SDS	0.1%

8. Non isotopic (Psoralen biotin) labeling kit (Ambion, USA)
9. Biodetect kit (Ambion USA)
10. 5-Bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium (BCIP-NBT) detection solution (Bangalore Genei, India)

The digested DNA samples (digested with restriction enzymes N*de* I and S*ac* II) were loaded onto the agarose gel 0.8% (w/v) and run at 80 V until the dye front reaches ¾ of the gel. After electrophoresis the gel was stained with ethidium bromide for 15 min and observed on the transilluminator.

After examination the gel was soaked for 45 min in several volumes of 1.5 M NaCl and 0.5 N NaOH mixtures with constant gentle agitation. The gel was soaked in several volumes of 0.2 N HCl for 10 min and rinsed briefly with triple distilled water. The DNA was neutralized by soaking in 1 M Tris (pH 7.4) and 1.5 M NaCl at room temperature for 30 min with mild agitation. The neutralization solution was changed three times with 15 min. interval.

2.7.18.3. Capillary transfer of DNA from agarose gel to nylon membrane

The transfer tank was filled with 75ml of 10XSSC buffer on each side. A Whatmann No. 3 filter was placed on the platform of the tank. The side of the filter paper was dipped into the buffer. The filter paper was rolled gently with a glass rod to remove air bubbles. Six Whatmann No. 3 sheets and nylon membrane was cut to the exact size of the gel. The nylon membrane was dipped in deionized water and then incubated in 10X SSC for five min. Two Whatmann No. 3 sheets were dipped in 10X SSC and placed in the middle of the platform. The gel was placed on the top of the filter in inverted fashion. The right side of the gel was nicked to serve as an identification mark. Parafilm strips were placed all around the gel to avoid short circuit of buffer during transfer. The nylon membrane was placed with its right side cut over the gel, so that the cut side matches with that of the gel. Remaining four Whatmann No. 3 filter sheets were dipped in 10X SSC and placed over the membrane. Air bubbles were removed by rolling a glass rod in each step. Stacks of paper towels were placed over the filter paper and applied a weight of about 500gms over the entire assembly. The transfer process was allowed for 24hrs with intermittent changes of paper towels and transfer buffer.

2.7.18.4. Disassembling the blot

The weight, paper towels and Whatmann No. 3 filter paper were removed. The positions of the wells were marked on the membrane with a pencil. The membrane was peeled off

carefully and gently submerged it in 6X SSC. After five min. the membrane was blotted with Whatmann No. 3 filter paper. The gel was restained in ethidium bromide to check the transfer efficiency.

2.7.18.5. Immobilizing the DNA:

After allowing the membrane to dry, it was placed inside a polythene bag and placed it over a UV transilluminator for two min. to allow cross-linking of DNA.

2.7.18.6. Pre-hybridization

The membrane was put in a hybridization jar to which 15 ml prewarmed (68^0C) hybridization buffer was added and incubated overnight at 68^0C in a hybridization oven (Shell Lab model-1004-2E) at constant and slow rotation.

2.7.18.7. Preparation of BrightStar Psoralen-Biotin conjugated probe

Psoralens are planar tricyclic compounds, which have a natural affinity for nucleic acids and intercalate between bases in a manner similar to ethidium bromide (User manual Psoralen Biotin Labeling Kit, Ambion USA). Upon irradiation with long wave UV light, psoralens become covalently attached to the nucleic acid, preferentially to thymidines, and also to uridines and cytidines. Ambion's BrightStar Psoralen-Biotin Nonisotopic Labeling Kit uses the intercalating property of psoralens to efficiently label nucleic acids with biotin, using a BrightStar Psoralen-Biotin conjugate.

A clean untreated 96 well plate was placed on an ice bath. Using Quiagen PCR purification Kit (Quiagen India, Genetix) the PCR product of *hpt* II gene was purified. Purified PCR product was denatured by heating to 100°C for 10 min, and then rapidly cooled the mixture in a dry ice bath to freeze. Subsequent steps were carried out under low light in a dim room. Centrifuged the vial containing the lyophilized BrightStar Psoralen-Biotin for ~15 seconds at 7,000 x g and reconstituted in 33 µl DMF. The frozen sample was thawed by rolling it between gloved hands, and immediately added 1 µl of the BrightStar Psoralen-Biotin to 10 µl of the nucleic acid solution in a microfuge tube. The sample was mixed and transferred to a well in the 96 well plate (on ice). The nucleic acid solution should have a final concentration of 0.5–50 ng/µl. A 365 nm UV light

source was held on the plate directly over the sample and irradiated the sample for 45 minutes. The sample was diluted to 100 µl by adding 89 µl of TE Buffer and mixture was transferred to a clean microfuge tube. Added 200 µl of water-saturated n-butanol and vortexed well and microcentrifuge for 1 minute at 7,000 rpm. The top n-butanol layer was removed. The trace of n-butanol was removed by extracting with 2 volumes of water-saturated diethyl ether, microcentrifuging and pipetting off the ether. The biotin-labeled nucleic acid was stored at –80°C for long-term storage.

2.7.18.8. Hybridization

Hybridization buffer was discarded from jar. The probe was diluted 10 fold with 10mM EDTA, denatured by incubating in boiling water bath for 10 min and snap cooling on ice. The denatured probe was added to the hybridization buffer and mixed it immediately. The membrane was incubated with the probe at 68^0C for 6 h with mild agitation in a hybridization oven .

2.7.18.9. Post-hybridization washes

The membrane was washed twice in 50 ml of post hybridization washing buffer I for 5 min at room temperature. Membrane was washed again in 50 ml of post hybridization washing buffer II for 15 min at 68^0C under mild agitation.

2.7.18.10. Detection of hybridization signals

Detection of hybridization signals were done with Ambion Biodetect Kit (Nonisotopic Detection Kit, Ambion, USA) Membrane was rinsed twice for 10 min at room temperature in Ambion wash buffer. Subsequently incubated the membrane in blocking buffer twice for 20 min duration. Streptavidin-alkaline phosphatase was prepared by gently and thoroughly mixing together 10ml blocking buffer and 1µl Streptavidin-alkaline phosphatase (Ambion USA) (It is important to mix with the blocking buffer before adding to membrane) and incubated the membrane for 45 min. The membrane was washed three times (15 min each time) in 1X Ambion wash buffer. The membrane was immersed in 10 ml BCIP-NBT detection solution (Bangalore Genei, India) For the development of color; the membrane was kept in the dark without shaking overnight.

Reaction was stopped by washing the membrane for 5 min with 50 ml of deionized water. The results were documented by photography of the wet membrane following which the membrane was air-dried and stored in dry place.

2.8. Transformation of coffee for down regulation of caffeine biosynthesis

2.8.1. Coffee genotype and *Agrobacterium* strain used:

Certified seeds of *C. canephora* viz., S274 was used. Somatic embryos were obtained as explained in sections 2.1 to 2.4. *Agrobacterium tumefaciens* strain EHA 101 carrying different constructs designed for post-transcriptional gene silencing (PTGS) was used to transform *Coffea canephora.*

2.8.2. Constructs used for Silencing caffeine biosynthesis

The binary vector pCAMBIA 1301 was used as the primary vector and co-suppression (pSAT 201), antisense (pSAT 202), and inverse repeat (pSAT 222) constructs (Fig 2.2 a, b, c) containing homologous sequence of *N* methyl transferase were used in transformation of coffee to down regulate caffeine biosynthesis. The constructs were obtained from Mr. Satyanarayana KV & Dr. Arun Chandrashekar CFTRI, Mysore. A 339 bp region from the second exon of the genomic clone for one of the *Coffea canephora* NMT gene (Accession no: AY918126) was cloned in sense, antisense and head to head invert repeat fashion (hpRNA) with respect to CaMV35S promoter in pCAMBIA1301 binary vector. The two arms of invert repeat structures were separated by a spacer of 90 bases comprising of multiple cloning site of the cloning vector.

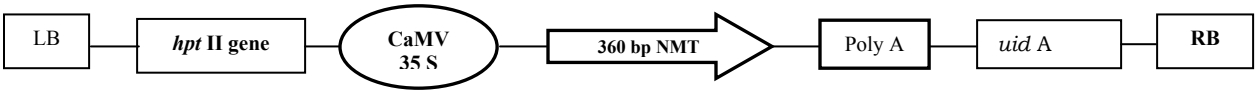


Fig 2.2a: Sense construct pSAT 201

Fig 2.2b: Antisense construct pSAT202

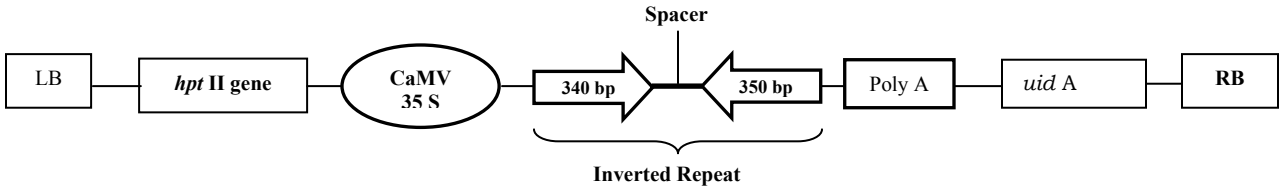


Fig 2.2c. Inverse repeat construct pSAT 222

Fig 2.2: The T-DNA maps of different constructs used to down regulate caffeine biosynthesis.

2.8.3. Mobilization of different constructs to *Agrobacterium tumefaciens*

The gene constructs were mobilized to *Agrobacterium* strains by freeze-thaw method of An *et al* (1988) as described in earlier section.

2.8.4. Transformation of *Coffea* with constructs targeted to silence *N*-methyl transferase.

Sonication-assisted *Agrobacterium* mediated transformation (Santarem *et al* 1998) was adopted for transformation. The somatic embryos were infected with *A. tumefaciens* as explained in section 2.7.15.

2.8.5. Molecular and biochemical analysis of plants transformed with constructs targeted for silencing caffeine synthesis

2.8.5.1. GUS assay

GUS assay was performed as in section 2.7.15. (p-73)

2.8.5.2. Isolation of genomic DNA from coffee

Genomic DNA was isolated from control and transgenic plants using the Gen Elute Plant Genomic DNA isolation kit (Sigma, USA).

2.8.5.3. Polymerase chain reaction (PCR) for transgenic coffee

PCR was performed as in section 2.7.17. (p-73).

2.8.5.4. Analysis of *N* methyltransferase transcript levels:

Total RNA was extracted using a total RNA extraction kit (RNeasy kit, Ambion, USA). All the plastic wares were treated with 0.1% diethyl pyrocarbonate (DEPEC) (Sigma USA) and the working area, electrophoresis tank and other required materials were treated with RNase Zap (Ambion, USA). The control and transgenic tissues were harvested, frozen in liquid nitrogen and RNA was extracted immediately. Quality and concentration of RNA were checked on denaturing agarose gel. All the RNA samples were subjected to DNase (Ambion, USA) treatment to avoid possible artifact amplifications from contaminant genomic DNA.

N methyltransferase gene specific primers were designed across the intron. First-strand cDNAs were synthesized from 2 µg of total RNA in 20 µL final volume, using MuLV reverse transcriptase (Ambion USA) and oligo-dT(18) primer (Sigma USA) following the manufacturer's instructions. To quantify template quantities, the RT-PCR reaction was stopped in the early exponential phase (28^{th} cycle) to maintain initial differences in target transcript quantities. PCR was performed as in section 2.7.17. (p-73) using the primer sequences NMTF- 5' CGAGGAGTCCATGCATTTTT 3' and NMTR- 5' CCTCCTCAACCA TGCACTTT 3'. Ten microlitres from each PCR reaction was fractionated on a 1.5% (w/v) agarose gel in Tris-acetate EDTA buffer and stained with

0.5% (w/v) ethedium bromide. The gels were photographed with a Digital Imaging System (HeroLab, GMBH, Germany).

2.8.6. Estimation of 7-methyl xanthine, caffeine, theobromine and theophylline in transgenic plantlets

Alkaloid estimation was carried out in 18 months old transgenic plantlets. 7-methyl xanthine, caffeine, theobromine and Theophylline were extracted in 80% ethyl alcohol. Estimation was carried out by high performance liquid chromatography on a Bondapak C-18 column (5µ x 25cm) with 50mM sodium acetate (pH 5.0), methanol, tetrahydrofuran in the ratio 91:8:1 as a mobile phase and eluted at room temperature. Each sample replicated thrice for HPLC and average values were expressed in % dry weight (DW) of the tissue. Parameters were controlled by a Shimadzu LC 10–AS liquid chromatograph equipped with a dual pump and a UV spectrophotometric detector (Model SPD-10 A) set at 270 nm. The recorder Shimadzu C –R7A chromatopac was set at a chart speed of 2.5cm/min. Injection volume was 10uL, injected with Rheodyne 7125 injector. Peak identification was achieved by comparing with the retention time of standards (SIGMA, USA) and confirmed by spiking the samples with standards. HPLC analysis was carried out in triplicate injections for each transgenic plant and the mean value is presented.

2.9. Optimization of electroporation mediated gene delivery: analysis of *N* methyl transferase and 11s globulin promoters in coffee

2.9.1. Optimization of electroporation mediated gene delivery to *Coffea* somatic embryos

Electroporation method was adopted to transfer DNA to coffee tissues. Electroporation was carried out according to Fernandez and Menendez (2003), with some modifications in pretreatment for cell wall degradation. Endosperm tissues from three months old fruits, *in vitro* callus, globular and torpedo stage somatic embryos were used for electroporation mediated DNA delivery. Pretreatment of somatic embryos were carried out according to Fernandez and Menendez (Fernandez and Menendez, 2003). Somatic embryo samples were incubated for 6 hours and endosperm tissues for 1 hour in enzymatic solution

containing 2% cellulase (Onozuka R-10 Yakult Pharmaceutical Industry Co., Ltd, Japan) and 1% pectinase (Sigma chemical Co, USA) in buffer 5 mM 2-(N-morpholine) ethanesulphonic acid (MES), 0.5 M mannitol and 25 mM $CaCl_2$ at pH 5.8. Subsequently the samples were washed thoroughly with electroporation buffer containing 70 mM aspartic acid, 5 mM calcium gluconate, and 5 mM MES and 0.5 mM mannitol (pH 5.6). The endosperm tissues were not treated with enzyme solution and taken with 100µg/ml plasmid DNA in 350 µl electroporation buffer. The tissues were incubated one hour at 4^0C prior to electroporation using an electroporator (BioRad Gene Pulser Xcell system). Electroporation was carried out with single electric pulse of 500 V/cm (Fernandez and Menendez, 2003), discharged from a 900-µF capacitor. Electroporation without plasmid DNA served as a negative control. The embryos were incubated for an additional hour at 4^0C after discharge. All the vectors were maintained in *E. coli* DH5α and plasmid DNA isolation was carried out according to (Sambrook *et al* 1989). Influence of spermidine was assessed by adding it in the electroporation buffer at a concentration of 0.2mM.

2.9.2. Constructs used

The plasmid vector pCAMBIA 1301 and pCAMBIA 1381 was procured from Center for the Application of Molecular Biology to the International Agriculture of Canberra, Australia (CAMBIA). All the promoter::GUS fusion constructs used in this study were obtained from Mr. Satyanarayana & Dr. Arun Chandrashekar CFTRI, Mysore. A 745 bp NMT promoter (gi:59710567) was used in making promoter::GUS construct pPCTS745 in binary vector pCAMBIA 1381 (Satyanarayana *et al* 2005) (Fig 2.3a). Similarly 959 bp 11S globulin promoter from coffee was isolated (gi:76365132) and was used in making promoter::GUS construct pPCGB959 in binary vector pCAMBIA 1381 (Fig 2.3b). The vector pCAMBIA 1301 contains the selectable marker gene hygromycin phosphotransferase (*hpt* II) under the control of the CaMV 35S promoter and CaMV 35S terminator; *β*-glucuronidase (*uid* A) gene with a catalase intron under the control of CaMV 35S promoter and NOS terminator (Fig 2.3c). pCAMBIA 1381 contains a promoter less *uid* A construct and used as a negative control. (Fig 2.3d) The constructs were introduced into *Coffea canephora* somatic embryos and endosperm by tissue electroporation. Electroporation of tissues without plasmid DNA, with pCAMBIA 1301

and with promoter less *uid* A construct pCAMBIA 1381 were used as controls. pCAMMBIA 1301 vector was digested and linearized with B*am* H1 using the protocol explained in p.74, section 2.7.18.1.

Apart from the transient assays attempts were made to regenerate the transgenic secondary embryos electroporated different constructs. The electroporated tissues were cultured on medium containing hygromycin 3-20 mgl^{-1}. The selection and regeneration of secondary embryos were performed as explained in section 2.7.14, p. 72. A total of 15 explants in 10 replicates were subjected to electroporation for each construct. The efficiency of electroporation-mediated transformation was determined by the number of somatic embryos/endosperm tissues showing GUS expression and expressed in terms of percentage transformation frequency. The mean value of 10 replicates has been presented in the results.

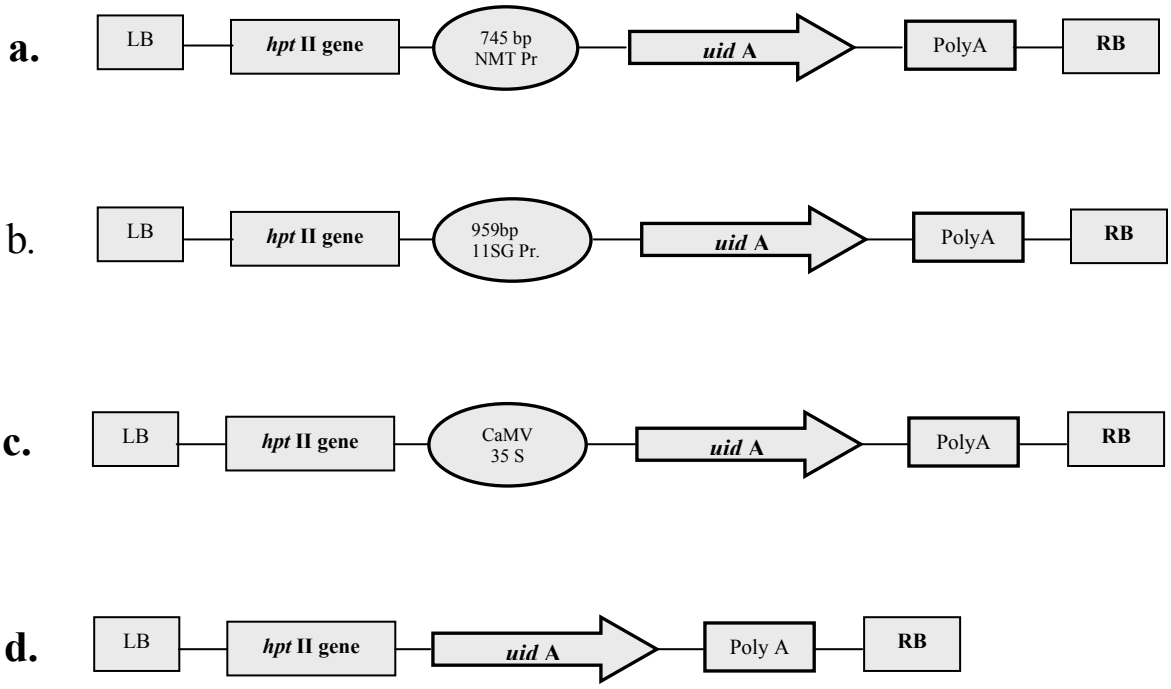


Figure 2.3: The T-DNA region of constructs of (a) pPCTS745, (b) pPCGB959, (c) pCAMBIA 1301, (d) pCAMBIA 1381 used for optimization of electroporation

2.10. Localization of *N* methyl transferase of caffeine biosynthetic pathway through localization of GUS reporter gene driven by NMT promoter in coffee endosperm.

2.10. 1. Explants and DNA delivery

Three months old green unripe fruits were collected and the endosperm tissues were used in electroporation experiments. Electroporation was carried out as explained in section 2.9.1 (Fernandez and Menendez 2003). Electroporation of endosperm without plasmid DNA served as a negative control.

2.10.2. Constructs used

The NMT promoter was isolated by PCR based genome walking (Satyanarayana *et al* 2005). This construct comprised a 938 bp PCR product having 29 bases of adapter and 909 bases of the coffee genomic fragment (accession no. DQ010010). The construct contained +83 bp region comprised of 75 bases of first exon and 8 bases of the first intron (AB048794). This promoter fragment was in frame with a cassette containing the *uid* A gene and the NOS terminator in pCAMBIA 1381 digested with E*co*R1/B*am*H1. The resulting translational fusion construct was designated as pPCTS938 (Fig 2.4a).

Similarly, another construct was made in such a way that the first exon region was avoided in the *N*-terminal fusion to the *uid* A gene. An 896 bp E*co*R1/B*am*H1 promoter fragment was fused in frame to the *uid* A gene in pCAMBIA 1381 (Fig 2.4d) digested with E*co*R1/B*am*H1. The resulting translational fusion construct was designated as pPCTS855 (Fig 2.4b). These constructs were obtained from Mr. Satyanarayana KV & Dr. Arun Chandrashekar CFTRI, Mysore.

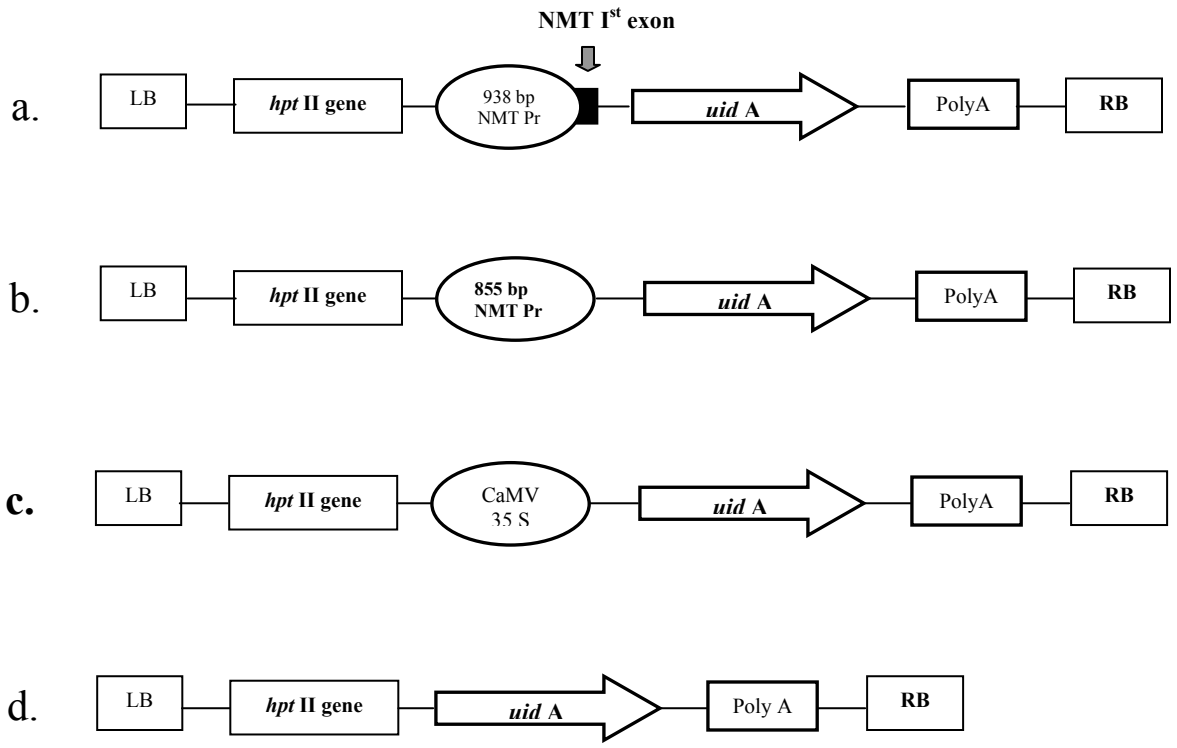


Figure 2.4: The T-DNA region of constructs of (a) pPCTS938, (b) pPCTS855, (c) pCAMBIA 1301, (d) pCAMBIA 1381 used for study of sub cellular targeing of NMTs.

2.10.3 GUS assay

GUS assay was performed as explained in earlier section (p.73, section 2.7.15). The samples were fixed in formaldehyde: acetic acid: 70% (v/v) ethanol (5:5:90) prior to free hand sectioning and observed in an Olympus light microscope (BX40).

2.10.4 Cellular localization of chlorogenic acid

The method of Reeve (1968) was followed for staining chlorogenic acid. Freehand sections were first treated with aqueous solution containing 10% sodium nitrite, 2% urea and 1% glacial acetic acid. NaOH 2% was added to the sections after the appearance of a

yellow color. A deep cherry red color appeared in 2-3 min. The sections were observed using an Olympus light microscope (BX40).

2.10.5 Immunocytolocalization of the NMT enzymes

Immunocytolocalization of the NMT enzymes was performed using rabbit polyclonal anti-Coffee theobromine synthase-2 (CTS2) antibodies (kind gift from Dr. Kouichi Mizuno, Faculty of Bioresource Sciences, Akita Prefectural University Japan). Immunolocalization was performed as explained by Anil *et al* (Anil *et al* 2000). The sections were observed under Olympus microscope equipped with flourescens filters (Olympus, BX40. Emission wavelength 450-480, Excitation wavelength 510-550).

2.11. Bio-safety measures

All the work was carried out according to the guidelines of IBSC (Institutional Bio-Safety Committee, CFTRI, Mysore). The transgenic work was carried out with permission from RCGM (Regulatory Committee for Genetic Modifications, Department of Biotechnology, Government of India). The transgenic work was confined to the *in vitro* laboratory level. ISO 14001 guidelines of CFTRI were followed for the disposal of contaminants and transgenic waste.

2.12. Statistical analysis

Experiments were carried out in triplicates and the data was presented in terms of mean and standard error.

CHAPTER III
Results and Discussion

3.1. ESTABLISHMENT OF *IN VITRO* CULTURES

Background:

A highly efficient *in vitro* plant regeneration system is very essential for carrying out genetic transformation work. Somatic embryogenesis is a highly useful method for a large-scale propagation of species of economic interest. Somatic embryos are widely considered to be of single cell origin; hence this is advantageous for transformation studies. Moreover, the process of somatic embryogenesis offers a mean to propagate large numbers of transgenic plants over a short period of time. In *Coffea* sp, somatic embryogenesis has been reported nearly 3 decades ago by Staritsky (1970) and constitutes a model system for perennial species (Sondahl and Loh 1988). With this view, various growth regulators were used to achieve plant regeneration through direct and indirect somatic embryogenesis. Secondary embryogenesis was also attempted for multiplication of primary embryos.

This section deals with optimization of growth regulators to achieve plant regeneration through somatic embryogenesis in *Coffea sp.*

3.1.1. *Coffea* seed germination:

The seeds of *Coffea canephora* cv S 274, CXR and *Coffea arabica* cv Hemavathy were germinated (Fig 3.1 a & b) in 30-40 days in ¼ MS basal medium and vitamins with 2% sucrose, 40 mg l^{-1} cysteine HCl. The germination percentage varied between 40-85% (Table 3.1). The surface sterilization of seeds did not eliminate the systemic fungal / bacterial contamination. Incorporation of cefotaxim 500mgl^{-1} and bavistin 100mgl^{-1} in the culture medium reduced the germination percentage but the contamination was prevented. Fungal contamination was very prevalent during the cotyledon emergence stage. Browning of the tissues during germination of seeds was the major problem and this was solved by using PVP 0.5% (w/v) and activated charcoal 0.5% (w/v) in the culture medium (Fig 3.1, Table 3.1). Thus prevention of fungal or bacterial contamination and tissue browning was crucial for normal germination to get healthy seedlings that could be used for *in vitro* establishment of callus and somatic embryo cultures. The initial seed germination accured in 40 days and plantlets reached to cotyledonary stage in 90-120 days.

Table 3.1: Influence of different media adjuvants on seed germination percentage

Treatment	% Germination[1]	% Browning	Percentage of Fungal / Bacterial contamination during germination	
			Radical stage	Cotyledon stage[2]
¼ MS + 2% Sucrose	65	40	40	95
¼ MS + 2% Sucrose + 0.5% PVP	85	30	40	85
¼ MS + 0.5%PVP + 0.5% Activated Charcoal + 2% Sucrose	80	10	40	85
¼ MS + 0.5% PVP + 0.5% Activated charcoal + 2% Sucrose + Cefotaxime 500mgl^{-1} + Bavistin 100mgl^{-1}	40	45	10	50

[1]data was recorded during germination stage after 40 days of culture.

[2]data was recorded during cotyledonary stage after 120 days of culture.

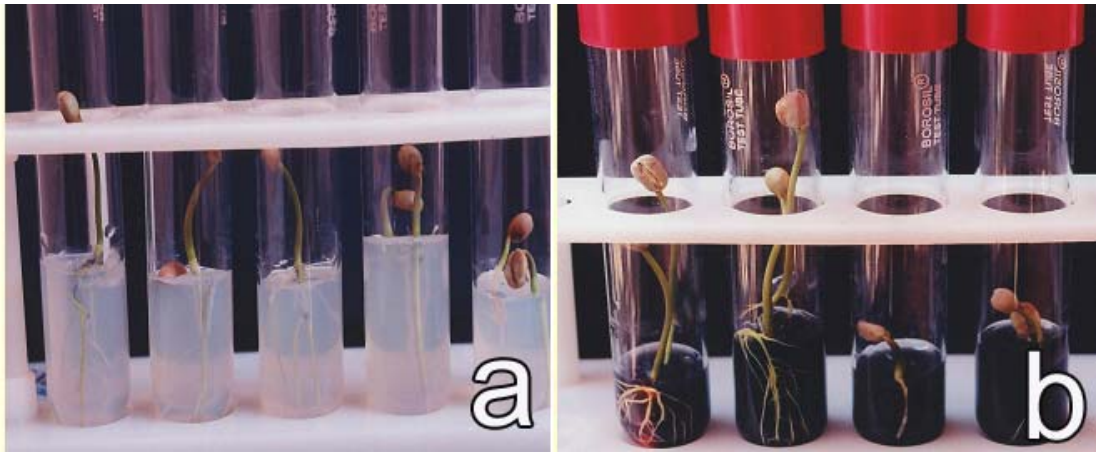


Fig 3.1: Germination stages of *Coffea sp*.

(a) Germination of seedlings in medium containing ¼ MS salts + 2% sucrose + 0.5% PVP (b) Germination of seedlings in medium containing ¼ MS salts + 0.5% PVP + 0.5% activated charcoal + 2% sucrose + cefotaxime + bavistin.

3.1.2. Callus formation

The earliest signs of callus formation from cotyledonary leaves as well as from hypocotyls were observed within two weeks in callus induction medium (p.53, section 2.1.4) containing half strength MS basal salts with 9.8 µM 2-iP, 2.2 µM 2,4-D and 4.9 µM IBA, producing white to greenish, hard callus. Callus formation was observed in cotyledonary leaf explants in both the cultivars cv. S-274 and cv. CxR. Maximum response for callus induction i.e. 68% was obtained in medium comprising 2,4-D, IBA and 2 iP (Table 3.2).

Table 3.2. Callus initiation in *Coffea canephora* cv S-274

*Hormonal treatment			% Explants showing callus initiation
2,4 D mgl^{-1}	IBA mgl^{-1}	2-iP mgl^{-1}	
0.5	0.5	2	68
1	0	2	36
0	0	0	-

*Media: ½ MS salts and B5 vitamins. Data recorded on 45th day of culture.

Further studies on embryogenesis suggested that, the callus were of two types with distinct morphological variations (Fig 3.2). The non-embryogenic callus was morphologically brownish black and spongy. The embryogenic callus was creamish white and friable in nature (Fig 3.2). Induction of embryogenic callus was sparse in hypocotyl explants. Sub-culturing the callus in the same medium did not produce somatic embryos. This callus was grown in callus multiplication medium and sub-cultured on the 45th day. Rapid multiplication of calli obtained in medium comprising 2,4-D 1mgl^{-1} and BA 4mgl^{-1} (p.53, section 2.1.4). Two kinds of callus were obtained in callus multiplication medium i.e., embryogenic callus and non-embryogenic callus. The non-embryogenic cell line was unable to undergo embryogenesis subsequently. At the end of 75 days of culture the explants showed 52% embryogenic callus and 28% non-embryogenic callus (Table 3.3).

Table 3.3: Multiplication of first generation callus in *Coffea canephora* cv S-274

*Hormonal treatment		% Explants producing embryogenic callus	% Explants producing non embryogenic callus
2,4 D mgl^{-1}	BA mgl^{-1}		
1	4	52	28
1	1	31	40
0	0	-	15

*Media: ½ MS salts and B5 vitamins. Data recorded on 75th day of culture.

3.1.3. Somatic embryogenesis

Globular somatic embryos were obtained (85% embryogenesis) from embryogenic calli in medium comprising IAA 0.5$mgl^{-1}$and BA 0.25mgl^{-1} (Table 3.4). Only embryogenic callus was produced in medium (p.53, section 2.1.5) comprising BA alone at 2mgl^{-1} (Table 3.4). These globular embryos matured and turned to torpedo stage when cultured on ½ strength MS medium with B5 vitamins for 45-60 days.

Table 3.4. Embryogenesis response of *Coffea canephora* callus cultured for 45 days

*Hormonal treatment		Percentage explants producing globular embryos
IAA mgl^{-1}	BA mgl^{-1}	
0.5	0.25	85
0	2	10[c]
0	0	-

*Media: ½ MS salts and B5 vitamins

[c]Embryogenic callus with vigorous multiplication was observed.

3.1.4. Discussion

In vitro germinated seedlings are good source to carry out experiments on callus initiation, somatic embryogenesis and micropropagation. *In vitro* germination of coffee seeds is a difficult task due to the presence of fungal and bacterial contamination, which is difficult to eliminate by surface sterilization. In plant tissue culture, contamination is caused by the microorganisms that inhabit either the surface of the explants (exogenous) or the ones present in the tissues of the explants (endogenous). It has been well established that the exogenous microorganisms are effectively eliminated by surface disinfection (Bonga 1982).

The induction of somatic embryos is very sensitive to culture conditions such as the composition of the medium, the physical environment of the culture, the genotype and the explant source (Fuentes *et al* 2000). Two general types of somatic embryogenesis may occur in coffee, direct embryogenesis, in which embryos originate directly from tissues in the absence of conspicuous callus proliferation and indirect embryogenesis, in which callus proliferation and embryogenic tissue precede embryo development (Sondahl and Sharp 1977, Dublin 1981, Pierson *et al* 1983). Direct embryogenesis reduces the time required for plant regeneration, which may be beneficial to minimize culture-induced genetic changes (Fuentes *et al* 2000). For large-scale micropropagation, indirect somatic embryogenesis, i.e. embryogenic callus (or tissue) induction that allows somatic embryo formation in high frequency is generally recommended. Embryogenic callus initiation needs a long time of culture on cytokinin medium, but once induced; embryogenic callus proliferates producing large amounts of somatic embryos continuously (Yasuda *et al* 1995) and similar observations were made in the present study.

Summary

- Somatic embryogenesis was achieved in *Coffea* sp.
- Embryogenic and non-embryogenic callus were obtained distinctly.
- Further studies were planned to identify the role of polyamines and calcium in determination of embryogenic nature in *Coffea* sp

3.2. INFLUENCE OF POLYAMINES ON SECONDARY EMBRYOGENESIS.

Background:

Endogenous and exogenously administered hormones play a crucial role in somatic embryogenesis. Polyamine pools and ethylene pathway are interlinked and known to redirect the cell towards embryogenesis when present in optimum concentrations (Feirer *et al* 1984). Polyamines (PAs) viz. putrescine, spermidine and spermine are known to play important role in various cellular process (Reviewed by Bais and Ravishankar 2002). Chemically they are non-protein, strait chain, aliphatic amines. Polyamines are known to be involved in DNA replication, cell division, protein synthesis, responses to abiotic stress, rhizogenesis, flower development, *in vitro* flower induction (Reviewed by Bais and Ravishankar 2002). Polyamines are reported to inhibit ethylene biosynthesis in plants (Apelbaum *et al* 1981). Evidence from several studies has indicated that, polyamines play a crucial role in somatic embryogenesis and regeneration of several plant species (Reviewed by Bais and Ravishankar 2002, Kumar and Rajam 2004).

Studies were carried out to elucidate the role of polyamines in regulating somatic embryogenesis in *Coffea canephora.* Polyamine inhibitors and polyamines were incorporated in the culture medium to assess its role in somatic embryogenesis.

3.2.1. Secondary embryogenesis under the influence of polyamines

Incorporation of 50mM putrescine in the culture medium (p.54, section 2.2.1) containing 0.5 mgl^{-1} IAA and 0.25 mgl^{-1} BAP resulted in 58% response for secondary embryos from primary embryos when compared to control with 42% response (Figure 3.2c, 3.3). These embryos were creamish green, bold and showed vigorous development (Figure 3.2c). Incorporation of silver nitrate in the culture medium containing 0.5 mgl^{-1} IAA and 0.25 mgl^{-1} BAP resulted in 65% response for embryogenesis (Figure 3.3). When polyamine biosynthetic pathway was inhibited by DFMO and DFMA, 83% reduction in secondary embryogenic response from embryos was observed when compared to control (Figure 3.2d, 3.3). Most of the explants turned brown and the embryos formed in this medium did not mature to plantlets (Figure 3.2d)

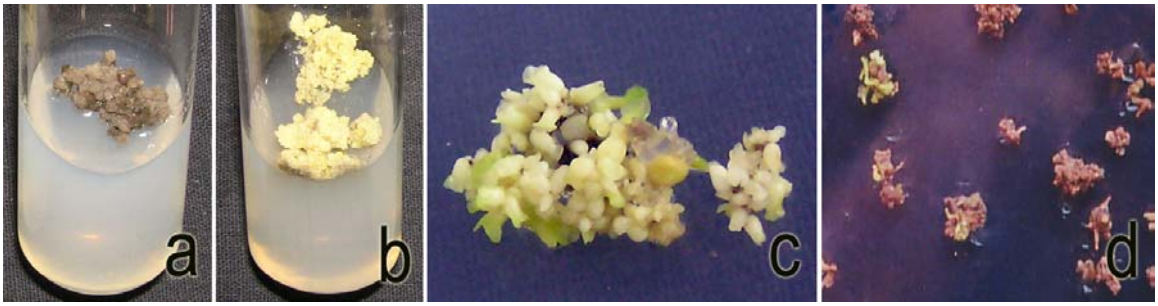


Fig 3.2. Response of *Coffea canephora in vitro* cultures for embryogenesis under the influence of putrescine and polyamine synthesis inhibitors.

(a) Non-embryogenic callus, b. Embryogenic callus, c. Somatic embryogenesis under the influence of 50mM putrescine, d. Inhibition of embryogenic response under the influence of polyamine biosynthetic pathway inhibitors DFMA and DFMO 1mM each.

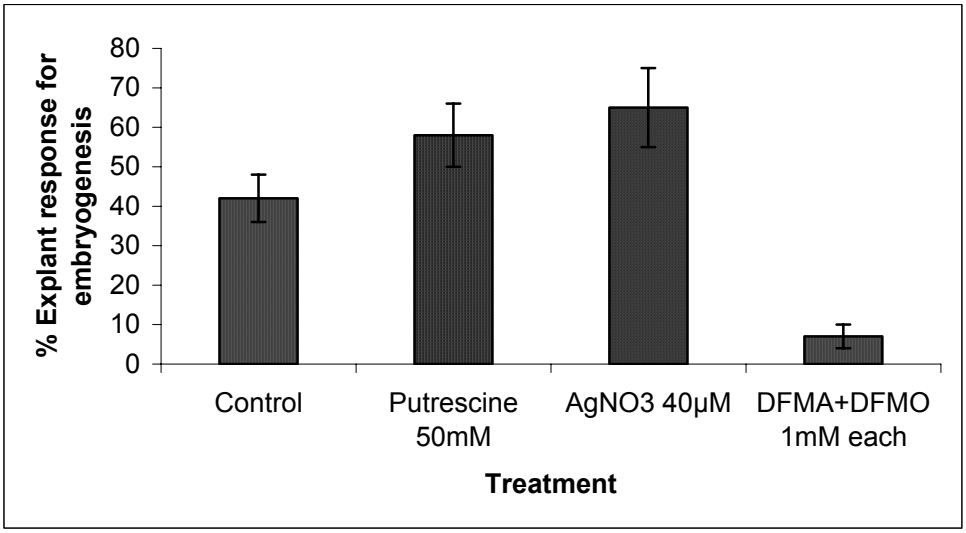


Fig 3.3. Influence of putrescine 50mM, Ethylene action inhibitor $AgNO_3$ 40µM and polyamine biosynthetic pathway inhibitors DFMA and DFMO 1mM each on somatic embryogenesis response in *Coffea canephora*

3.2.2. Endogenous polyamine pool

The total polyamine estimation (p.54, section 2.2.2) data revealed difference in the endogenous spermine levels (Fig.3.4). The embryogenic callus contained 11 fold more

spermine and 3.3 fold higher spermidine when compared to non-embryogenic callus. There was no significant difference in the putrescine levels in embryogenic and non-embryogenic callus.

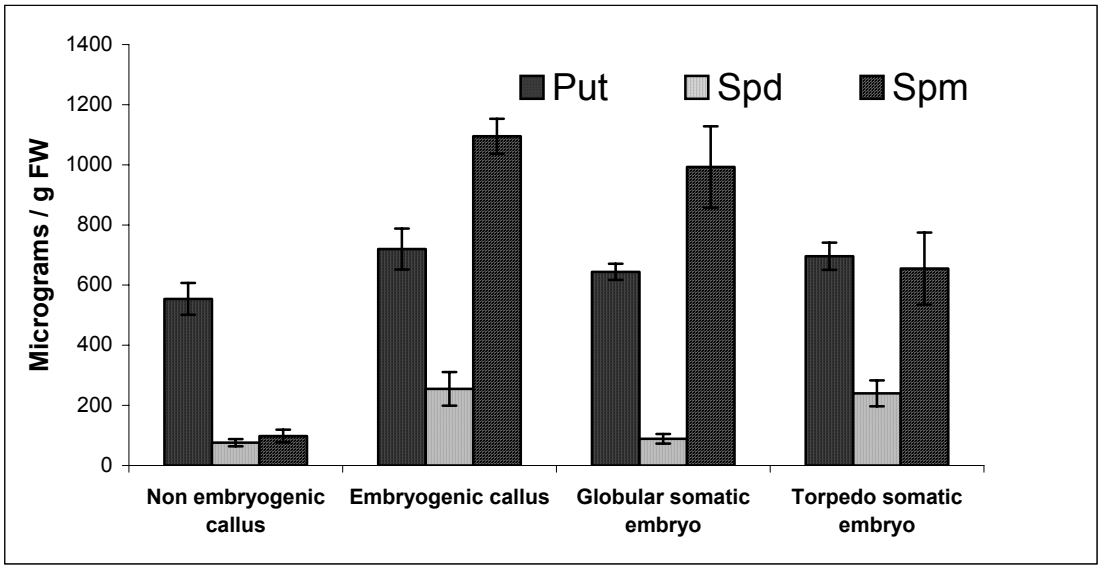


Fig 3.4. Endogenous polyamine levels during embryogenesis and callus lines of *Coffea canephora* (put-putrescine, spd- spermidine, spm-spermine).

3.2.3. Influence of polyamines on caffeine content

There was no significant difference in caffeine levels in polyamine treated cultures. However treatment with DFMA and DFMO resulted two-fold increase in the caffeine levels (Fig 3.5). Incorporation of silver nitrate in the culture medium resulted in decrease in the caffeine level to 0.48% DW when compared to the control with 0.6% DW caffeine in the calli. Incorporation of silver nitrate and polyamine synthesis inhibitors resulted in 0.76% DW caffeine content (Fig 3.5).

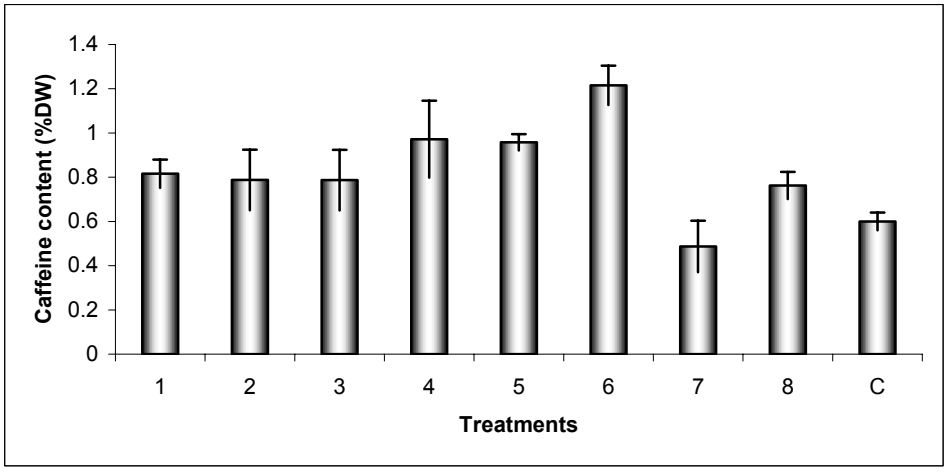


Fig 3.5 Influence of putrescine, silver nitrate and polyamine biosynthetic pathway inhibitors on caffeine content in *Coffea canephora* callus cultures.

Bars: 1-Put 50mM, 2-Spm 50mM, 3-Spd 50mM, 4-DFMA 1mM, 5-DFMO 1mM, 6-DFMA+DFMO 1mM each, 7-$AgNO_3$ 50µM, 8-DFMA 1mM +DFMO 1mM + $AgNO_3$ 50µM, c-Control.

3.2.4. Discussion

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 in many plant species (Thomas, 1993). Endogenous and exogenously administered hormones play a crucial role in somatic embryogenesis. Polyamine pools and ethylene pathway are interlinked and known to redirect the cell towards embryogenesis when present in optimum concentrations (Feirer *et al* 1984). Polyamines viz. putrescine, spermidine and spermine are known to play important role in various cellular process (Reviewed by Bais and Ravishankar 2002). Chemically they are strait chain, aliphatic amines. Polyamines are known to be involved in DNA replication, cell division, protein synthesis, responses to abiotic stress, rhizogenesis, flower development, *in vitro* flower induction (Reviewed by Bais and Ravishankar 2002). Polyamines are reported to inhibit ethylene biosynthesis in plants (Apelbaum *et al* 1981). Evidence from several studies have indicated that, polyamines play a crucial role in somatic embryogenesis and regeneration of several plant species (Reviewed by Bais and Ravishankar 2002, Kumar and Rajam 2004)

Putrescine is synthesized from ornithine as well as from arginine through ornithine decarboxylase and arginine decarboxylase respectively and is used as a precursor together with the aminopropyle moiety derived from *S* adenosyl-L-methionine (SAM) after decarboxylation, to synthesize spermidine and spermine (Evans and Malmberg 1989). Apart from this silver nitrate is known to regulate morphogenesis through various means. It is known to be a potent inhibitor of ethylene action (Beyer 1976) and known to increase polyamine pools.

The accumulation of spermine during embryogenesis may be essential for the shift from callus to embryogenesis as evident from the present study. A fine balance of different polyamines may be required for embryogenesis. This is evident from the experimental results that treatment with polyamine biosynthesis inhibitors resulted in drastic reduction in embryogenesis (Fig 3.2, 3.3). Similar response was observed in different plant systems (Reviewed by Bais and Ravishankar 2002). Polyamines are reported to promote shoot multiplication (Patil *et al* 1999, Bais *et al* 2001), *in vitro* flowering in *Cichorium intybus* (Bais *et al* 2000). In the present study, exogenous administration of putrescine increased embryogenesis, suggesting the promotive role of polyamines in embryogenesis in *Coffea canephora*. In an earlier study Beatriz and co workers (1994) studied the effect of exogenous administration of polyamines in the concentration range of 0, 50 and 100µM. The results indicated that there is significant difference in embryogenesis. However incorporation of 50µM spermidine with high dose of putrescine resulted in the promotion of embryogenesis in *C. canephora* genotype N-123 (Beatriz *et al* 1994). In preliminary experiments, it was found that low concentration of polyamines (50-100µM) does not elicit any morphogenetic response and this was supported by the earlier work (Beatriz *et al* 1994). It was found that higher concentrations of polyamines are required for embryogenesis in *Coffea canephora* and the promotive effect of polyamines were confirmed by incorporation of polyamine inhibitors in the culture medium (Fig 3.2, Fig 3.3).

Polyamines are known to elicit morphogenetic responses when incorporated in high concentration (Bais *et al* 2001). The results presented here indicate that incorporation of 50mM putrescine promotes embryogenesis (Fig 3.3) and 1mM each polyamine synthesis inhibitors significantly inhibits embryogenesis response (Fig 3.3). These results are in

agreement with earlier reports (Feirer *et al* 1984, Bais *et al* 2001) where polyamines play a crucial role in embryogenesis and plant morphogenesis.

Silver nitrate was found to promote embryogenesis in *Coffea canephora*. Therefore many experiments were conducted to understand the role of silver nitrate in embryogenesis. The results are presented in the subsequent sections dealing with influence of silver ions on primary and secondary embryogenesis. Ethylene is known to inhibit embryogenesis and morphogenesis in various plant systems. Silver ions are potent inhibitor of ethylene action (Beyer 1976) and have been found to enhance plant regeneration in different systems (Bias *et al* 2000, Hyde and Phillips 1996). Pua *et al* (1996) clearly described the synergistic effect of $AgNO_3$ and putrescine on shoot regeneration in Chinese radish. Polyamines also known to inhibit ethylene biosynthesis (Apelbaum *et al* 1981) and this may be one of the reasons for its ability to enhance somatic embryogenesis.

The results demonstrated that the caffeine content was increased in callus grown in medium containing polyamine synthesis inhibitors. Miyazaki and Yang (1987) reported the influence of putrescine and $AgNO_3$ on the competitive utilization of *S* adenosyl-L-methionine. Utilization of SAM by putrescence for its conversion to spermidine would possibly result in a lower availability of SAM for ethylene biosynthesis (Bias *et al* 2001). Reports on somatic embryogenesis in carrot (Roustan *et al* 1990) indicate that the potent ethylene action inhibitor $AgNO_3$ helps in increasing arginine decarboxylase (ADC) activity, which in turn increases the levels of endogenous polyamines in carrot embryogenic cultures. There is strong evidence that there is competition for SAM between the biosynthetic pathways of polyamines and ethylene (Miyazaki and Yang 1987). The formation of caffeine is closely associated with SAM because the three-methylation steps in the caffeine biosynthetic pathway use SAM as methyl donor (Reviewed by Ashihara and Suzuki 2004). The increase in caffeine levels in embryos cultured on polyamine synthesis inhibitors may be due to more availability of *S* adenosyl-L-methionine for caffeine biosynthesis. These results also indicate that SAM may be playing a crucial role in regulating caffeine biosynthesis in coffee beans and down regulation of SAM may lead to low caffeine coffee plants. The possible pathways were depicted in Figure 3.6.

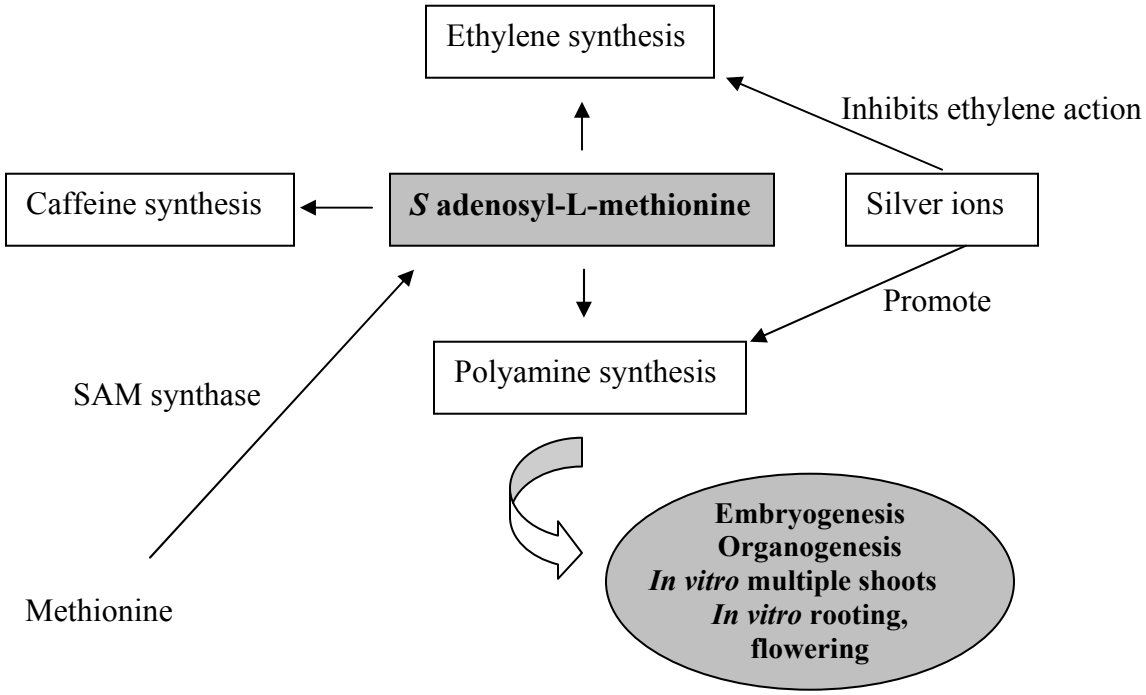


Figure 3.6: Interplay of polyamine and caffeine biosynthesis pathway and its modulation by *S*-adenosyl L methionine.

3.2.5. Summary

- Higher concentration of polyamines are required for embryogenesis in *Coffea canephora* and the promotive effect of polyamines were confirmed by incorporation of polyamine inhibitors in the culture medium.
- Caffeine accumulation in *Coffea* callus cultures found to be altered by the regulation of polyamine biosynthesis pathway.

3.3. TDZ INDUCED DIRECT EMBRYOGENESIS IN *C. ARABICA* AND *C. CANEPHORA*

Background :

The origin of somatic embryos in *Coffea* may be either direct from explants (Yasuda *et al* 1985, Hatanaka *et al* 1991, 1995) or indirect via embryogenic callus formation (Sondahl and Sharp 1977, Dublin 1981, Van Boxtel and Berthouly 1996, Pierson *et al* 1983, Nishibata *et al* 1995, Sandra *et al* 2000). In spite of many reports on somatic embryogenesis of *Coffea*, the induction of somatic embryogenesis and plantlet formation are very sensitive to culture conditions such as nutrient and hormonal composition of the medium (Staristsky and Van Hassel 1980). 1-phenyl-3-(1,2,3- thiadiazol-5-yl) urea (thidiazuron - TDZ) has received considerable attention as a potent regulator of *in vitro* propagation system and as an effective stimulus for the development of adventitious shoots and somatic embryos in a wide variety of plants (Huetteman and Preece 1993, Lu 1993).

In the present section, results on the effect of TDZ on somatic embryogenesis of *Coffea* sp are presented.

3.3.1. Direct embryogenesis under the influence of TDZ

The media used for induction of direct embryos were presented in (p.55, section 2.3) *C. arabica and C. canephora* both produced direct embryos from leaf and hypocotyl explants (Fig 3.7). *C. arabica* showed direct somatic embryogenesis in 70% *in vitro* leaf explants (161.6± 2.5 embryos) (Tab 3.5 Fig 3.7a). In *C. canephora* cv S-274, 102.8 ± 6.8 and 16.5 ± 1.5 embryos were produced respectively from in *in vitro* stalk of regenerated plants (Tab 3.6, Fig. 3.7d) and cotyledonary leaf cultured on medium A (Tab 3.7 Fig 3.7c). *In vitro* leaf explants cultured on medium A containing 9.08 µM TDZ showed increased direct somatic embryogenesis when compared to *in vivo* leaf explants (Tab 3.5, 3.7). Direct somatic embryos (180.1 ± 3.0) were produced from *in vitro* leaf explants of *C. canephora* cv S-274 on MS with 2% sucrose and TDZ 9.08 µM (Tab 3.5, Fig 3.7b). In *C. canephora* cv S-274, only 22% *in vivo* cotyledonary leaf explants produced embryos (16.5 ± 1.51) on medium A (Tab. 3.7). The higher sucrose levels in medium B (with 3% sucrose) reduced embryogenesis in *C. canephora in vitro* leaf and stalk explants (Tab.

3.5, 3.6 and 3.8). Almost all of *C. canephora* cv S-274 and 64% of *C. arabica in vitro* leaf explants produced light yellow friable callus and greenish white non friable callus respectively on medium C. In *C. canephora* cv S-274 *in vitro* stalk explant and cotyledonary leaf explants produced light yellow friable callus which becomes embryogenic after culturing on Heller's (Heller 1953) medium for 3 months in dark. The callus obtained from *in vivo* coteledonary leaf explants of *C. arabica* was white non embryogenic. In both *C. arabica* and *C. canephora* cv S-274 the ability to form embryos directly from hypocotyls explants was almost similar (Tab. 3.8). *C. canephora* Cv. CXR explants showed good response for direct somatic embryogenesis (Tab 3.9) on MS medium supplemented with B5 vitamins and 9.08 µM TDZ (Fig. 3.7c). A maximum of 76.4 ±2.8 embryos and 91.6 ± 12.3 embryos were produced from *in vitro* leaf and cotyledonary leaf explants (Tab. 3.9). But some of the cotyledonary leaf explants showed greenish yellow color callus from margins.

3.3.2. Plant regeneration from somatic embryos

Greenish tubular stage embryos (Fig 3.7e) were physically removed from the explants and placed on embryo development medium resulted in individual plantlets (80-85%) (Fig. 3.7f). They produced shoots after 30–45 days. All the regenerated plantlets appeared to be morphologically normal. The regenerated plantlets were rooted on half strength MS basal medium within 45 days (Fig. 3.7f).

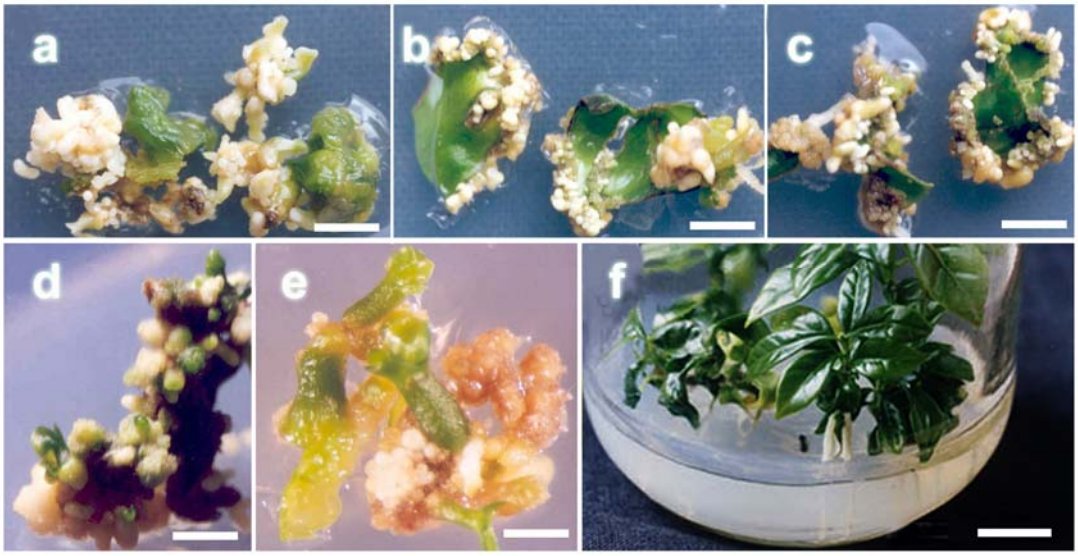


Fig 3.7: Direct somatic embryogenesis and plant regeneration from *Coffea* sp.

Fig 3.7a Direct somatic embryogenesis from leaf explants of *C . arabica* on medium a comprising MS basal medium supplemented with 2% Sucrose and TDZ 9.08 μM (bar 10mm)

Fig 3.7b Direct somatic embryogenesis from leaf explants of *C .canephora* cv S-274 on MS with 2% sucrose and TDZ 9.08 μM (bar 10 mm)

Fig 3.7c Direct somatic embryogenesis from cotyledonary leaf explant of *C. canephora* cv. CXR on MS medium containing B5 vitamins , 2% sucrose and supplemented with TDZ 9.08 μM (bar 10 mm)

Fig 3.7d Direct somatic embryogenesis from *in vitro* stalk segments of *C. canephora* cv S-274 on MS medium containing 2% sucrose and TDZ 9.08 μM (bar 4 mm)

Fig 3.7e Development of somatic embryos originated from leaf explants of *C. canephora* cv S-274 into plantlets. (bar 4 mm)

Fig 3.7f Regenerated plantlet of *C. canephora* (bar 10 mm)

Table. 3.5 Effect of TDZ on *Coffea* sp somatic embryogenesis from *in vitro* leaf explants

Medium[#]	Supplements			No. of embryos per explant	
	Sucrose % (w/v)	TDZ (µM)	CW (v/v)	*C. arabica*	*C. canephora* cv S-274
A	2	9.08	0	161.6 ± 2.5	180.1 ± 3.0
B	3	9.08	0	24.3 ± 1.6	36.0 ± 0.6
C	2	9.08	10	13.3 ± 0.5	0
D	2	0	10	0	0
E	2	-	-	0	0

Incubation= 45 days; [#] = MS medium, E= control. CW=coconut water.

Medium A- MS basal medium, 2% sucrose (w/v) supplemented with 9.08 µM TDZ,

Medium B- MS basal + 3% sucrose (w/v) + 9.08 µM TDZ,

Medium C- MS basal + 10% (v/v) coconut water (CW) + 2% (w/v) sucrose + 9.08 µM TDZ

Medium D- 2% (w/v) sucrose + 10% (v/v) CW.

Medium E – MS basal and 2% sucrose (Control)

Table. 3.6 Effect of TDZ on *Coffea* sp somatic embryogenesis from *in vitro* stalk explants of regenerated plants

Medium[#]	Supplements			No. of embryos per explant	
	Sucrose %(w/v)	TDZ (µM)	CW (v/v)	*C.arabica*	*C.canephora* cv S-274
A	2	9.08	0	39.9 ± 1.2	102.8 ± 6.8
B	3	9.08	0	14.5 ± 0.8	40.5 ± 1.6
C	2	9.08	10	0	0
D	2	0	10	0	0
E	2	-	-	0	0

Incubation= 45 days, [#] = MS medium and supplements as given in Table 3.5.

Table. 3.7 Effect of TDZ on *Coffea* sp somatic embryogenesis from *in vivo* cotyledonary leaf explants

Medium[#]	Supplements			No. of embryos per explant	
	Sucrose %(w/v)	TDZ (µM)	CW (v/v)	*C.arabica*	*C.canephora* cv S-274
A	2	9.08	0	12.6 ± 0.8	16.5 ± 1.5
B	3	9.08	0	12.0 ± 0.2	20.2 ± 0.6
C	2	9.08	10	0	0
D	2	0	10	0	0
E	2	-	-	0	0

Incubation= 45 days, [#] = MS medium and supplements as given in Table 3.5.

Table. 3.8 Effect of TDZ on *Coffea* sp somatic embryogenesis from *in vivo* hypocotyl explants

Medium[#]	Supplements			No. of embryos per explant	
	Sucrose %(w/v)	TDZ (µM)	CW (v/v)	*C.arabica*	*C.canephora* cv S-274
A	2	9.08	0	21.4 ± 0.5	36.5 ± 1.4
B	3	9.08	0	24.2 ± 0.2	26.0 ± 0.4
C	2	9.08	10	0	0
D	2	0	10	0	0
E	2	-	-	0	0

Incubation= 45 days, [#] = MS medium and supplements as given in Table 3.5.

Table. 3.9 Effect of TDZ on somatic embryogenesis of different explants of *Coffea canephora* cv. CXR

Medium with supplements			No. of embryos per explant		
Salts	TDZ (µM)	CW (v/v)	Cotyledon	hypocotyl	*In vitro* leaf
MS	9.08	0	91.6 ± 12.3	27.9 ± 0.7	76.4 ± 2.8
½ MS	9.08	0	88.4 ± 13.2	42.5 ± 0.8	60.0 ± 4.2
MS	9.08	10	26.5 ± 1.0	33.5 ± 0.9	44.2 ± 0.8
½ MS	0	10	-	0	0
MS	-	-	-	0	0

Incubation period = 45 days

3.3.3. Discussion

In general TDZ at very low levels induces morphogenetic responses and direct somatic embryogenesis was evident in geranium (Qureshi and Saxena 1992) alfalfa (Iantcheva *et al* 1999), water melon (Compton and Gray 1992) and muskmelon (Gray *et al* 1992). Both *C. arabica* as well as *C. canephora* responded for direct embryogenesis on medium supplemented with TDZ. It has been reported that the induction of somatic embryogenesis is commonly associated with modulations of auxins by TDZ (Capelle *et al* 1983, Visser *et al* 1992, Hutchinson *et al* 1996).

Various investigators have suggested the process of high frequency somatic embryogenesis in both *C. arabica* and *C. canephora* (Hatanaka *et al* 1991, 1995, Etienne-Barry *et al* 1999, Sandra *et al* 2000). In all these cases, callus intermediates were involved and take long duration for the embryo induction. Callus derived somatic embryos are known to exhibit somaclonal variations in regenerated lines.

Summary

- Direct somatic embryogenesis was successfully achieved for the first time using TDZ.
- Healthy regenerates were obtained using TDZ as a sole growth hormone.
- This study was highly useful in further work on genetic transformation studies of *Coffea canephora*.

3.4. SOMATIC EMBRYOGENESIS UNDER THE INFLUENCE OF SILVER NITRATE

Background and brief objective:

In recent years basic studies on ethylene regulation opened new vistas for applied research in the area of micro-propagation, somatic embryogenesis, *in vitro* flowering, growth promotion, fruit ripening, sex expression etc. Silver nitrate has proved to be a very potent inhibitor of ethylene action and has been used in plant tissue culture. Few properties such as low cost of silver nitrate, easy availability, solubility in water, specificity, stability and non-toxicity to plants make it very useful for various applications in exploiting plant growth regulation and morphogenesis *in vivo* and *in vitro*. Silver ion mediated responses seems to involve polyamines, ethylene and calcium mediated pathways and plays a crucial role in regulating physiological process including morphogenesis. The molecular basis for regulation of morphogenesis under the influence of silver nitrate is completely lacking. Silver ions are capable of generating ethylene insensitivity in plants (Zhao *et al* 2002).

In this section the results of experiments on somatic embryogenesis under the influence of silver nitrate is presented.

3.4.1. Influence of silver nitrate on somatic embryogenesis

Silver nitrate is well known for its ability to promote growth in *in vitro* plant cultures. The study was focused on the effect of silver nitrate in coffee *in vitro* cultures of *Coffea*. In *C. canephora and C. arabica* the leaf and hypocotyl explants gave rise to embryos after 3 weeks of culture on medium (p.56, section 2.4) containing 40-70µM silver nitrate. *C. arabica* and *C. canephora* 68.7 ± 3.3 embryos (Fig 3.8a, Table 3.10) and 144.1 ± 7.3 embryos (Fig. 3.8b, Table. 3.10) from *in vitro* stalk explants. *In vitro* leaf explants of *C. arabica* and *C. canephora* did not respond well for embryogenesis under silver nitrate treatment (Table 3.10). However profuse callus induction was observed from *in vitro* leaf explants.

3.4.2. Influence of silver nitrate on secondary somatic embryogenesis

Secondary embryogenesis was observed in coffee embryos cultured on silver nitrate supplemented medium. Small globular green embryos formed secondary embryos on medium containing 40-70μM $AgNO_3$ in both *C. arabica* and *C. canephora* (Table 3.11). Around thirty percent of the torpedo stage embryos responded for secondary embryogenesis (Table 11).

A maximum of 7.7±1.6 *C. canephora* secondary embryos were produced per clump of primary embryos inoculated on to the medium containing 40μM $AgNO_3$. At higher concentration of $AgNO_3$, the tubular embryos developed into plantlets without producing any new embryos.

When matured embryos were used as explants for further multiplication, most of them directly developed into plantlets at lower concentration of silver nitrate (10μM) or in the absence of silver nitrate. In *C. canephora* only higher silver nitrate concentration (60 μM) supported plantlets formation from tubular primary embryos.

In both *C. arabica and C. canephora* 60-100% explants produced direct somatic embryos when *in vitro* stalk of the regenerated plantlets are used as explants. Direct somatic embryogenesis was observed from hypocotyl explants of regenerated plantlet of *C. canephora* on medium containing 40μM silver nitrate (Fig.3.8c). Similarly direct somatic embryogenesis from hypocotyls segments were observed from regenerated plantlets of *C. arabica* on medium supplemented with 40μM silver nitrate (Fig. 3.8d). Direct somatic embryogenesis and yellow friable callus formation was noticed from *in vitro* leaf explants of *C. canephora* on medium containing 70μM silver nitrate (Fig. 3.8e). Direct somatic embryogenesis and yellow friable callus formation was also observed from *in vitro* leaf explants of *C. arabica* on medium containing 70μM silver nitrate (Fig. 3.8f). Almost 100% of *C. arabica* hypoctoyl explants responded for direct somatic embryogenesis on medium containing 20-70μM $AgNO_3$ but 40μM $AgNO_3$ concentration induced higher rate of embryo multiplication (68.76± 3.3) (Fig. 3.8d). The *in vitro* leaf explants did not responded well for direct somatic embryogenesis (Table. 3.11). When 70μM AgNO3 was used in the medium direct somatic embryogenesis was noticed in 24% of *C. canephora* explant (Fig.3.8e). Only 12% explants responded in case of *C. arabica* at 70μM AgNO3. Only 2-3 embryos per leaf explant were produced on medium containing 40μM $AgNO_3$

in *C. arabica* (Fig. 3.8f). The yellow friable callus that was obtained from peripheral portions of leaf explants was embryogenic in nature upon further culturing in embryo induction medium. All the embryos were of matured type and regenerated into plantlets specifically on embryo development media (Fig. 3.8g).

Table. 3.10 Effect of silver nitrate on somatic embryos production in cultured explants of *Coffea* sp.

Genotype and type of explant	$AgNO_3$ conc. (µM)	Explants producing secondary embryos (%)	Average number of embryos per explant	Explants producing callus (%)
C. canephora cv. 274				
In vitro stalk	0.0	-	-	-
	10.0	60.0	19.6 ± 2.8	-
	20.0	80.0	25.6 ± 2.6	-
	40.0	100.0	144.1 ± 7.3	-
	60.0	100.0	47.1 ± 5.3	-
	70.0	68.0	27.2 ± 3.4	
In vitro leaf	0.0	-	-	-
	10.0	-	-	32.0 [a]
	20.0	-		40.0 [a]
	40.0	20.0	2.6 ± 0.8	40.0 [a]
	60.0	20.0	4.4 ± 0.5	60.0 [a]
	70.0	24.0	7.0 ± 0.8	60.0 [b]
C. arabica cv Hemavathy				
In vitro stalk	0.0	-	-	-
	10.0	40.0	4.0 ± 1.1	-
	20.0	100.0	13.7 ± 1.9	-
	40.0	100.0	68.7 ± 3.3	-
	60.0	100.0	10.2 ± 1.7	-
	70.0	100.0	3.6 ± 1.2	-
In vitro leaf	0.0	-	-	-
	10.0	-	-	72.0 [a]
	20.0	-	1.5 ± 0.5	80.0 [a]
	40.0	12.0	2.0 ± 0.0	84.0 [a]
	60.0	-	-	80.0 [b]
	70.0	-	-	72.0 [b]

Number of explants in each experiment= 25, incubated for 45 days in dark

[a] yellow friable callus (embryogenic), [b] non embryogenic callus

Table. 3.11 Effect of silver nitrate on secondary embryogenesis of *Coffea* embryos

Genotype and nature of explant	$AgNO_3$ conc. (μM)	Embryo clumps responded (%)	Average no. of secondary embryos per primary embryo inoculum
C. canephora cv. 274			
Globular stage embryos	0.0	-	-
	10.0	100.0	-
	20.0	100.0	-
	40.0	63.3	5.0 ± 1.0
	60.0	20.0 [c]	28.2 ± 5.3
	70.0	-	8.0 ± 1.0
Torpedo stage embryos	0.0	-	-
	10.0	73.3	-
	20.0	30.0	2.5 ± 0.8
	40.0	33.3	7.7 ± 1.6
	60.0	100.0 [c]	-
	70.0	60.0 [c]	-
C. arabica cv Hemavathy			
Globular stage embryos	0.0	-	-
	10.0	73.3	-
	20.0	83.3	6.5 ± 1.8
	40.0	46.6	40.5 ± 8.2
	60.0	100.0 [c]	5.7 ± 1.1
	70.0	83.3 [c]	7.5 ± 2.5
Torpedo stage embryos	0.0	-	-
	10.0	6.6 [c]	-
	20.0	33.3 [c]	-
	40.0	20.0 [c]	-
	60.0	-	-
	70.0	-	-

[c]- callus induction

No. of explants used in each experiment=30, Incubation in dark for 45 days.

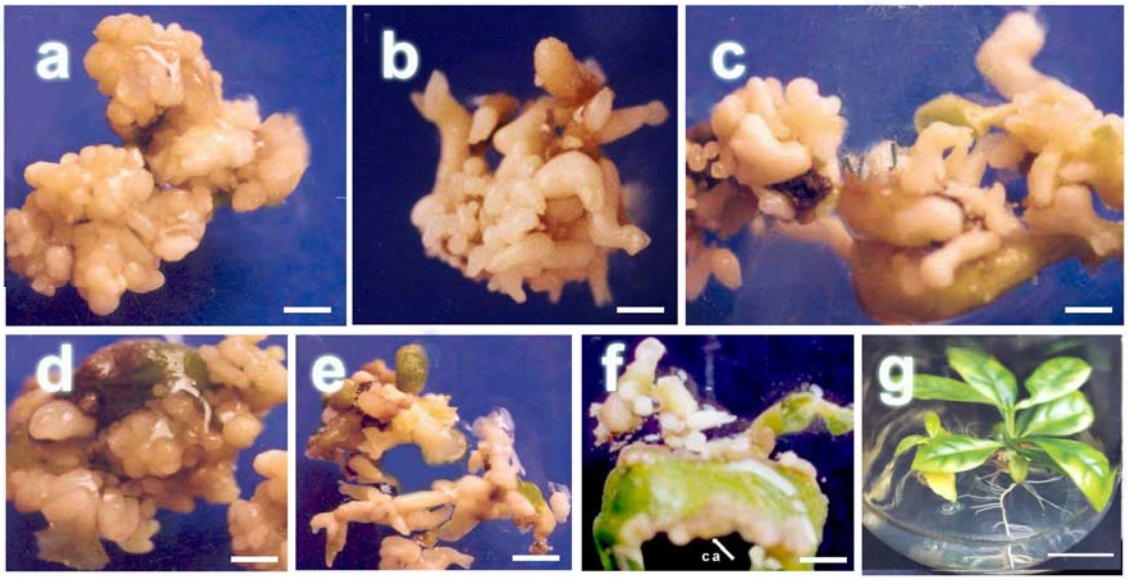


Fig 3.8. Embryogenesis under the influence of silver nitrate

Fig.6a Development of secondary embryos from small green globular embryos of *C. arabica* on medium containing 10 µM silver nitrate (bar = 10 mm)

Fig.6b Development of secondary embryos from small green globular embryos of *C. canephora* on medium containing 60 µM silver nitrate (bar = 10 mm)

Fig.6c Direct somatic embryogenesis from hypocotyl explants of regenerated plantlet of *C. canephora* on medium containing 40 µM silver nitrate (bar = 10 mm)

Fig.6d Direct somatic embryogenesis from hypocotyls segments of regenerated plantlets of *C. arabica* on medium supplemented with 40 µM silver nitrate (bar = 10 mm)

Fig.6e Direct somatic embryogenesis and yellow friable callus formation from *in vitro* leaf explants of *C. canephora* on medium containing 70µM silver nitrate (bar = 10 mm)

Fig.6f Direct somatic embryogenesis and yellow friable callus formation from *in vitro* leaf explants of *C. arabica* on medium containing 70µM silver nitrate (bar = 10 mm)

Fig.6g Regenerated plantlets of *C. arabica* (bar = 30 mm)

3.4.3. Discussion

In general the combination of auxin and cytokinin in culture media was adopted to obtain moderate to high proliferation of embryos not only from leaf explants and hypocotyl explants. The overall response entirely depends on the nature of explants, and also the genotype.

There are no clear reports on how silver nitrate affects coffee somatic embryogenesis. Strong evidence indicates that, silver nitrate is an inhibitor of ethylene action (Beyer

1976) and also influences endogenous ABA levels (Kong and Yeung 1995). Silver ions are capable of generating ethylene insensitivity in plants (Zhao *et al* 2002). Ethylene insensitive mutations (Hall *et al* 1999) and silver ions are thought to perturb the ethylene binding sites (Rodriguez *et al* 1999). The ethylene receptor ETR1 contains one ethylene-binding site per homodimer within ethylene binding mediated by a single copper ion (Cu) present in the ethylene-binding site. The replacement of copper cofactor by silver also serves to lock the receptor into a conformation such that it continuously represses ethylene responses (Zhao *et al* 2002). However there are no further reports on the molecular mechanisms on silver ion mediated ethylene action inhibition in plants.

The positive effect of silver nitrate on somatic embryogenesis in the present study is further supported by similar studies in other systems like *Hordeum vulgare* (Cho and Kasha 1989), and carrot (Roustan *et al* 1990). Various reports are available on indirect method of somatic embryogenesis from leaf based callus in both *C. canephora* (Van Boxtel and Berthouly 1996), and *C. arabica* (Nishibata *et al* 1995, Yasuda *et al* 1995) wherein, low to high frequency somatic embryogenesis could be possible but it requires 6-15 months period by using either liquid media alone or agar media. Very few reports are available on direct somatic embryogenesis from leaf explants of both *C.arabica* (Yasuda *et al* 1995) and *C. canephora* (Hatanaka *et al* 1991, Yasuda *et al* 1995, Sandra *et al* 2000). According to Yasuda *et al* (1985, 1995) 4-6 months time is required for somatic embryogenesis in *C. canephora*, similarly eight months time is required for direct somatic embryogenesis on improved medium as reported by Hatanaka *et al* (1991). By using silver nitrate in the culture medium it was possible produce somatic embryos in 2-3 months.

3.4.4. Summary

- Silver nitrate 40-60μM promotes somatic embryogenesis in coffee.
- Rapid embryogenesis in 2-3 months.
- The results indicate that regulation of ethylene perception pathways leads to enhancement in embryogenesis responses. The results may be useful in transformation of coffee.

3.5. INVOLVEMENT OF CALCIUM IN SOMATIC EMBRYOGENESIS

Background:

Embryogenesis is the starting point of the life cycle for both plants and animals. In plant cells cytosolic calcium (Ca^{2+}) is a key second messenger and demonstrated to respond to a number of stimuli. The amplitude, duration, frequency and location of calcium signal change according to the induced response (Poovaiah and Reddy, 1993). The environmental stimuli and signalling events that trigger and regulate plant embryogenesis are largely unknown. It is well known that auxins and cytokinins induce changes in cytosolic free calcium in plant cells, modifying Ca^{2+} fluxes through the plasma membrane (Felle, 1988). The activity of many protein kinases can respond to $[Ca^{2+}]_{cyt}$ signals directly (Poovaiah and Reddy, 1993). These can be categorized in four classes viz, Ca^{2+} dependent protein kinases (CDPKs), CDPK related proteins (CRKs), Calmodulin (CaM) dependent protein kinases (CaMKs) and chimeric Ca^{2+} and CaM-dependent protein kinases (CCaMKs) (White and Broadley, 2003). They are implicated in pollen development, control of cell cycle, phytohormone signal transduction, light regulating gene expression, gravitropism, thigmotropism, nodulation, embryogenesis, cold acclimation, salinity tolerance, drought tolerance and response to pathogens (White and Broadley, 2003). These observations give CDPKs credibility as key intermediates in Ca^{2+} mediated signaling in plants.

The objective of the present study was to elucidate the role of calcium in somatic embryogenesis and to identify CDPKs specific to embryogenesis.

3.5.1. Influence of calcium in regulating embryogenic callus formation

Callus initiation was observed from 70-80% explants in primary callus initiation medium supplemented with 0.5 mg l^{-1} 2,4-D, 0.5 mg l^{-1} IBA and 2 mg l^{-1} 2-iP (Fig 3.9a). Callus multiplication was achieved in CM medium supplemented with 1 mg l^{-1} 2,4-D and 4 mg l^{-1} BAP (Fig 3.9 b, c, d, e). Callus multiplication was carried out under different calcium levels in the culture medium. Further studies on embryogenesis suggested that, the callus were of two types with distinct morphological variations. The fast growing callus was non embryogenic and this kind of callus was produced in medium devoid of calcium and in medium comprising less than 3mM calcium in the medium. Yellow

powdery non-embryogenic callus was produced in medium containing 1mM calcium (Fig 3.9b). White spongy non-embryogenic callus was produced in medium devoid of calcium (Fig 3.9c). Brownish black spongy non-embryogenic callus was noticed in medium containing calcium channel blocker verapamil (Fig 3.9d). Only 12±3 explants responded for callus multiplication in calcium free medium. Prolonged sub-culturing of callus in the same medium revealed that all the callus clumps were non embryogenic (Table 3.12). Incorporation of 0.1 mM EGTA resulted in complete inhibition embryogenesis from embryogenic callus. Multiplication of callus under light (2000Lux) completely inhibited the formation of embryogenic callus as well as secondary embryos. Supplementation of low calcium (0.5mM and 1mM) also produced only non-embryogenic callus (Table 3.12). Incorporation of 3mM (CM-4 medium) and 5mM (CM-5 medium) calcium in the medium resulted in formation of 38±6 and 60±17 percent embryogenic callus (Table 3.12). Formation of globular embryos was observed after 60 days of culture in medium supplemented with 3-5 mM calcium.

3.5.2. Influence of calcium in regulating primary and secondary embryogenesis

Globular somatic embryos were produced by further culturing of embryogenic callus in the same medium (Fig 3.9e). Embryos were produced in medium supplemented with 5 mM calcium in 20% of the explants. Secondary embryogenesis did not require high level of calcium in the culture medium. Maximum response was observed for secondary embryogenesis at 1mM level of calcium (Fig 3.9f, Fig. 3.10). More than 80 % response for secondary embryogenesis was noticed under 1-5 mM exogenous calcium treatment (Fig 3.10). On an average 25-30 secondary somatic embryos were induced from each torpedo stage embryo (Fig. 3.11). Globular embryos did not respond well for secondary embryogenesis. Calcium chloride 50mM level did not support embryogenesis response. Tissue browning was observed in 15 days of culture and this may be due to the toxic effect of calcium at high concentration (50mM). The involvement of calcium channels in secondary somatic embryogenesis response was determined by exogenous addition of calcium channel blocker. Calcium channel blocker verapamil hydrochloride drastically reduced (Fig. 3.12) the primary embryogenesis as well as secondary embryogenesis

response. Almost 50% reduction in embryogenesis was observed in cultures subjected to 0.1 mM verapamil (Fig 3.12).

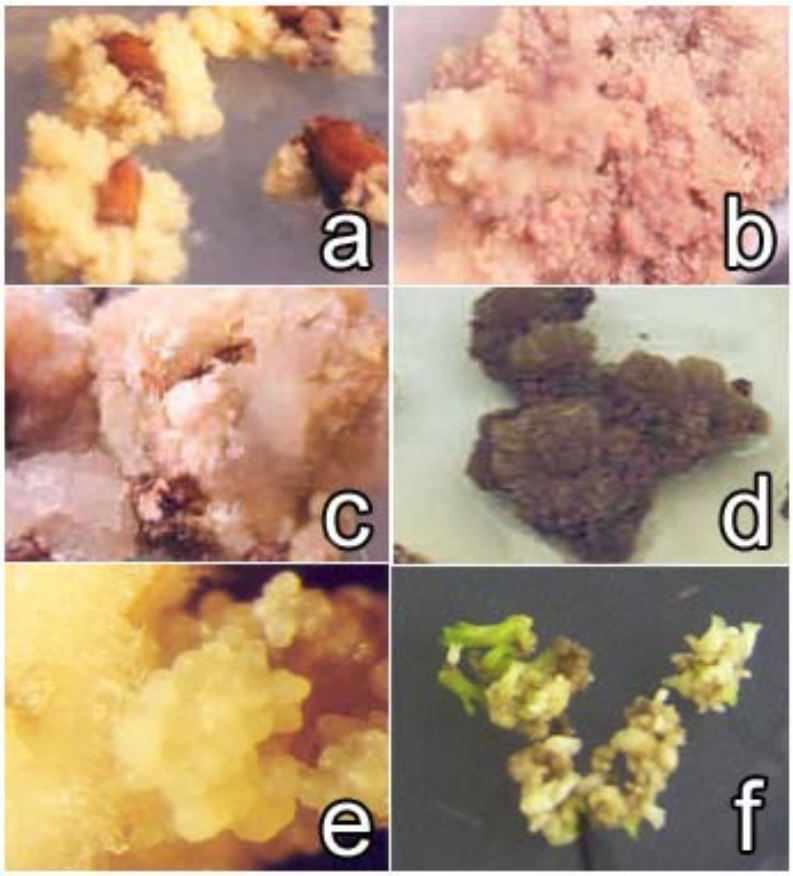


Fig 3.9. Influence of calcium on regulating embryogenesis

Fig 3.9a: Callus initiation from hypocotyl explants in medium (CI medium) supplemented with 0.5 mg l^{-1} 2,4-D, 0.5 mg l^{-1} IBA and 2 mg l^{-1} 2-iP.

Fig 3.9b: Yellow powdery non embryogenic callus in medium containing 1mM calcium.

Fig 3.9c: White spongy non embryogenic callus in medium devoid of calcium

Fig 3.9d: Brownish black spongy non-embryogenic callus was noticed in medium containing calcium channel blocker verapamil.

Fig 3.9e: Yellow friable embryogenic callus in medium containing 5mM calcium.

Fig 3.9f: Formation of secondary embryos under 1mM calcium.

Table 3.12 : Influence of calcium and EGTA on induction of embryogenic and non-embryogenic callus in *Coffea canephora*

*Medium	Calcium	Percentage explants responding for			
		Callus induction	Embryogenic callus	Non embryogenic callus	Callus with globular embryos
CM-1	0	12±3	-	12±3	-
CM-2	0.5	15±3	-	15±3	-
CM-3	1	17±5	-	17±5	-
CM-4	3	45±7	38±6	5±3	7±3
CM-5	5	87±10	60±17	10±3	20±5

*Calcium free medium containing MS salts, B5 vitamins + 1 mg l^{-1} 2,4-D and 4 mg l^{-1} BAP

The cultures were incubated for 45 days in dark

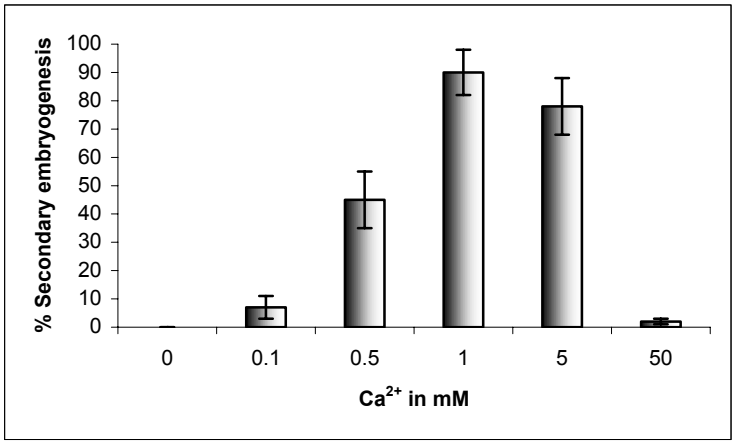


Fig 3.10 Effect of exogenous calcium level on secondary somatic embryogenesis from torpedo stage primary embryos.

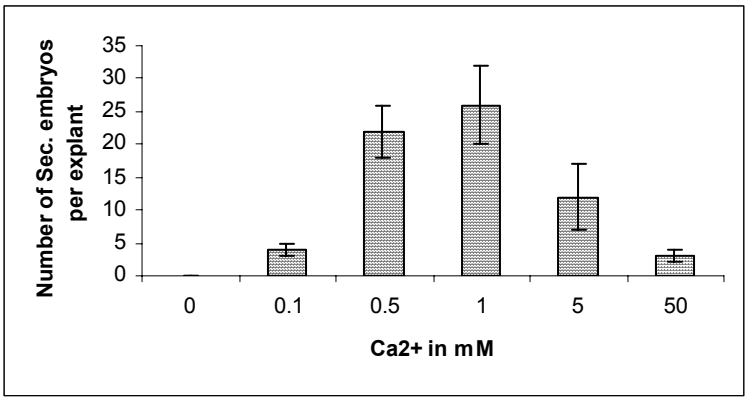


Fig 3.11 Effect of exogenous calcium level on the number of secondary somatic embryos from torpedo stage primary embryo.

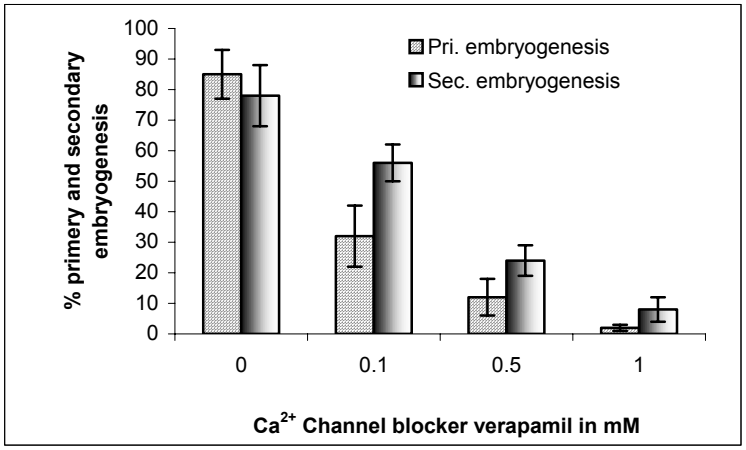


Fig 3.12 Effect of calcium channel blocker verapamil on primary and secondary somatic embryogenesis.

3.5.3. Identification of ccCDPK

With a view to elucidate involvement of calcium mediated signalling in embryogenesis, attempts were made to detect calcium dependent protein kinases in callus cultures. Immunodetection (p.58, section 2.5.1) studies revealed a 58kDa Ca^{2+}-dependent protein kinase (ccCDPK) isoform accumulation exclusively in embryogenic callus and was absent in non-embryogenic callus (Fig 3.13). This isoform was also present in somatic embryos and secondary somatic embryos but not present in leaf and embryogenic callus. Further characterization was carried out by autophosphorylation (p.59, section 2.5.7) and substrate phosphorylation (p.60, section 2.5.8) assays. Addition of micromolar levels of

calcium in the assay buffer resulted in autophosphorylation of 58kDa ccCDPK (Fig 3.14). Elimination of calcium in the assay mixture did not resulted in the phosphorylation of 58kDa protein. A 75 kDa autophosphorylated protein was observed in embryogenic callus (Fig 3.14). However the phosphorylation was not calcium dependent and this protein did not cross react with antiCDPK antibodies (Fig 3.13) and thus may not be one of the CDPK isoforms. Histone IIIS was used to detect substrate phosphorylation activity. The results revealed strong phosphorylation of histone IIIS in all the samples however, the phosphorylation was more intense in assay mixture containing calcium (Fig 3.15)

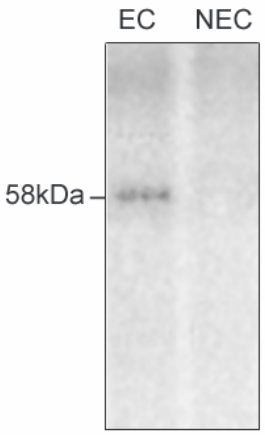


Fig 3.13: Western blot depicting the accumulation of 58kDa ccCDPK exclusively in embryogenic callus. (EC- embryogenic callus, NEC-non embryogenic callus)

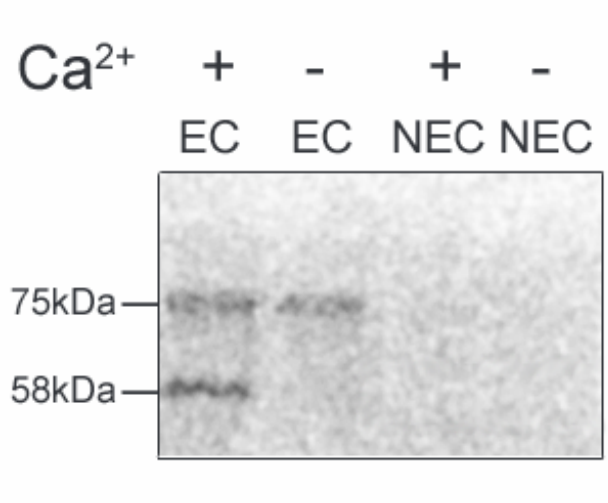


Fig 3.14. The 58 kDa ccCDPK shows calcium dependent autophosphorylation activity in embryogenic callus.

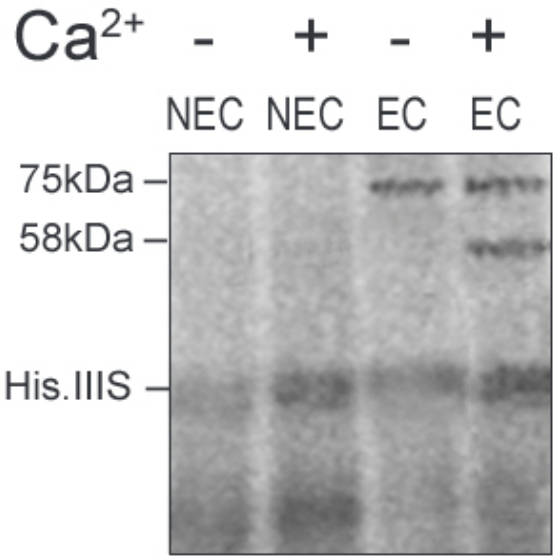


Fig 3.15 Calcium dependent histone IIIS phosphorylation activity in non-embryogenic and embryogenic callus.

3.5.4. Immunolocalization of ccCDPK

Somatic embryos were produced from callus (Fig 3.16a) and primary embryos (Fig 3.16b). Longitudinal and transverse sections of somatic embryos of coffee showed strong immunofluoroscent localization of ccCDPK in their cytoplasm. ccCDPK was found to be located in vascular regions and uniformly distributed in parenchyma cells of somatic embryos (Fig 3.17a,b). Negative controls wherein the sections were incubated with normal rabbit sera did not show fluorescence.

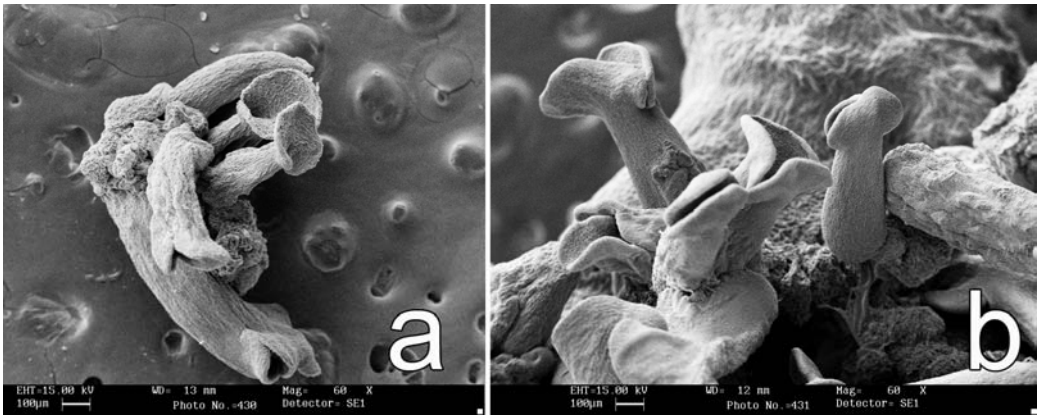


Fig:3.16 a. Scanning electron micrographs of embryos arising from callus mass. b. Secondary somatic embryos from a tubular primary embryo

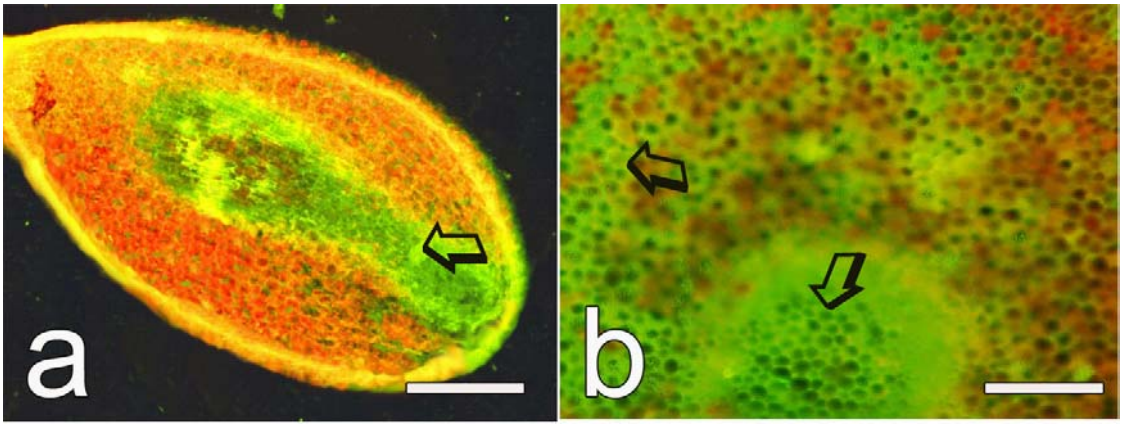


Fig 3.17 Immunolocalization of ccCDPKs in somatic embryos.

3.17a. Longitudinal section depicting the accumulation of ccCDPKs in the central vascular region and evenly distributed in the parenchyma cells (bar 0.5mm). 3.17b. Transverse section depicting the accumulation of ccCDPK (bar 200µm).

3.5.5. Discussion

In plants, embryogenesis is not strictly dependent on fertilization, because many species naturally produce asexually derived embryos in the seed (apomixis) or can be induced to form somatic embryos in tissue culture and apomictic development is characterized by the avoidance of both meiosis and egg cell fertilization to produce an embryo that is genetically identical to the maternal parent (Komamine *et al* 1992). Asexually derived embryos also can be induced to form *in vitro* from a wide range of somatic and gametophytic donor tissues (Komamine *et al* 1992). The combination of donor tissue and induction treatment by growth regulators or hormones determines whether the embryos develop directly from single cells or indirectly through an intermediary callus phase (Komamine *et al* 1992). The induction of *in vitro* embryogenesis from differentiated tissues probably arises from a transient spatio-temporal reprogramming of regulatory genes that control zygotic/somatic embryo development.

In plant cells no second messengers has been demonstrated to respond to more stimuli than cytosolic calcium (Ca^{2+}). The amplitude, duration, frequency and location of calcium signal change according to the induced response (Poovaiah and Reddy 1993). The environmental stimuli and signalling events that trigger and regulate plant embryogenesis are largely unknown. It is well known that auxins and cytokinins induce

changes in cytosolic free calcium in plant cells, modifying Ca^{2+} fluxes through the plasma membrane (Felle 1988).

The activity of many protein kinases can respond to $[Ca^{2+}]_{cyt}$ signals directly. These can be categorized in four classes viz, Ca^{2+} dependent protein kinases (CDPKs), CDPK related proteins (CRKs), Calmodulin (CaM) dependent protein kinases (CaMKs) and chimeric Ca^{2+} and CaM-dependent protein kinases (CCaMKs) (Reviewed by White and Broadley 2003). CDPKs have been mainly identified in plants as a critical Ser/Thr protein kinase family and Harmon *et al* (1994) first characterized a family of Ca^{2+} - dependent protein kinases (CDPKs) from soybean. The CDPKs are capable of converting $[Ca^{2+}]_{cyt}$ signals into biochemical and genetic consequences through the phosphorylation of diverse target proteins. They are implicated in pollen development, control of cell cycle, phytohormone signal transduction, light regulating gene expression, gravitropism, thigmotropism, nodulation, embryogenesis, cold acclimation, salinity tolerance, drought tolerance and response to pathogens (Reviewed by White and Broadley 2003). These observations give CDPKs credibility as key intermediates in Ca^{2+} mediated signaling in plants.

CDPKs possess a characteristic structure in which an *N*-terminal serine/ threonine protein kinase domain is fused to a carboxyterminal calmodulin-like domain containing EF-hand calcium-binding sites (Cheng *et al* 2002, Harmon *et al* 2001). Therefore, CDPKs do not depend on the interaction with exogenous calmodulin but can be activated directly by Ca^{2+} binding. A junction domain between the kinase and calmodulin-like domain functions as a pseudo-substrate autoinhibitor that inhibits phosphorylation in the absence of Ca^{2+} and keeps the CDPK in a state of low activity (Harmon *et al* 1994). Activated CDPKs alter protein phosphorylation or relative gene expression through transduction of calcium signals (Cheng *et al* 2002). Consequently, the biological properties and locations of different CDPKs are proposed to determine their precise cellular activities during specific plant growth and development stages. However, a number of CDPK gene family members remain to be identified and their precise physiological roles are still largely to be elucidated.

Plant CDPK family is represented by many genes, with 12 subfamilies comprising 34 isoforms in *Arabidopsis* (Hrabak *et al* 2003). To understand precise roles for CDPKs in

signal transduction pathways associated with growth and development or morphogenesis, it is necessary to clarify its kinase characteristics and sub-cellular localizations. In the present study a strong correlation between exogenous incorporation of Ca^{2+} and somatic/secondary embryogenesis is established. We have also identified CDPK isoforms which, appear in embryogenic callus, primary and secondary somatic embryogenesis with autophosphorylation and substrate phosphorylation activities.

The present study indicates that coffee somatic embryogenesis and secondary embryogenesis is highly influenced by the calcium concentrations in the culture medium. Work on the carrot *in vitro* system has shown that Ca^{2+} enhances embryogenic frequency (Jansen *et al* 1990) and its deprivation arrests somatic embryo formation (Overvoorde and Grimes, 1994).

Increasing evidence suggests that changes in cytosolic Ca^{2+} levels and phosphorylation play important roles in the regulation of embryogenesis. However, other class of protein kinases responsible for Ca^{2+} signaling during secondary embryogenesis need to be identified. Anil *et al* (2000) reported the accumulation of swCDPK isoforms during embryogenesis and regulated spatio-temporally during developmental stages in *Santalum album*.

Expression of some CDPKs are induced by physical stress, salt stress, by $CaCl_2$ in mung bean (Botella *et al* 1996), by osmotic stress in sorghum (Pestenacz and Erdei, 1996), and by phytohormones, methyl jasmonate, wounding, fungal elicitors, chitosan, and NaCl in tobacco leaves (Yoon *et al* 1999). Calcium dependent autophosphorylation and substrate phosphorylation activities were observed for 58kDa ccCDPK. Similar observation has been made with native CDPK (Anil *et al* 2000) and recombinant CDPK (Lee *et al* 1998).

The presence of light during callus induction phase completely inhibits the formation of embryogenic callus and secondary embryos in coffee. Absolute dark conditions are required for embryogenesis in *Coffea canephora*. Immunodetection studies revealed that the 58kDa ccCDPK was not observed in callus grown under 2000Lux light intensity. In nature the zygotic embryo formation takes place in dark and similar phenomenon may be operating in *in vitro* systems too. This phenomenon may be due to the fact that several CDPK mRNAs are down-regulated by light, including *Curcubita pepo* CpCPK1 (Ellard-Ivey *et al* 1999), *Oryza sativa* OsCPK2 (Breviario *et al* 1995), *Zea mays* ZmCPK7 and

ZmCPK9 (Saijo *et al* 1997). However we cannot rule out the fact that several other genes responsible for regulation of morphogenesis also may be activated in the absence of light. The role of autophosphorylation in regulating CDPK activity remains unclear. autophosphorylation of groundnut CDPK was suggested to be a prerequisite for its activity (Chaudhuri *et al* 1999) The presented results do not demonstrate the significance of autophosphorylation however, the 58kDa ccCDPK is capable of phosphorylating other proteins in a calcium dependent manner which would lead to activation of morphogenetic responses and may direct the cellular machinery towards embryogenesis.

3.5.6. Summary

- Optimum concentration of calcium is required for somatic embryogenesis.
- A 58kDa ccCDPK is found only in embryogenic callus and shows autophosphorylation activity in calcium dependent manner.
- The results may be useful in unraveling the mechanisms involved in calcium-mediated activation of signaling pathways leading to morphogenetic responses.

3.6. OPTIMIZATION OF *A. RHIZOGENES* MEDIATED GENETIC TRANSFORMATION IN *NICOTIANA TABACUM*: ASSESSMENT OF PHYSICAL AND CHEMICAL TREATMENTS.

Background:

Various species of bacteria are capable of transferring genes to higher plant species (Broothearts *et al* 2005). Among them, most widely studied ones are *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. *A. rhizogenes* are also capable of transferring the T-DNA of binary vectors in trans, thereby facilitating the selection of transgenic plants from screened hairy roots (Reviewed by Christey, 2001). A number of factors in *Agrobacterium* mediated transformation process can limit transformation of a particular plant. These include the genotype, wounding of plant tissue, synthesis of phenolic *vir* gene inducers by the plant, bacterial attachment, T-DNA transfer into the plant cytoplasm, T-DNA nuclear translocation and T-DNA integration (Reviewed by Gelvin, 2000). In order to enhance transformation rates, improvements have been made in various steps involved in genetic transformation. Till date, there are no reports available on influence of physical and chemical treatments on enhancement of transformation rates in *A. rhizogenes* mediated transformation. As a prelude to *Coffea* transformation experiments, the parameters were optimized using a model system *Nicotiana tabacum*, which is a very rapid system for transformation experiments.

In this section the results of experiments on the influence of physical and chemical factors such as ultrasonication, cell wall degrading enzyme treatment calcium ions and acetosyringone on transformation efficiency in *Nicotiana tabacum* as a model system is presented.

3.6.1. Optimization of transformation

Hairy roots were formed only from wounded regions. Each type of infection and wounding method (p.60, section 2.6.2 to 2.6.7) showed unique pattern of hairy root induction with varying percentage of transformation frequency (Fig 3.18). Infection of leaf explants by manual wounding resulted in induction of hairy roots originating from the mid vein region (Fig 3.18a). However, sonication treatment alone and with

acetosyringone and calcium ion treatments resulted in induction of hairy roots from all over the surface of the leaf explants (Fig 3.18b,c,d,e,f)

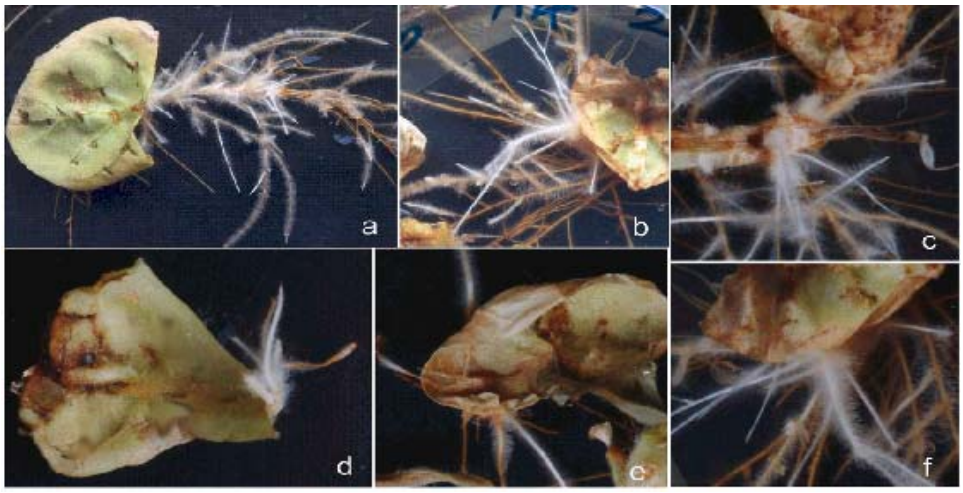


Figure 3.18. Induction of genetically modified hairy roots under different treatments.

a – Induction of hairy roots by manual wounding, b – Induction of hairy roots by ultrasonication, c –Induction of hairy roots by ultrasonication and 100µM acetosyringone treatments, d – Induction of hairy roots by ultrasonication and 0.1% cellulase treatments, e– Induction of hairy roots by ultrasonication and 0.1% each cellulase and pectinase treatments, f– Induction of hairy roots by ultrasonication and 10 mM calcium chloride treatments.

Persisting *A. rhizogenes* contamination was eliminated by frequent subcultures on medium containing antibiotics (p.63, section 2.6.8). Bacteria free root tips were cultured on liquid medium. Profuse growth of the hairy roots were observed in liquid MS medium devoid of growth regulators and antibiotics.

3.6.2. Confirmation of transgenic nature of hairy roots

The transgenic nature of hairy roots was confirmed by PCR (p.63, section 2.6.9) using *rol* A specific primers in bacteria free hairy roots DNA. A 308bp *rol* A expected size fragments was obtained only in hairy roots and absent in normal roots (Fig 3.19). Culturing the hairy root samples in LB medium did not show the bacterial growth indicating the absence of live *A. rhizogenes.* PCR with *vir* C primers revealed the absence of contaminating *A. rhizogenes.*

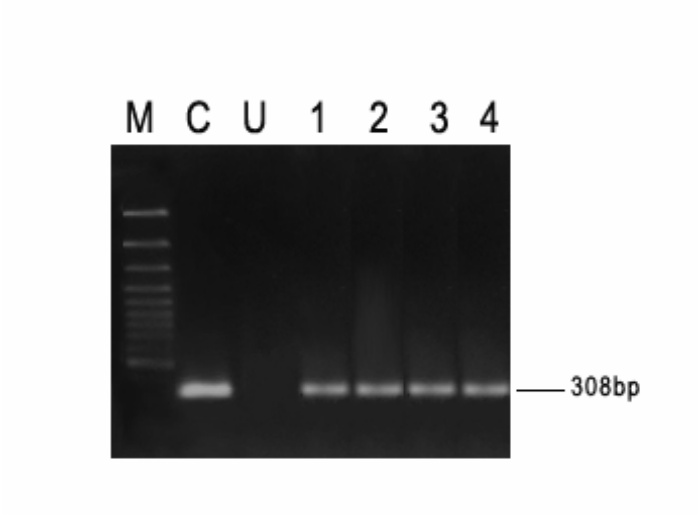


Fig 3.19. PCR amplification of 308 bp fragment of *rol* A gene in *Nicotiana tabacum* hairy root.

Lanes M- 100bp Marker.

C- TempleT-DNA from *Agrobacterium rhizogenes* plasmid.

U- Untransformed roots of *Nicotiana tabacum*.

1- 4 – TempleT-DNA from *Nicotiana tabacum* hairy roots.

3.6.3. Comparison of different treatments

Manual wounding resulted in 21% transformation frequency (data analysis p.64, section 2.6.10). The control explants inoculated with only LB medium devoid of *Agrobacterium rhizogenes* did not show induction of roots. Macerating enzyme treatment in combination with sonication resulted in reduced transformation frequency (Fig 3.20, 3.21, 3.22). Sonication treatment of leaf explants resulted in 2.2 fold increase in terms of transformation frequency when compared to manual wounding (Fig 3.20). Sonication assisted transformation resulted in 46% transformation frequency (Fig 3.20). There was slight increase in transformation frequency when 20mM calcium chloride was used in co cultivation medium. Sonication with 5mM $CaCl_2$ treatment resulted in 2.5 fold increase with a transformation frequency of 52%. Where as 60% transformation was obtained at 10mM $CaCl_2$ (Fig 3.23). Sonication with 100 μM acetosyringone treatment resulted in 4.1 fold increase with 86% transformation frequency (Fig 3.24) This treatment was found to be the best for enhancing the transformation frequency. Among all theses treatments, sonication with macerating enzyme treatment resulted in 1.5 to 5.25-fold decrease in

transformation frequency compared to manual wounding treatment (Fig 3.20, 3.21, 3.22). Less than 6% explants showed induction of hairy roots. Use of cellulase or pectinase alone or in combination severely damaged the explants and the explants showed bacterial over growth. In general sonication in combination with acetosyringone gave excellent results in terms of transformation frequency as well as number of transformation events in each individual explants.

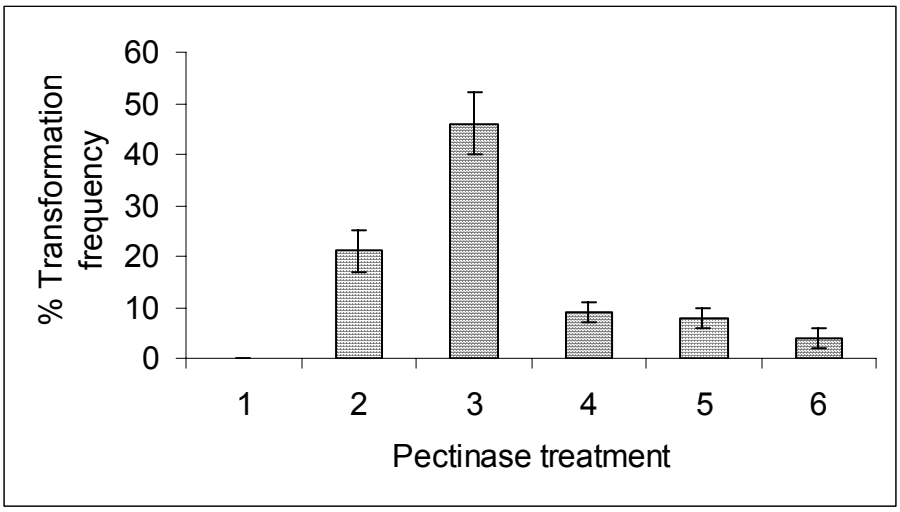


Figure 3.20 Influence of ultra sonication with pectinase enzyme treatment on transformation frequency.

Legends: 1-Control (No *Agrobacterium rhizogenes* infection), 2-Mannual wounding prior to co cultivation, 3-Sonication treatment prior to co cultivation, 4-Sonication with 0.1% pectinase enzyme treatment prior to co cultivation, 5-Sonication with 0.5% pectinase enzyme treatment prior to co cultivation, 6-Sonication with 1% pectinase enzyme treatment prior to co cultivation.

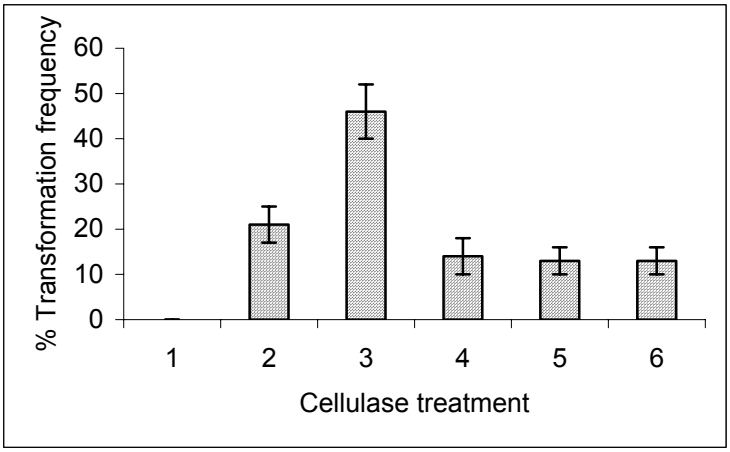


Figure 3.21. Influence of ultra sonication with cellulase enzyme treatment on transformation frequency.

Legends: 1-Control (No *Agrobacterium rhizogenes* infection), 2-Mannual wounding prior to co cultivation, 3-Sonication treatment prior to co cultivation, 4-Sonication with 0.1% cellulase enzyme treatment prior to co cultivation, 5-Sonication with 0.5% cellulase enzyme treatment prior to co cultivation, 6-Sonication with 1% cellulase enzyme treatment prior to co cultivation.

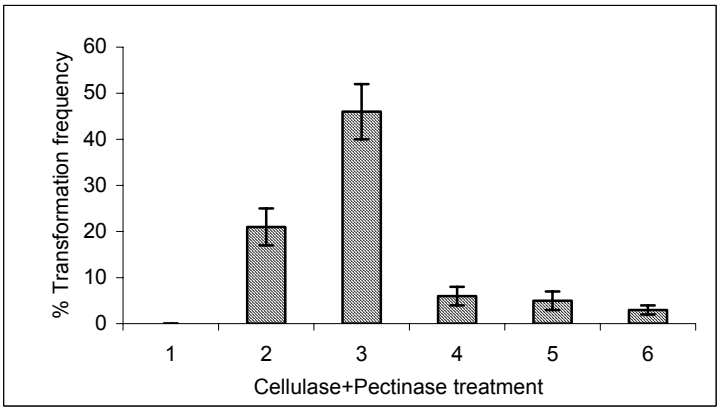


Figure: 3.22. Influence of ultra sonication with cellulase and pectinase enzyme treatment on transformation frequency.

1-Control (No *Agrobacterium rhizogenes* infection), 2-Mannual wounding prior to co cultivation, 3-Sonication treatment prior to co cultivation, 4-Sonication with 0.1% cellulase and pectinase enzyme treatment prior to co cultivation, 5-Sonication with 0.5% cellulase and pectinase enzyme treatment prior to co cultivation, 6-Sonication with 1% cellulase and pectinase enzyme treatment prior to co cultivation.

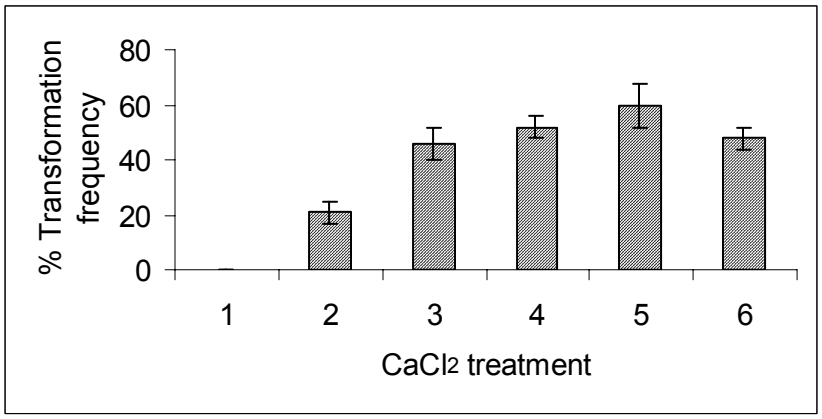


Figure 3.23. Influence of ultra sonication with calcium ion treatment on transformation frequency.

1-Control (No *Agrobacterium rhizogenes* infection), 2-Mannual wounding prior to co cultivation, 3-Sonication treatment prior to co cultivation, 4-Sonication with 5mM $CaCl_2$ in the co cultivation medium, 5- Sonication with 10mM $CaCl_2$ in the co cultivation medium, 6-Sonication with 20mM $CaCl_2$ in the co cultivation medium.

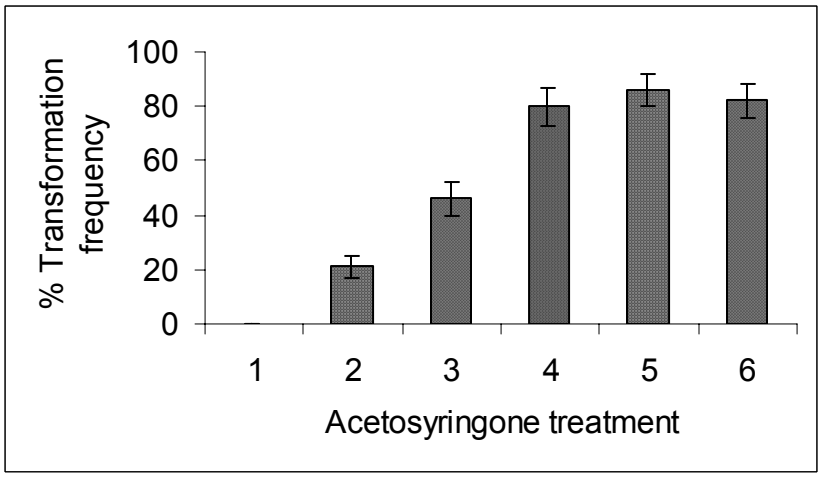


Figure 3.24. Influence of ultra sonication with acetosyringone treatment on transformation frequency.

1-Control (No *Agrobacterium rhizogenes* infection), 2-Mannual wounding prior to co cultivation, 3-Sonication treatment prior to co cultivation, 4-Sonication with 50µM acetosyringone in the co cultivation medium, 5- Sonication with 100µM acetosyringone in the co cultivation medium, 6-Sonication with 150µM acetosyringone in the co cultivation medium.

3.6.4. Discussion

Tobacco serves as an excellent model system to study the factors influencing genetic transformation. *Agrobacterium rhizogenes* is used to express *rol* genes and also to deliver foreign genes to susceptible plants. A number of reports available on enhancing the transformation rate in *A. tumefaciens* mediated gene transfer. There is increasing need for studies on enhancement in gene transfer efficiency in other bacteria such as *A. rhizogenes*, *Rhizobium* sp NGR234, *Sinorhizobium meliloti* and *Mesorhizobium loti* which are capable of gene transfer to higher plants (Broothearts *et al* 2005). However this is the first report on the use of physical and chemical treatments by which the transformation efficiency by *A. rhizogenes* can be enhanced. This would be useful in genetic transformation of other recalcitrant plants.

Wounding is a prerequisite for the genetic transformation process through *Agrobacterium* and may aid in the production of signal phenolics (Reviewed by Gelvin, 2000) and enhances the accessibility of putative cell-wall binding factors (Reviewed by Gelvin *et al* 2000) to the bacterium. Acetosyringone is one such compound used successfully to enhance transformation in various plant species in *A. tumefaciens* mediated genetic transformation. Similar observations have been made in the present study.

The cell wall disruption caused by the lower energy ultrasonic frequency utilized in the present study is apparently very useful for *Agrobacterium rhizogenes* mediated transformation. Meurer and others (1998) reported the enhancement in transgene delivery to soybean cotyledonary nodes by *Agrobacterium tumefaciens* mediated transformation. Sonication has been used to enhance *Agrobacterium tumefaciens* mediated transformation of many different plant species (Trick and Finer 1997, Santarem *et al* 1998). The synergistic effect of ultra-sonication with *A. rhizogenes* mediated transformation was tested in the present study. This is the first report in which ultrasonication has been tried and found to enhance transformation rates in *A. rhizogenes* mediated genetic transformation.

Calcium is an essential plant nutrient and required for various signalling pathways (White and Broadly 2003, Sanders *et al* 1999). Since calcium ions are known to increase the permeability of the biological membranes, its effect on gene transfer was tested in higher plant.

Macerating enzymes such as cellulases, pectinases, which are regularly used in protoplast isolation procedures (Alibert *et al* 1994), could represent a less disruptive method to remove the cell wall barrier. Following the digestion, the area where *Agrobacterium* can attach to plant cells might increase for enhanced transformation. In order to test this hypothesis, the effect of macerating enzymes on the transformation efficiency was tested in tobacco leaves. In order to assess the effects of macerating enzymes, the enzyme treatment and mechanical wounding of the explant by sonication was compared. But the results clearly demonstrated that, the macerating enzyme treatment drastically reduces the transformation efficiency.

Acetosyringone has been known to enhance transformation efficiency due to activation of *vir* genes in *A. tumefaciens* (Reviewed by Gelvin, 2000). Therefore it could be presumed that, the enhancement in transformation by acetosyringone treatment may be due to activation of *vir* genes which is absolutely required for the T-DNA delivery to plant tissues. Tang (2003) demonstrated that, incorporation of additional virulence genes and sonication treatment can enhance *Agrobacterium tumefaciens* mediated transformation in loblolly pine.

3.6.5. Summary

- Wounding of host tissue by ultrasonication and treatment with acetosyringone followed by exposure to *A. rhizogenes* results in enhanced transformation frequency in *Nicotiana tabacum*.
- This may be useful in transfer of genes to recalcitrant plants using *A. rhizogenes* and further used in transformation of *Coffea*.

3.7. *A. RHIZOGENES* MEDIATED GENETIC TRANSFORMATION STUDIES ON *COFFEA CANEPHORA.*

Background:

Coffee breeding by conventional methods is a low efficiency and time consuming process due to their long cycles. Hence, genetic transformation is a desirable technique for genetic improvement of coffee (Hatanaka *et al* 1999). 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 programmes including genetic transformation. Genetic transformation of *Coffea* sp. has been achieved using electroporation of protoplasts (Barton *et al* 1991), biolistic methods (Van Boxtel *et al* 1995, Rosillo *et al* 2003), *Agrobacterium tumefaciens* (Hatanaka *et al* 1999, Leroy *et al* 2000, Ogita *et al* 2004) and *Agrobacterium rhizogenes* (Sugiyama *et al* 1995). Many reports have shown the use of *Agrobacterium rhizogenes* for expression of the *rol* genes and to deliver foreign genes to susceptible plants (Christey 2001). *A. rhizogenes* harbouring binary vectors have been used successfully in plant transformations (Christey 2001). The results of the previous study on optimization of transformation in *N. tabacum* were taken into account for further work on *Coffea* transformation.

In this section the results of experiments involving development of a transformation system in *Coffea canephora* using *A. rhizogenes* harbouring its native Ri plasmid and a binary vector pCAMBIA 1301 is presented.

3.7.1. Sensitivity tests for selection of transformed tissue

Regeneration was not observed in medium containing 3-50 mgl^{-1} hygromycin. The minimum regeneration inhibition concentration was determined to be 5 mgl^{-1}. However in all the transformation experiments, up to 20 mg l^{-1} hygromycin was chosen as the ideal level for the successful selection of the transformants because it prevents regeneration and also kills the untransformed tissues (Table 3.13). Tissue browning was observed under higher concentration of hygromycin. Transfer of transformed tissues to different medium with increasing concentration of hygromycin in the medium gradually gives enough time for the transformed cells to survive and regenerate.

Table. 3.13 Determination of minimum inhibitory concentration of hygromycin for selection of transgenic secondary embryos

Medium* with hygromycin (mg l^{-1})	Secondary embryogenesis (%)		Browning (%)	
	2 weaks	4 weaks	2 weaks	4 weaks
Control	0	90	0	5
1	0	65	0	10
2	0	25	0	10
3	0	0	0	50
5	0	0	20	60
10	0	0	40	75
20	0	0	90	95
30	0	0	100	100
40	0	0	100	100
50	0	0	100	100

*Half strength MS salts + BA 0.25 mg l^{-1} + IAA 0.5 mg l^{-1} + B5 vitamins + 2% sucrose

3.7.2. Transformation of competent *E. coli* cells

Transformed colonies of *E. coli* strain DH5α was obtained under kanamycin selection. Isolation of plasmid and agarose gel electrophoresis revealed the presence of 13 kb pCAMBIA 1301 plasmid (Plasmid map p.65, Fig 2.1) in kanamycin resistant colonies (Fig.3.25)

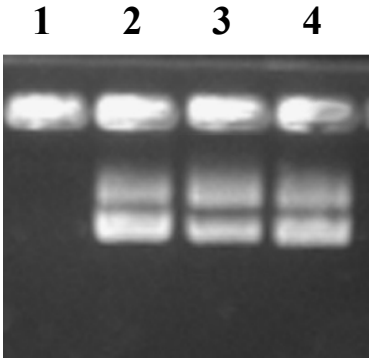


Fig 3.25 Isolation of plasmid pCAMBIA 1301 from E. coli strain DH5α and agarose gel electrophoresis

Lanes:

1- Control kanamycin sensitive colonies, which do not receive the plasmid.

1 to 3 – Kanamycin resistant transforments showing 13 kb pCAMBIA 1301 vector.

3.7.3. Mobilization of binary vectors to *Agrobacterium rhizogenes*

The isolated plasmid was mobilized to *Agrobacterium rhizogenes*. PCR was performed to confirm the presence of Ri native plasmid and pCAMBIA 1301 binary vector. PCR with plasmid for the plant selection marker hygromycin phoshphotransferase gene (*hpt* II) confirmed the presence of the introduced plasmid vector in *Agrobacterium rhizogenes* (Fig 3.26). PCR for the *rol* A gene confirmed the presence of Ri plasmid in *Agrobacterium rhizogenes* (Fig 3.27).

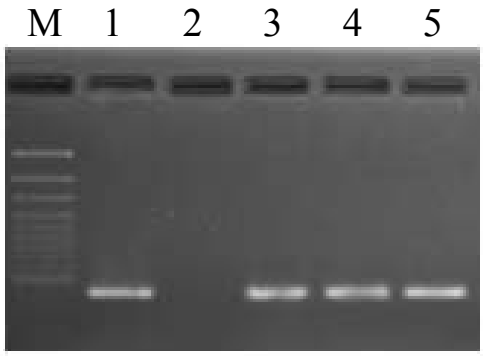


Figure 3.26: PCR amplification of *hpt* II gene from *A. rhizogenes* transformed with pCAMBIA1301.

Lanes:

M- 100bp marker.

1- Control DNA from *E. coli* strain DH5α harboring pCAMBIA 1301 vector.

2- Control DNA from untransformed *A. rhizogenes.*

3-5 DNA from *A. rhizogenes transformed* with binary vector pCAMBIA1301.

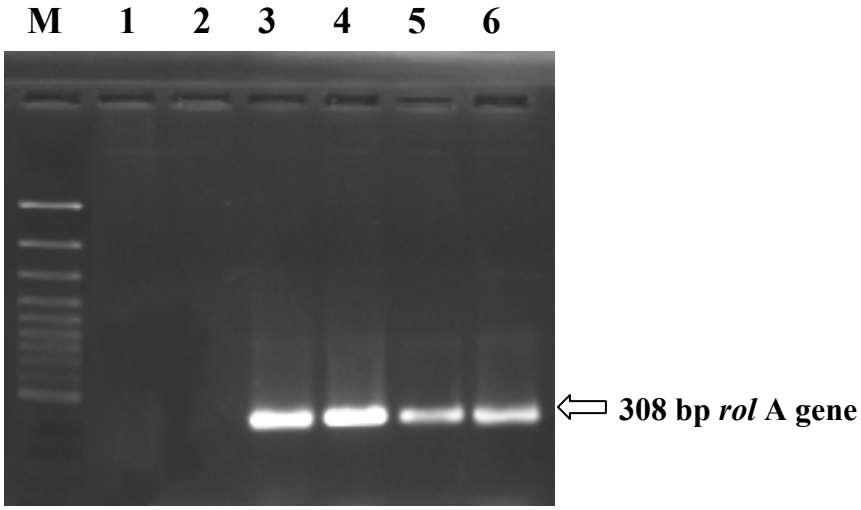


Figure 3.27 : PCR amplification of *rol* A gene from *A. rhizogenes*.

Lanes:

M- 100bp marker.

1, 2 -Control DNA from *E. coli* strain DH5α.

3, 4 DNA from wild *A. rhizogenes*

5, 6 DNA from *A. rhizogenes* transformed with binary vector pCAMBIA1301.

3.7.4. *Transformation using A. rhizogenes*

The hypocotyl segments co-cultivated with wild type *A. rhizogenes* remained green and callused slightly in the wounded portions. Hairy roots were initiated after 23 days (Fig. 3.28a) from *A. rhizogenes* co-cultivated explants. The transformation efficiency was approximately upto 3%. These roots were grown in solid MS medium containing 500 mg l^{-1} cefotaxime and 300 mg l^{-1} potassium clavulanate to eliminate *A. rhizogenes*. Roots were not induced from control-uninfected explants. These experiments demonstrated the susceptibility of *Coffea canephora* to *A. rhizogenes* mediated transformation.

3.7.5. Transformation using *A. rhizogenes* harbouring a binary vector

Hairy roots of *Coffea canephora* did not elongate even after 6-10 weeks of culture and regeneration of plants from the roots was not successful. In other experiments, the explants were co cultivated with *A. rhizogenes* harboring pCAMBIA 1301 (Materials and methods Fig. 2.1). Tubular, mature somatic embryos at the mature stage were used for

sonication-assisted transformation (p.72, section 2.7.14). After 24 hrs of co-cultivation, colonization of *A. rhizogenes* was observed at the wounded regions. The co-cultivated somatic embryos were directly grown on medium comprising half strength MS salts and B5 vitamins, 0.45 mg l^{-1} IAA, 0.25 mg l^{-1} BA, 2% sucrose and either 0.5 mg l^{-1} thidiazuron (TDZ), 60 µM AgNO3, 1 mg l^{-1} 2ip or 5µM triacontanol and 5-20 mg l^{-1} hygromycin. The uninfected control explants could not regenerate in medium supplemented with 5mg l^{-1} hygromycin (Fig. 3.28b) Direct induction of hygromycin resistant secondary embryos was observed after 3 months of culture (Fig. 3.28c). After 10 months of selection on hygromycin containing medium, the plants were cultured under hormone free medium to facilitate growth. Plantlets were cultured on MS medium with 10 mg l^{-1} hygromycin devoid of other antibiotics for 18 months (Fig. 3.28d). The use of TDZ, triacontanol and silver nitrate in the culture medium was found to be very useful for somatic embryogenesis in *Coffea canephora*. Silver nitrate in combination with BA and IAA proved to be good for *in vitro* shoot growth of both *C. arabica* and *C. canephora* (Giridhar *et al* 2003). The results of the regeneration experiments helped in increasing the yield of transgenic embryos. PCR and Southern blot confirmed the transgenic nature of plants regenerated on medium containing hygromycin. The transformation efficiency varied and ranged upto 3%. Altogether 36 putatively transgenic secondary somatic embryos survived and developed on 20mg l^{-1} hygromycin selection, for a period of 8-10 months.

Secondary embryos and plantlets growing on 10mg/l hygromycin selection medium were subjected to GUS assay (p.73, section 2.7.15) to confirm the expression of transgenes in the T_0 lines. Strong expression of intron *uid* A gene was obtained in transgenic plants. The expression was greater in the stem region (Fig. 3.28e) and localized expression was observed in the leaves also. Untransformed control somatic embryos and plants were not stained under similar reaction conditions (Fig. 3.28f). In all the assays mixture 20% methanol was added to prevent false positive endogenous "GUS like" activity (Kosugi *et al* 1990). The results confirmed the expression of transgenes in *Coffea canephora* delivered using *A. rhizogenes*.

In general the transgenic plants devoid of *rol* genes were normal in appearance (Fig 3.28g) without any abnormalities associated with the *rol* genes. However, the plants

harbouring *rol* genes resist elongation in culture medium and the leaves were brittle and wrinkled (Fig 3.28h). The transgenic plantlets are presently growing in *in vitro* condition and ready for green house transfer.

Slow growth of embryogenic callus, embryogenesis and maturity during embryogenesis is a major hindrance in transformation experiments with *Coffea* sp. The study resulted in the development of a unique method, which could be used for gene transfer in *Coffea* (Vinod Kumar et al 2003 Indian Patent). This is the first report of use of direct secondary embryogenesis route using TDZ, triacontanol and silver nitrate in transformation of *Coffea canephora*. It remained difficult to obtain data on the hormonal regimes best suited for transgenic secondary embryo induction. As reported by earlier workers (Leroy *et al* 2000) *Coffea* sp are slow in *in vitro* response and many transformation experiments ended up in not getting any transgenic embryos.

Hygromycin was found to be the most suiTable for *Coffea* transformation. Minimum inhibitory concentration of hygromycin was found to be 5 mg l^{-1}. Sub optimal levels of selection pressure were employed initially in order to retain the embryogenic nature of the transformed cell. Further selection on 20 mg l^{-1} hygromycin concentration allowed the growth of only transformed plants.

One of the most commonly observed problems with any *Agrobacterium* mediated gene transfer is the over growth of *Agrobacterium* on explants, which causes irreversible damage to the tissue. The overgrowth was controlled by using potassium clavulanate, a β-lactamase inhibitor at concentration of 100 mg l^{-1} along with cefotaxime in the selection medium. Use of cefotaxime alone did not prevent the over growth of *A. rhizogenes*, so preliminary experiments were carried out to standardize the best antibiotic combination which will kill the *A. rhizogenes* but not interfere with regeneration.

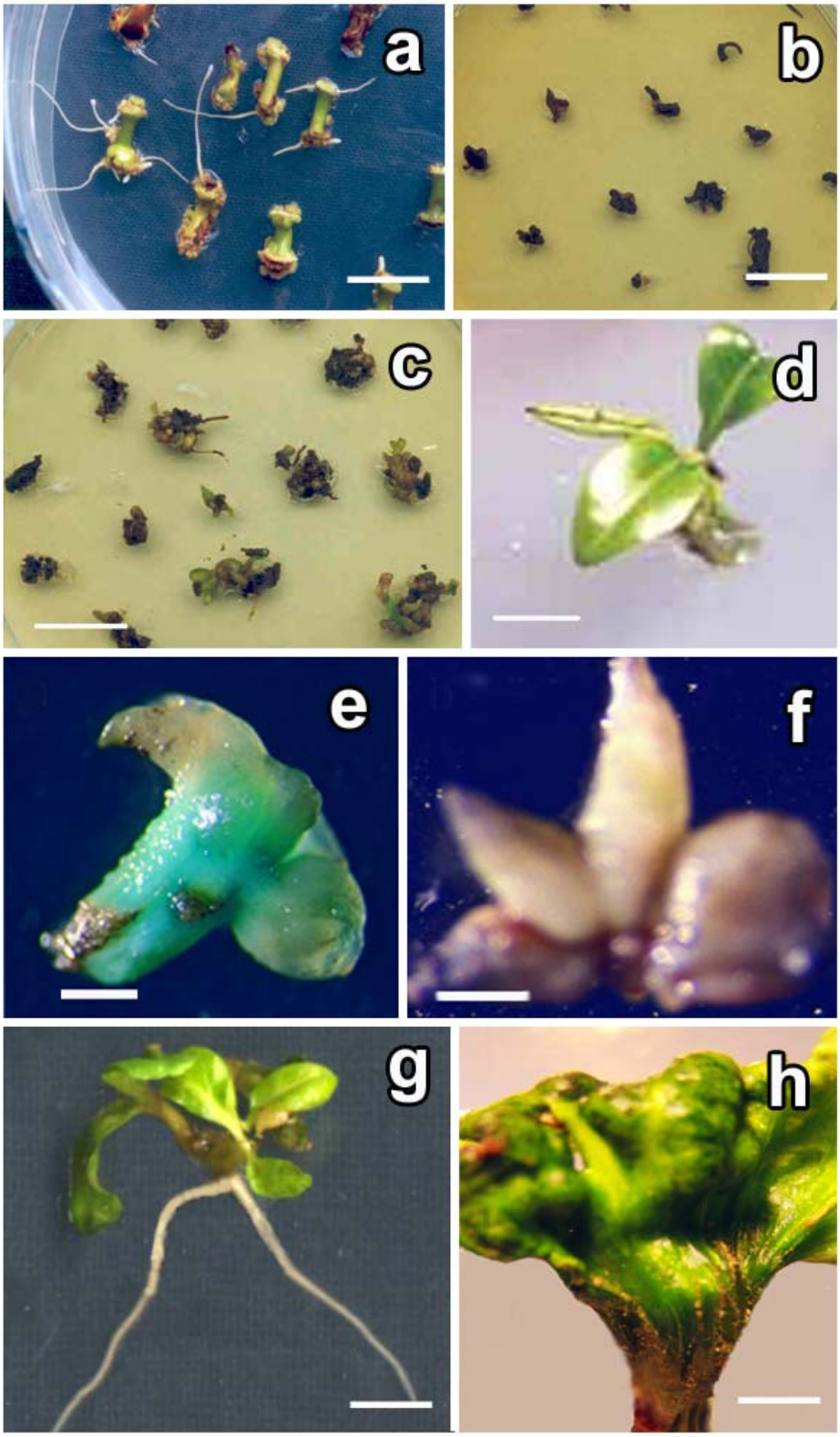


Fig 3.28. ***A. rhizogenes*** **mediated transformation of** ***Coffea canephora***

Fig. 3.28a: Induction of hairy roots from infected hypocotyls in hormone free media by *Agrobacterium rhizogenes* mediated transformation.

Fig. 3.28b: Complete killing of control untransformed primary somatic embryos in medium containing 10mg l^{-1} hygromycin. Bar-10mm

Fig. 3.28c: Regeneration of transgenic secondary embryos from primary somatic embryos after transformation with *Agrobacterium rhizogenes* harboring a binary vector with a reporter and a marker gene for the selection of transformed plants. Bar-10mm

Fig. 3.28d: 18-month-old transgenic plant. Bar-5mm

Fig. 3.28e: Expression of intron *uid* A gene. Bar-5mm

Fig. 3.28f: Control untransformed secondary embryos. Bar-3mm

Fig 3.28g: Normal transgenic plant harboring T-DNA from the binary vector and devoid of Ri-TDNA. (Bar-5mm)

Fig 3.28h: Phenotype of transgenic plant harboring T-DNA from both binary vector and Ri plasmid (Bar-2.5mm).

3.7.6. PCR analysis

Five, 16 month old plantlets were tested for the presence of the *rol* A gene from Ri-T-DNA and the *hpt* II gene from the binary vector T-DNA. PCR (p.73, section 2.7.17) using gene specific primers for hygromycin phosphotransferase resulted in amplification of 479bp fragment of the *hpt* II gene in all five hygromycin resistant plants (Fig. 3.29a). However, 3 of these plantlets were negative for presence of the *rol* A gene (Fig. 3.29b). These results clearly indicate the independent integration of T-DNA from the pCAMBIA 1301 binary vector and the Ri native plasmid from *A. rhizogenes*. It is evident from the present study that during transformation process co-transfer of T-DNA from both Ri plasmid and binary vector are not obligatory.

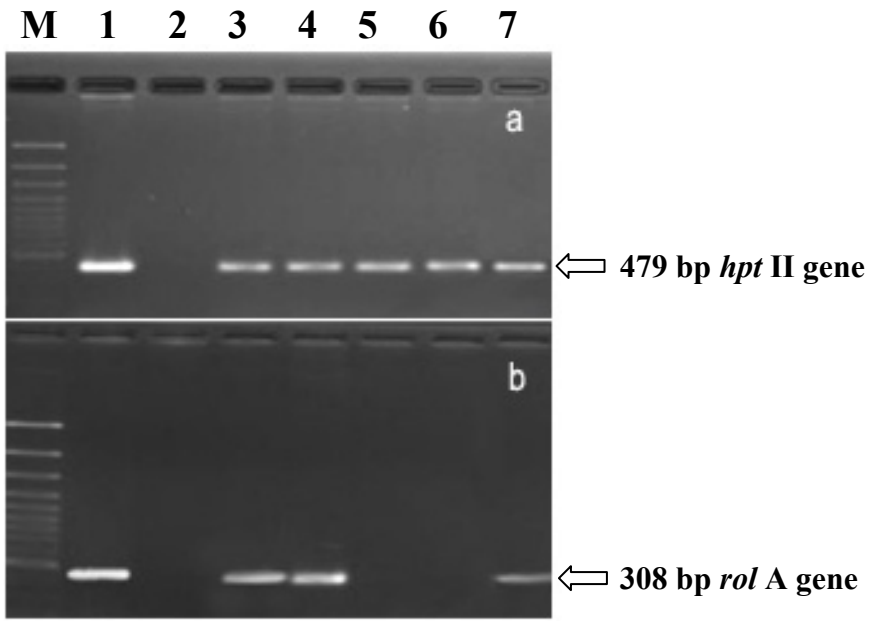


Fig 3.29: PCR for the detection of the *hpt* II and *rol* A gene.

PCR analysis was carried out using gene specific primers for the *hpt* II (a) gene and *rol* A (b) gene. Lanes,

M- 100bp marker,

1- DNA from *A. rhizogenes* harboring the binary vector.

2- DNA from the untransformed *C. canephora*.

3 to 7 - DNA from independent transformants selected under hygromycin.

3.7.7. Southern analysis

Southern analysis (p.74, section 2.7.18) further confirmed the transgenic nature and stable integration of T-DNA in hygromycin resistant plantlets. The presence of several fragments of variable size in some lines indicates insertion of multiple copies of the T-DNA into the plant genome (Fig. 3.30). The genomic DNA was digested with N*de* I and S*ac* II enzymes. N*de* I cuts once inside the *hpt* II gene and the probe and S*ac* II cuts just outside the T-DNA left border. No signals were observed in lanes containing untransformed DNA. The 1.4 Kb fragment detected in the Southern blot is the expected size fragment from the pCAMBIA 1301 vector. Since the S*ac* II site is absent in the T-DNA of the vector, all the large size fragments (greater than 1.4Kb) are possibly derived from the plant genome and not from any bacterial contamination.

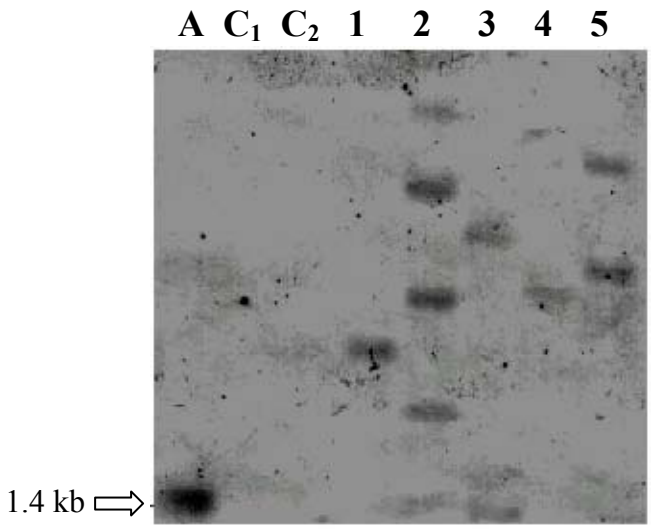


Fig. 3.30: Southern blot analysis of PCR positive, transformed plants.

Plasmid and genomic DNA was digested with N*de* I and S*ac* II, separated in agarose gel, transferred to nylon membrane and probed with *hpt* II coding regions. S*ac* II position is located at just outside the T-DNA left border. S*ac* II was selected in order to ensure the stable integration of T-DNA in the coffee genome and to ensure the absence of possible contamination of bacterial DNA. The expected size of the S*ac* II and N*de* I fragment in pCAMBIA 1301 is 1.4kb. Since S*ac* II is out side the T-DNA left border, all the fragments larger than 1.4kb must come from stable integration of T-DNA in the *Coffea canephora* genome. Lanes,

A- pCAMBIA 1301 vector.

C_1 and C_2 untransformed *Coffea canephora* plants.

1 to 5- DNA from PCR positive *Coffea canephora* T_0 transformants.

Though diverse species of bacteria are capable of gene transfer to plants (Broothaerts et al 2005), *Agrobacterium* sp are widely used for genetic modification of plants. *A. rhizogenes* mediated transformation is a potent tool for producing hairy roots and synthesis of secondary metabolites (Rao and Ravishankar 2002). *A. rhizogenes* can transfer the T-DNA of binary vectors in *trans*, thereby facilitating the selection of transgenic plants from screened hairy roots (Christey *et al* 1997). The integration of *rol* genes and T-DNA from the binary vector is independent (Shahin *et al* 1986). Generally the hairy root phenotype is considered as a primary marker in *A. rhizogenes* mediated genetic transformation. However, co-transformation with binary vector results in

complex T-DNA integration patterns (Karimi *et al* 1999) and some of the hairy roots may not contain the T-DNA from the binary vector. Manners and Way (1989) demonstrated the production of normal plants that contain T-DNA of the binary vector; devoid of T-DNA from the native Ri plasmid in *A. rhizogenes* mediated transformation of *Stylosanthes humilis*. Integrations occurring at independent loci, segregating through meiosis has been demonstrated in *Agrobacterium* mediated transformation (De Framond *et al* 1986). In this case, it may be possible to get normal plants devoid of Ri T-DNA in subsequent generations.

Transgenic coffee plants containing the *cry1A(c)* gene were produced using both the *Agrobacterium rhizogenes* and *Agrobacterium tumefaciens* systems (Leory *et al* 1997). In *Coffea* sp. the frequency of transgenic plants produced is considerably below that of many other transformation systems (Leroy *et al* 1997). Using the *Agrobacterium tumefaciens* system 0.4% of the somatic embryos infected gave transgenic plantlets (Leroy *et al* 1997). However in the present study it was possible to get comparatively higher transformation frequency ranging up to 3%. Sugiyama *et al* (1995) reported *Agrobacterium rhizogenes* mediated transformation system using *A. rhizogenes* strain IFO 14554 in *Coffea arabica var*. typica. Transgenic roots were obtained and subsequently plants were regenerated from the roots. All the plantlets showed typical *rol* gene phenotype with short internodes and stunted growth. In the present study the hairy root phenotype was not used as a marker during selection and thus able to produce plants which are devoid of *rol* genes and did not show hairy root phenotype. The plants developed into normal phenotype unlike those obtained by Sugiyama and co-workers (1995).

2.7.8. Summary

- The study resulted in the development of a protocol for genetic transformation of *Coffea canephora*. The results proved that *Agrobacterium rhizogenes* could be used to deliver transgenes present in T-DNA of binary vectors with *A. tumefaciens* T-DNA borders.
- Use of hormonal regimes and culture conditions can help to avoid an intervening hairy root phase in the regeneration of plants transformed with *A. rhizogenes*.

3.8. TRANSFORMATION OF COFFEE FOR DOWN REGULATION OF CAFFEINE BIOSYNTHESIS

Background:

Coffea species contain caffeine (a purine alkaloid) in the beans, leaves and in embryos. The sequential conversion of xanthosine to caffeine through 7-methylxanthosine, 7-methylxanthine and theobromine occurs through the action of *N*-methyltransferases (NMTs) that use *S*-adenosyl-L-methionine (SAM) as the methyl donor (Kato and Mizuno 2004, Uefuji *et al* 2003). The cDNAs encoding 7-methylxanthosine synthase (Uefuji *et al* 2003) theobromine synthases and caffeine synthase (Mizuno *et al* 2003a, Mizuno *et al* 2003b) have been cloned. There is high degree of similarity in the coding region of coffee NMT genes isolated so far, and differences exist in the 3´untranslated region (UTR). Excess intake of caffeine is known to cause health effects (Table 1.5). RNAi, co-suppression and antisense technologies are powerful tools of post-transcriptional gene silencing in sequence specific manner. In this report the efficiency of these strategies in down regulating caffeine biosynthesis was envisaged. Post-transcriptional gene silencing (PTGS), a sequence specific RNA degradation mechanism can be induced in plants by transforming them with antisense, co-suppression or hairpin RNA constructs (Wesley *et al* 2001). By choosing unique or conserved region, a single or all members of gene families, respectively can be silenced Wesley *et al* 2001, Allen *et al* 2004. Ogita *et al* 2004 reported the application of a specific 3´-untranslated sequence for one of the coffee NMT gene (CaMXMT-1) for design of RNAi construct.

In this section the results of experiments involving transformation *Coffea canephora* with different constructs bearing conserved region of *N* methyltransferase genes with a view to down regulate caffeine biosynthesis is presented.

3.8.1. Constructs used in transformation experiments

The binary vector pCAMBIA 1301 was used as the primary vector and co-suppression (pSAT 201), antisense (pSAT 202), and inverse repeat (pSAT 222) constructs (Fig 2.2) containing homologous sequence of *N* methyl transferase was used for transformation of coffee. The transformation efficiency was recorded by counting the number of putatively

transgenic secondary embryos selected on 20 mgl^{-1} hygromycin after a period of minimum 8 months of culture.

3.8.2. Transformation of *Coffea* with constructs targeted to silence *N*-methyl transferase.

In subsequent experiments only *A. tumefaciens* (strain EHA 101) was used to transform *Coffea* with constructs designed for post transcriptional gene silencing (PTGS) of *N* methyl transferase. Transgenic secondary embryos and plantlets were produced under hygromycin selection (Fig 3.31 a, b, c, d). Secondary embryos and plantlets growing on 10 mgl^{-1} hygromycin selection medium were subjected to GUS assay to confirm the expression of transgenes in the T_0 lines. Strong expression of intron *uid* A gene was obtained in transgenic plants. The expression was greater in the stem as well as in leaves (Fig. 3.32a). Untransformed control somatic embryos and plants were not stained under similar reaction conditions (Fig. 3.32b). The results confirmed the expression of transgenes in *Coffea canephora* delivered using *A. rhizogenes.*

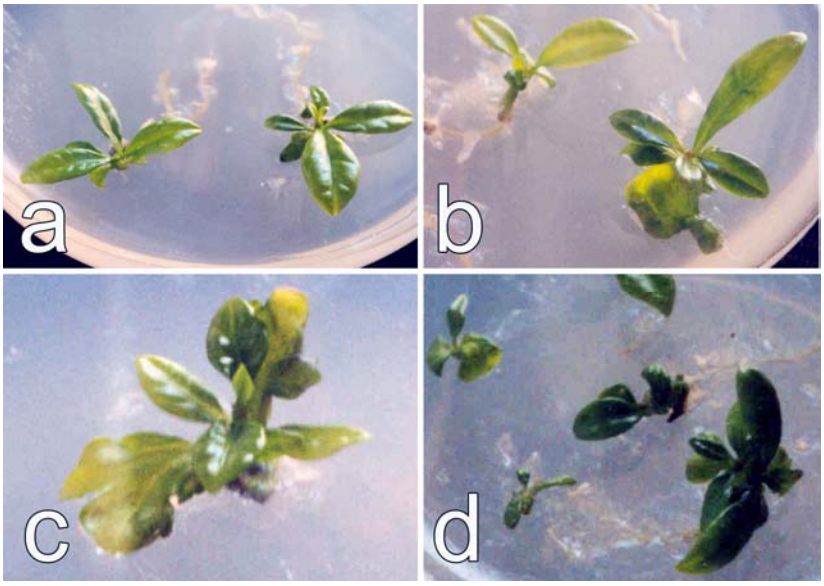


Fig 3.31. *Coffea canephora* plants transformed with different constructs for silencing caffeine synthesis.

Plants transformed with a: pCAMBIA 1301, b: pSAT 201, c: pSAT 202, d: pSAT 222

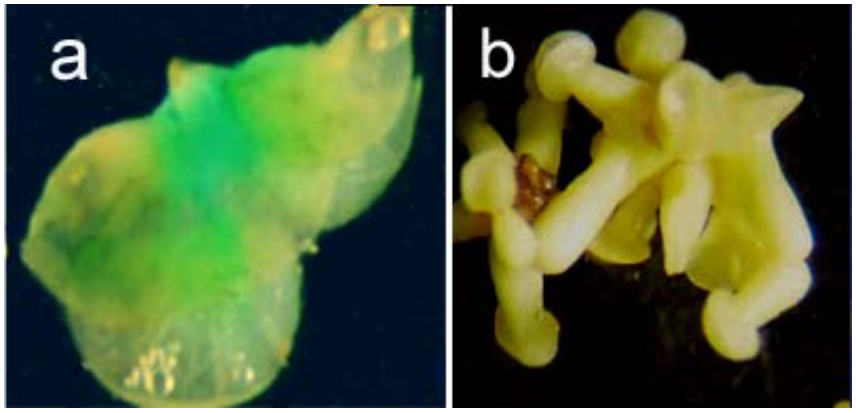


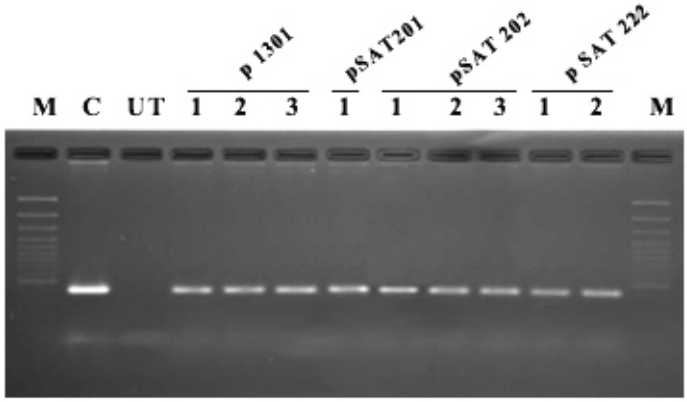
Fig. 3.32. GUS activity was detected in PCR positive transgenic embryos

a. *Coffea* leaf transformed with pSAT 222 (NMT invert repeat).

b. Control untransformed embryos.

3.8.3. PCR for the confirmation of transgenic nature.

The transgenic plantlets were selected under hygromycin confirmed for the presence of *hpt* II gene (Fig 3.33).



⇦ **479 bp *hpt* II gene**

Fig 3.33: PCR for the detection of the *hpt* II gene in plants transformed with different silencing constructs targeted to *N* methyltransferase.

Lanes; M- 100bp marker, C- DNA from *A. tumefaciens* harboring PTGS constructs, UT-DNA from the untransformed *C. canephora*.

3.8.4. Caffeine levels in transgenic coffee

The transgenic coffee plantlets were analyzed for caffeine content (p.55, section 2.3.3). The untransformed coffee plantlets and coffee transformed with control pCAMBIA 1301 construct showed a caffeine level ranging between 0.7-0.8% DW (Fig 3.34, 3.35). It is evident that the caffeine level does not vary due to *Agrobacterium* infection.

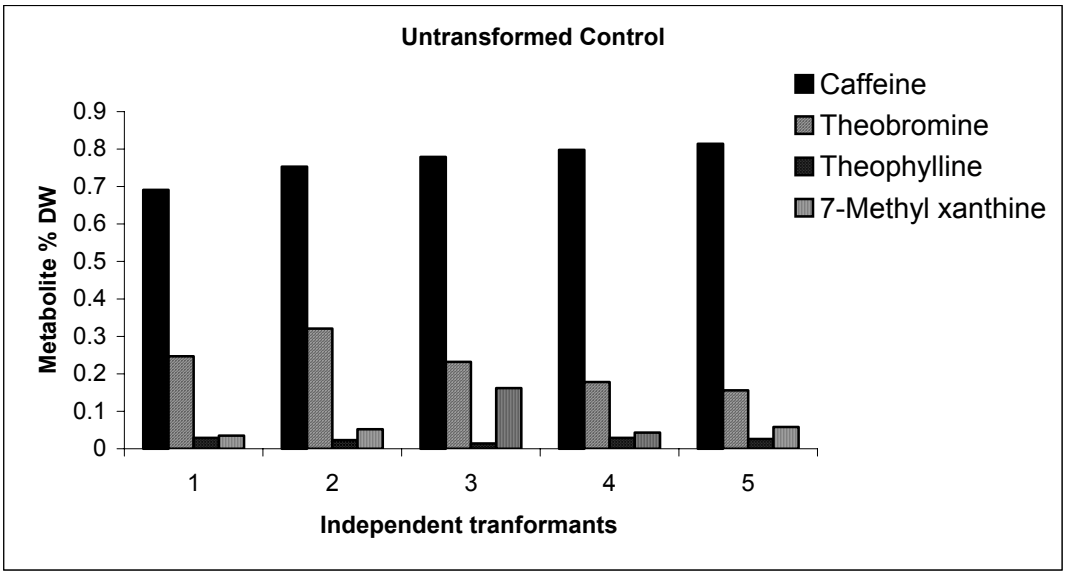


Fig 3.34. Caffeine and other purine alkaloid level in independent untransformed coffee plantlets (Control)

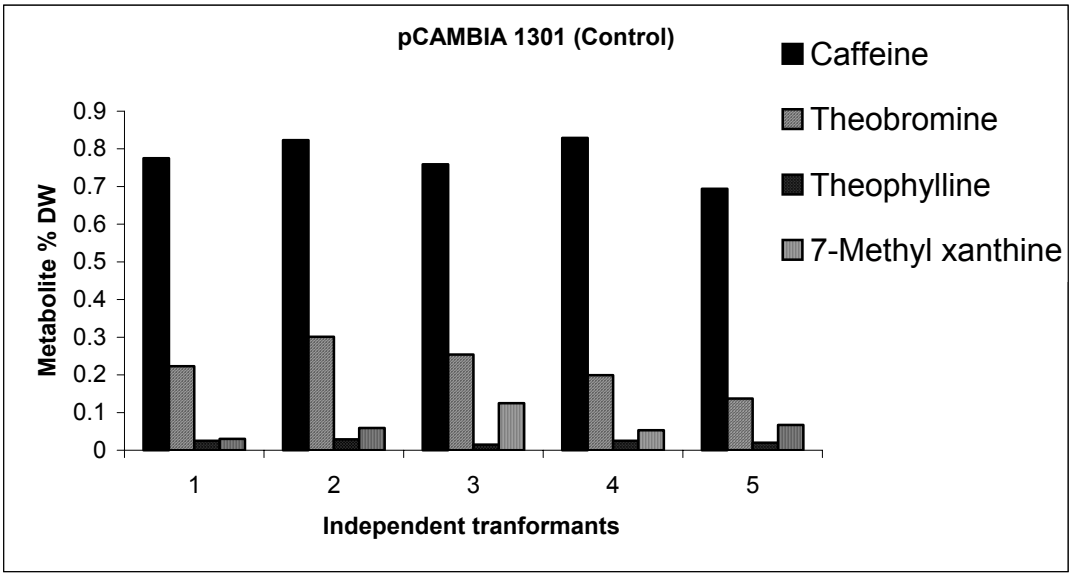


Fig 3.35. Caffeine and other purine alkaloid level in independent coffee plantlets transformed with *A. tumefaciens* harboring pCAMBIA 1301 (Control)

Transformants with sense construct pSAT 201 resulted in caffeine level ranging between 0.38-0.63% DW (Fig 3.36). The sense construct was not very effective in down regulation of caffeine synthesis. The sense construct resulted in 10-50% reduction in caffeine content when compared to the control (pCAMBIA1301). Despite this reduction in its precursor content, caffeine content was found to be 90% of that of control.

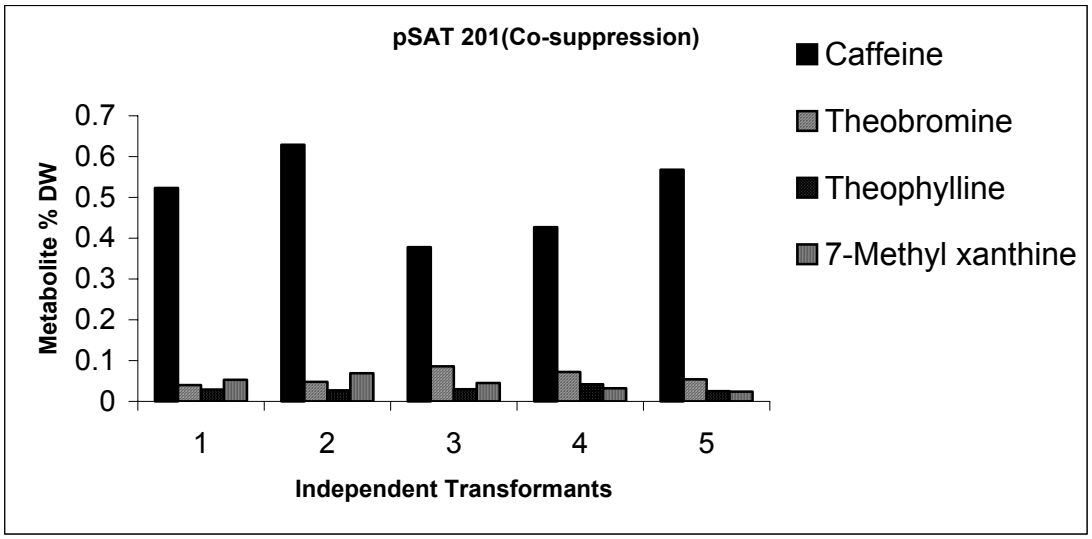


Fig 3.36. Caffeine and other purine alkaloid level in independent coffee plantlets transformed with *A. tumefaciens* harboring pSAT 201 construct. (NMT fragment in sense orientation with respect to the promoter)

Transformants with antisense construct, pSAT 202 resulted in caffeine levels ranging between 0.05-0.12% DW (Fig 3.37). The antisense construct resulted in reduction of caffeine, theobromine and 7-methylxanthine when compared to the control. There was 90% reduction in caffeine content in antisense transformants when compared to control. Effect of antisense RNA depends transcripts of antisense RNA and the homology between the sequence used in the antisense gene and target sequence (Lichtenstein and Nellen 1997). Extremely low levels of the three purine alkaloids estimated suggest that antisense to homologous sequence shared by several members of the multigene family is highly effective in suppressing the expression of the genes. Theophylline was observed only in control and sense transformants, while it was not detected with antisense constructs. It is known that theophylline is an intermediate in the catabolism of caffeine

rather than *de novo* caffeine biosynthesis. Therefore theophylline content was detected only when caffeine is accumulated.

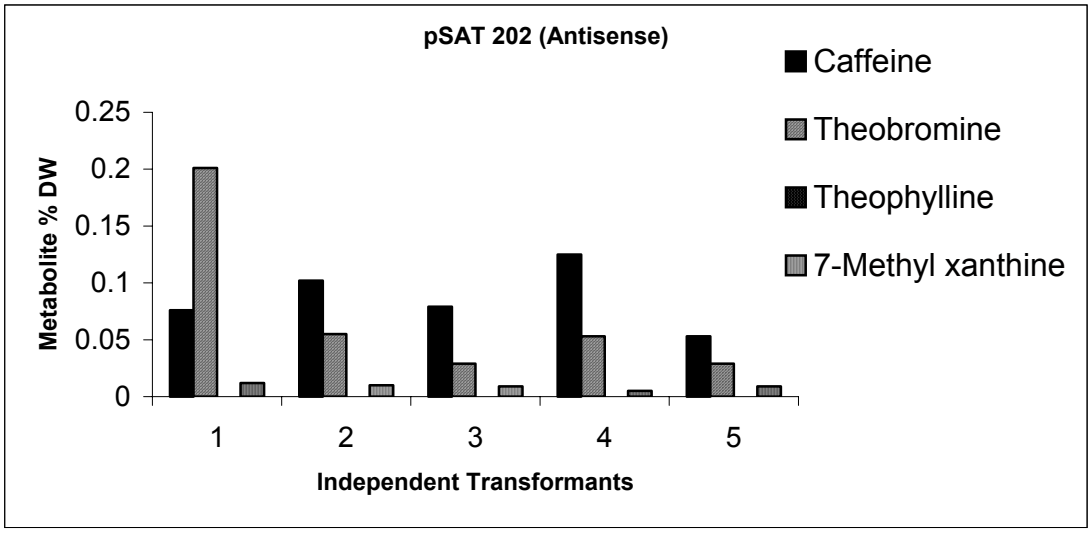


Fig 3.37. Caffeine and other purine alkaloid level in independent coffee plantlets transformed with *A. tumefaciens* harboring pSAT 202 construct. (NMT fragment in antisense orientation with respect to the promoter)

The pSAT 222 construct, which is an inverse repeat construct, expected to form dsRNA and induce RNAi. This was the most efficient in reducing the caffeine content and ranged up to 0.03% DW over control (Fig 3.38). Among the tested transgenic plants 3 of them did not show detecTable level of caffeine. RNAi was known to be more effective than other silencing strategies (Wesley *et al* 2001). Theophylline was not detected however; there was slight increase in theobromine and 7-methylxanthine contents in the RNAi transformants over control. In the present study, 100% reduction of caffeine was observed with RNAi when compared to 30% to 50% reduction reported by Ogita *et al* 2004. The RNAi construct made by Ogita *et al* (2004) comprised of 517 bp GUS gene fragment as spacer. In the present study a tighter loop of about 90 bp between the two arms of the invert repeat was adopted when compared to the 517 bp GUS gene fragment as spacer used by Ogita *et al* (2004). It is known that tightness of the hairpin loop can contribute to enhance RNAi silencing (Wesley *et al* 2001). Though, we have used homologous coding sequence, very little or no reduction in theobromine the immediate precursor of caffeine was noticed. These data clearly demonstrated reduction in caffeine

levels. However the extent of silencing varied in independent transformants. Theophylline was completely absent or not present in detecTable levels in transgenic plants with sense and inverse repeat construct where caffeine synthesis was blocked.

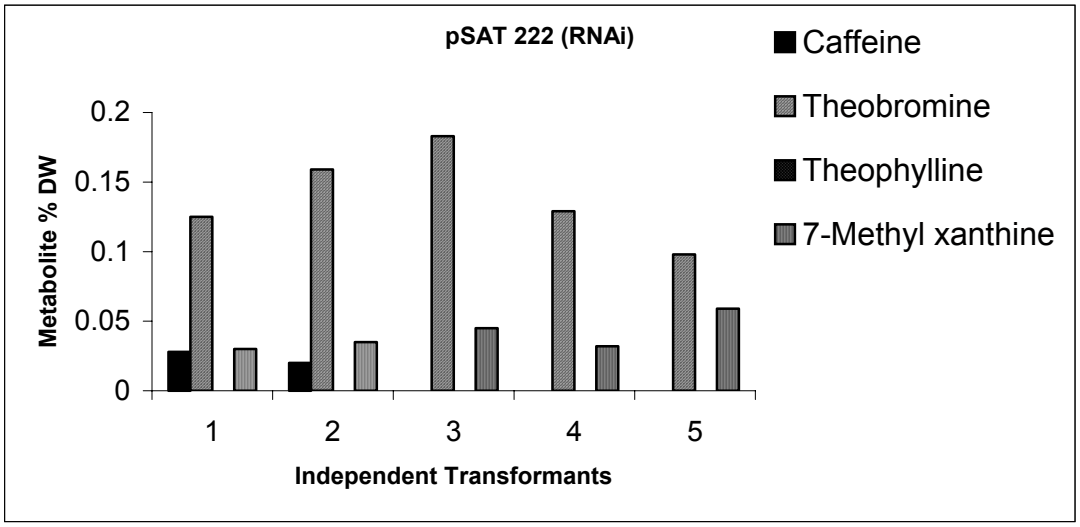


Fig 3.38. Caffeine and other purine alkaloid levels in independent coffee plantlets transformed with *A. tumefaciens* harboring pSAT 222 construct. (NMT fragment in inverse repeat orientation with respect to the promoter)

From the present study, it can be observed that though the co-suppression and RNAi constructs were not specific to individual NMTs, transformants were obtained that were mainly affected in only one of the three NMTs. This is in contrast to the earlier report (Ogita *et al* 2004), wherein non-specific RNAi effect was observed with specific RNAi construct. Ogita *et al* (2004) designed an RNAi construct containing 139-bp 3` untranslated region with sequence homology to *CaMXMT1* cDNA (AB048794). However down regulation of both *CaXMT1* and *CaMXMT1* transcripts were observed in transgenic lines. Three different kinds of constructs were used in the present study for down regulating caffeine synthesis. The efficiency of silencing varied with the type of construct used for PTGS.

3.8.5. Transcript levels of *N* methyl transferase

RT-PCR analysis (p.81, section 2.8.5.4) indicated reduction in *N* methyl transferase transcript levels in transgenic somatic embryos. The NMT transcripts were similar in

plants transformed with pCAMBIA 1301 and pSAT 201 (Fig. 3.39). Maximum reduction in transcripts of NMT was observed in coffee plants transformed with antisense and inverse repeat constructs. The primers CSF2 and CSR2 were not specific to individual NMTs and the transcript accumulated might comprise of several NMT genes having the conserved primer binding sites. Differences in the reduction rates of purine alkaloid content in the transgenic lines and concomitant reduction in the transcript levels for NMT genes was observed. Since the primer sequences for CSF2 and CSR2 were conserved in all the genes reported so far and possibly in the unknown genes also, the detection of transcripts in the case of RNAi despite of zero caffeine might be due to the accumulation of transcripts from the bypass pathway or unknown NMT genes.

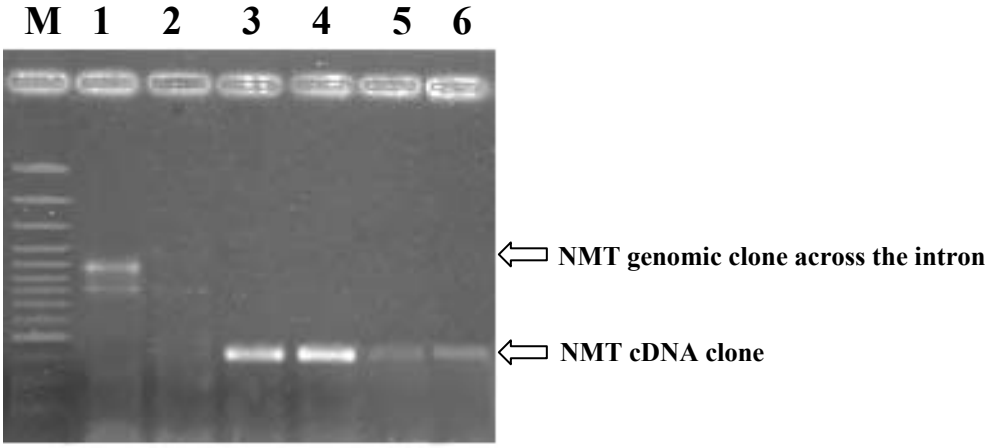


Fig 3.39. Analysis of *N*-methyl transferase transcript levels in transgenic *Coffea* plants by RT-PCR.

Lanes: M- Marker, Templet from, 1- *Coffea* genomic DNA, 2- *Coffea* RNA (without 1st strand cDNA synthesis), 3- cDNA from control plant transformed with pCAMBIA 1301, 4- cDNA from *Coffea* transformed with pSAT 201, 5- cDNA from *Coffea* transformed with pSAT 202, 6- cDNA from *Coffea* transformed with inverse repeat construct (pSAT 222).

Despite the fact that inverse repeat construct presented here was made with sequence conserved between all *N* methyl transferases of coffee, transformants were obtained that were mainly affected in third methylation step of the three NMTs. Such unexpected results are not unknown in RNAi mediated gene silencing. Allen *et al* 2004 observed the accumulation of rare alkaloids several steps upstream to the target gene codeinone

reductase (COR) in opium alkaloid biosynthesis pathway, which was silenced with chimeric RNAi construct; but did not observe the accumulation of immediate precursors as expected.

Naturally decaffeinated arabica coffee plants have been reported, where the low caffeine content observed was not due to enhanced degradation of caffeine, but more likely due to possible mutation in caffeine synthase gene (Silvarolla *et al* 2004). These plants accumulate theobromine indicating that these plants might be deficient in caffeine synthase, which acts on theobromine. The possibility of using coffee demethylases, human demethylases or demethylases from other sources may be another alternative to degrade caffeine in the plant. Recently our group has isolated the promoter for theobromine synthase from *Coffea canephora* (Satyanarayana *et al* 2005). The promoter sequence may 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). The 11S globulin promoter demonstrated its ability to drive transgene expression in coffee endosperm (section 3.9). Development of transgenic plants with silencing constructs driven by seed specific promoters such as 11S globulin promoter may be highly useful in down regulation of caffeine only in the seeds.

3.8.6. Summary

Low caffeine and zero caffeine coffee plants were obtained as evident from HPLC analysis and RT PCR. The transformants obtained in the present study were mainly affected in third methylation step of the three NMTs. These results may be highly useful in developing transgenic coffee with seed specific silencing.

3.9. OPTIMIZATION OF ELECTROPORATION MEDIATED GENE DELIVERY: ANALYSIS OF *N* METHYL TRANSFERASE AND 11S GLOBULIN PROMOTERS IN COFFEE.

Background:

The promoter for one of the *N* methyl transferase gene has been cloned and demonstrated the reporter gene expression in *Nicotiana tabacum* (Satyanarayana *et al* 2005). Similarly the promoter for gene encoding seed specific 11S globulin has been cloned (Marraccini *et al* 1999). 11S globulins are the major seed storage proteins in Coffee beans. These storage proteins are also found in low levels in somatic and zygotic embryos (Yuffa *et al* 1994) of coffee. Tissue specific promoters such as 11S globulin could be very good candidate promoter, which can be used for silencing caffeine biosynthesis in tissue specific manner in endosperm and embryos. As a prelude to this, it is essential to study the function of isolated promoter in Coffee tissues especially in somatic embryos where caffeine is synthesized and down regulation of this pathway can be analyzed in the early stage of transgenic plants.

Electroporation is a DNA delivery technique, which utilizes a high intensity electric pulse to create transient pores in the cell membrane, hence facilitates the uptake of DNA.

This section deals with development of a rapid transformation protocol by optimizing electroporation in somatic embryos, endosperm tissues to analyze reporter gene expression driven by 11S globulin and *N* methyltransferase promoter in *C. canephora*.

3.9.1. Optimization of tissue electroporation protocol in *Coffea canephora*

A total of 150-200 explants were subjected to electroporation (p.82, section 2.9.1) for each experiment. The efficiency of electroporation-mediated transformation was determined by the number of somatic embryos with GUS expression and the data was expressed in terms of percentage transformation frequency.

The efficiency of DNA uptake of linear or circular plasmid pCAMBIA 1301 at 275, 500 and 750 V/cm field strengths has been estimated, 48 h after electroporation, by analyzing transient expression of the *uid A* gene in the cells. There was no visible GUS activity after a pulse of 275 V/cm. GUS activity was observed in somatic embryos electroporated at 500V/cm (Table 3.14). Gus assays demonstrated that the cell wall degradation using

enzyme treatment was absolutely required for delivery of plasmid vectors and expression of transgenes. The transformation frequency was varied between 0.4-4.8 % (Table 3.14).

Table 3.14 Influence of field strength and type of plasmid vector in electroporation mediated gene delivery to *Coffea canephora* somatic embryos

Type of plasmid	Field strength [a] V/cm	Percentage explants with GUS expression*	
		Without spermidine	With spermidine [#]
pCAMBIA 1301 circular	250	0	0
	500	2.6	4.8
	750	1.2	3.2
pCAMBIA 1301 linear	250	0	0
	500	2.5	4.5
	750	0.4	1.3

* The data represents mean values of 10 replicates and each replicate comprised 15 explants.

[#] Spermidine was added to the electroporation buffer. [a] Electroporation was carried out at constant capacitance of 900μF. GUS expression was not observed in promoterless *uid A* constructs pCAMBIA 1381 as well as in coffee tissues electroporated without plasmid DNA.

Table 3.15 Transient expression of *uid A* reporter gene driven by NMT and 11S globulin promoter in different electroporated tissues of *Coffea canephora*

Sl No	Construct	Nature of tissue used for electroporation	*Percentage explants with GUS expression
1	pCAMBIA 1301	Globular embryos	3.1
		Torpedo embryos	3.5
		Endosperm	0.5
3	pPCTS 745	Globular embryos	2.8
		Torpedo embryos	3.5
		Endosperm	12.0
4	pPCGB 959	Globular embryos	1.5
		Torpedo embryos	5.6
		Endosperm	32.0

*The data represents mean values of 10 replicates and each replicate comprised 15 explants.

No GUS expression was observed in untransformed tissues and promoterless *uid A* containing vector pCAMBIA 1381

3.9.2. Analysis of NMT and 11S globulin promoter in somatic embryos

No GUS expression was detected in somatic embryos electroporated without plasmid DNA and in somatic embryos electroporated with pCAMBIA 1381 vector which harbors promoterless *uid A* gene (Fig 3.40a). There was not much difference in transformation frequency in somatic embryos electroporated with linear or circular plasmid vector (Table 3.14). Addition of 0.2mM spermidine in the electroporation buffer enhanced transformation frequency by almost 2 fold (Table 3.14). Increased transformation efficiency was observed in torpedo stage embryos (Table 3.15). Among the tested tissue types for electroporation mediated gene delivery, endosperm tissues showed 12-32% transformation frequency and a very intense GUS staining (Table 3.15).

No GUS expression was noticed in control embryos transformed with promoterless *uid* A construct (Fig 3.40a). GUS expression was observed in somatic embryos transformed with *uid A* gene driven by coffee 11S globulin (Fig 3.40b) and CaMV 35S promoter (Fig 3.40c). Stable expression of NMT promoter was also observed Fig 3.40d.

3.9.3. Analysis of NMT and 11S globulin promoter in endosperm tissues

GUS activity was observed in immature endosperm tissues electroporated with *uid A* gene driven by CaMV 35S promoter (Fig 3.41a). Incorporation of spermidine found to enhance transformation frequency and more intense GUS activity (Fig 3.41b) was observed in these tissues when compared to tissues electroporated in the absence of spermidine (Fig 3a). The present study clearly demonstrated the ability of 11S globulin (Fig 3.41c) and NMT (Fig 3.41d) promoters to drive transgenes in endosperm tissues of *Coffea canephora.*

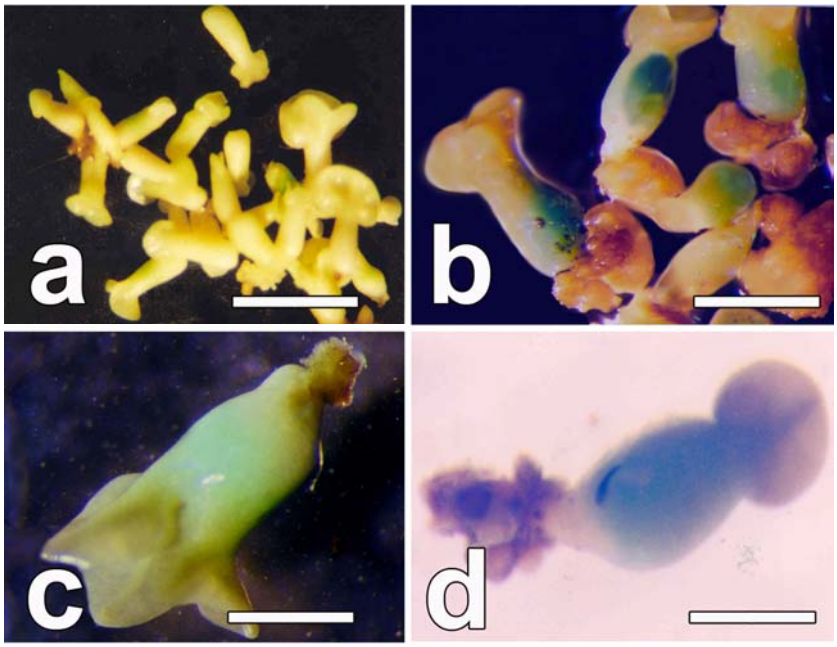


Figure 3.40: GUS staining in electroporated somatic embryos

Fig. 3.40a *Coffea* transformed with promoterless construct pCAMBIA 1381 (Bar- 3mm).

Fig. 3.40b *Coffea* electroporated with GUS gene driven by 11S globulin promoter (Bar- 1.5mm).

Fig. 3.40c *Coffea* electroporated with GUS gene driven by CaMV promoter (Bar- 0.8mm).

Fig. 3.40d Stable expression of GUS gene in regenerated transgenic secondary embryos of *Coffea* electroporated with GUS gene driven by NMT promoter (Bar- 0.8mm).

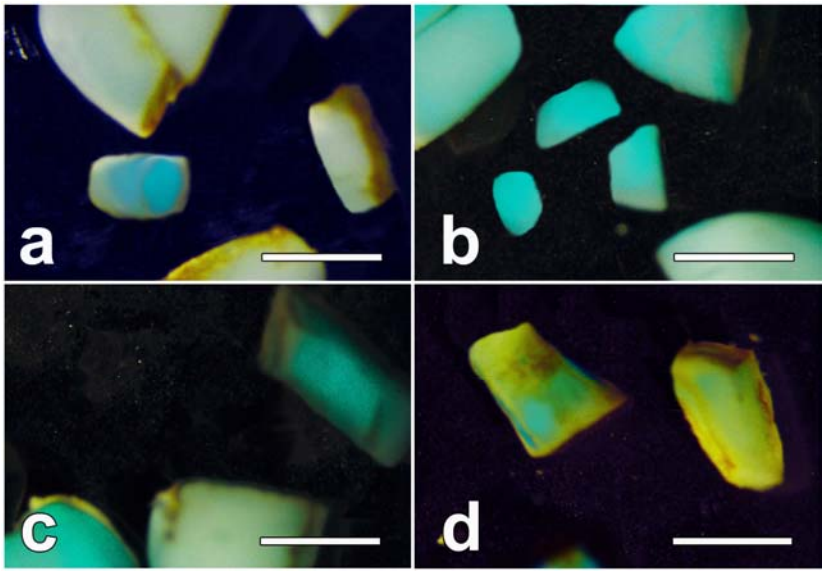


Figure 3.41: GUS staining in electroporated endosperm with control and different promoter constructs

Fig 3.41a *Coffea* endosperm tissues electroporated with GUS gene driven by CaMV promoter without spermidine treatment (Bar- 2mm).

Fig 3.41b *Coffea* endosperm tissues electroporated with GUS gene driven by CaMV promoter with spermidine treatment (Bar- 2mm).

Fig 3.41c *Coffea* endosperm tissues electroporated with GUS gene driven by NMT promoter (Bar- 2mm).

Fig. 3.41d *Coffea* endosperm tissues electroporated with GUS gene driven by 11S globulin promoter (Bar- 2mm).

3.9.4. Stable transformation using electroporation mediated gene delivery

Attempts were made to regenerate the tissues electroporated with NMT promoter. The somatic embryos were cultured on secondary embryogenic medium containing 5-20 mgl^{-1} hygromycin. Transgenic secondary embryos were produced after a period of 4 months. The stable expression of *uid A* reporter gene was observed in transgenic secondary embryos (Fig 3.40d). The overall efficiency of regeneration of transgenic secondary embryos was found to be upto 0.3%. However rapid browning of the tissues is a major hindrance for regeneration of somatic embryos from the electroporated tissues.

3.9.5. Discussion

These results are in accordance with the previous report of tissue electroporation where tissue electroporation has been used successfully in stable transformation of *Coffea arabica* (Fernandez and Menendez 2003). However the pretreatment step was suitably modified for the endosperm tissues. One hour pretreatment or electroporation without pretreatment with cell wall degrading enzymes found to be better and prolonged incubations resulted in blackening of the tissues.

The results clearly demonstrated that, the NMT promoter and 11S globulin promoter could drive the expression of reporter genes in somatic embryos and endosperm tissues of *Coffea canephora.*

Electroporation has been used successfully in various plant systems including sweet potato (Mitchell *et al* 1998), maize (D'Halluin *et al* 1992), (Luong *et al* 1995), rice (Tada *et al* 1990, Arencibia *et al* 1998) and wheat (Ke *et al* 1997, Kloti *et al* 1993). Arencibia *et al* 1998 reported use of spermidine in the electroporation buffer to enhance the transformation efficiency. Results presented here also indicate that spermidine helps in

increasing the transformation efficiency. Songstad *et al* (1993) showed transient expression of the GUS gene after electroporation in a buffer containing spermidine. It has been shown that spermidine can induce condensation and clustering of DNA molecules (Hansma *et al* 1998) and this may facilitate uptake of more DNA by the cells, and as a result, more intense transgene expression. These evidences prove the possibility of using electroporation as an efficient method for functional genomic studies in plants including *Coffea canephora.*

The cloning of the promoter for the gene involved in caffeine biosynthetic pathway (Satyanarayana *et al* 2005) opened the possibility of studying the molecular mechanisms that regulate the production of caffeine in different tissues of *Coffea* sp. The NMT 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) to develop caffeine free coffee plants.

This is the first report on demonstration of expression of reporter gene driven by coffee promoters in coffee embryos and endosperm. The results indicate the possibility of using 11S globulin promoter for driving the post-transcriptional gene silencing constructs to down regulate the gene expression in seed specific manner. Since NMT and 11s globulins express in somatic embryos also, it may be possible to analyze the down regulation of NMTs in the embryo stage and may be highly useful in perennial crop like coffee where seed setting requires more than 4 years.

3.9.6. Summary

In conclusion, an efficient method of electroporation mediated gene delivery in somatic embryos and endosperm tissues of *Coffea canephora* was developed. We have also demonstrated the expression of marker gene *uid* A driven by NMT and 11S globulin promoters in somatic embryos and endosperm tissues of *Coffea*.

3.10. LOCALIZATION OF *N* METHYL TRANSFERASE OF CAFFEINE BIOSYNTHETIC PATHWAY IN VACUOLAR SURFACE OF *COFFEA CANEPHORA* ENDOSPERM ELUCIDATED THROUGH LOCALIZATION OF GUS REPORTER GENE DRIVEN BY NMT PROMOTER.

Background:

The sequential conversion of xanthosine to caffeine through 7- methylxanthosine, 7-methylxanthine and theobromine occurs through the action of *N*-methyltransferases (NMTs) that use *S*-adenosyl-L-methionine (SAM) as the methyl donor (Kato and Mizuno 2004, Uefuji *et al* 2003). Information on cellular localization of coffee NMTs are lacking. It has also been reported that chlorogenic acid (CGA) and caffeine are bound together in the cell (Waldhauser and Baumann 1996). The free CGA and also the CGA-caffeine complex are stored intracellularly in the central vacuole (Martin *et al* 1987, Horman and Viani (1971) and caffeine accumulation to a certain extent correlates with the CGA concentration in the cells (Baumann and Rohrig 1989). The sub-cellular targeting of proteins mainly determined by the signal peptides and in coffee NMTs the 1st exon region may encode for the same.

The objective of this study has been to analyze expression of *uid* A reporter gene driven by NMT promoter in the presence and absence of the first exon (75bp) of the NMT gene in relation to the localization of coffee NMT enzymes in the cells of coffee endosperm.

3.10.1. Cellular localization of GUS reporter gene driven by NMT promoter with and without the first exon.

Electroporation of coffee endosperm was carried out with constructs containing the *uid* A gene driven by NMT promoter bearing the 1st exon and with construct lacking the 1st exon (p.85, section 2.10.2). GUS expression was not detected in endosperm electroporated without plasmid DNA and in endosperm electroporated with pCAMBIA 1381 vector harboring a promoterless *uid A* gene (Fig 3.42a). GUS expression was observed in peripheral cells of the endosperm transformed with *uid A* gene driven by coffee NMT promoter containing the 1st exon (Fig 3.42b) and also in those tissues transformed with the NMT promoter constructs devoid of the 1st exon (Fig 3.42c). The results clearly demonstrated that the NMT promoter could drive the expression of

reporter genes in endosperm tissues of *Coffea canephora.* GUS activity was observed in positive control endosperm electroporated with pCAMBIA 1301 wherein the *uid* A gene was driven by CaMV 35S promoter (Fig 3.42d).

Interestingly in tissues electroporated with constructs containing the 1st exon sequence, activity of GUS was targeted to the external surface of the vacuole (Fig 3.42e). Deletion of the 1st exon resulted in a diffused expression of *uid* A gene in the cytoplasm, not localized to the areas around the vacuole.

3.10.2. Localization of caffeine-chlorogenic acid complex

Staining for chlorogenic acid (CGA) according to the method of Reeve (p.86, section 2.10.4) (Reeve 1968) revealed localization of CGA in the vacuole (Fig 3.42g).

3.10.3. Immuno-cytolocalization of NMTs

The NMTs of coffee were traced using antibodies raised against coffee NMTs and found to be localized surrounding the vacuole (Fig 3.43a and 3.43b) as seen with GUS expression driven by NMT promoter containing the first exon (Fig 3.42e).

3.10.4. *In silico* analysis of the first exon of NMT and other methyl transferase genes

In silico analysis of the 1st exon sequence of the coffee NMT revealed homology to that of methyl transferase from many different plant species. Two motifs "LHMN" and "GETSYAKNS" were particularly conserved (Fig 3.44). The *N* terminal of the tea caffeine synthase is FMNRGEESSYAQNSQFTQV (Kato *et al* 1999).

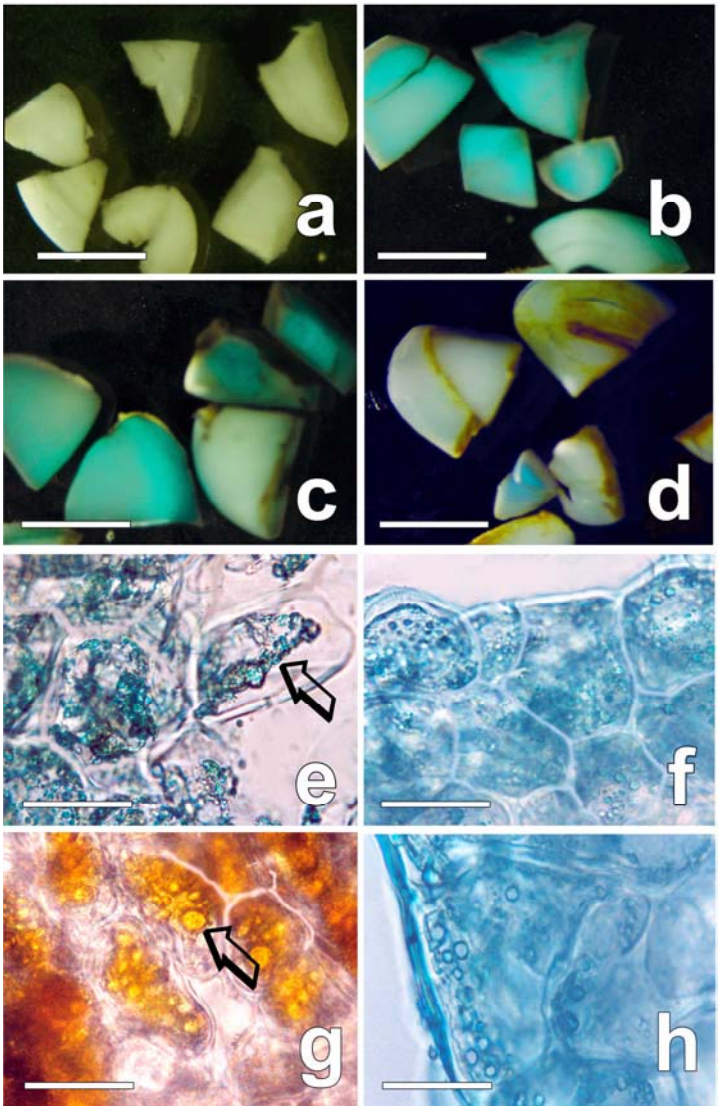


Figure 3.42a: ***Coffea*** **transformed with promoterless** ***uid*** **A construct pCAMBIA 1381 (Bar-2.5mm).**

Figure 3.42b: ***Coffea*** **electroporated with GUS gene driven by NMT promoter containing the first exon (Bar-2.5mm).**

Figure 3.42c: ***Coffea*** **electroporated with GUS gene driven by NMT promoter without the first exon (Bar-2.5mm).**

Figure 3.42d: ***Coffea*** **electroporated with GUS gene driven by CaMV promoter (Bar-2.5mm).**

Figure 3.42e: Sub cellular localization of GUS expression in endosperm tissues electroporated with GUS gene driven by NMT promoter containing the first exon (Bar-30µm).

Figure 3.42f: Sub cellular localization of GUS expression in endosperm tissues electroporated with GUS gene driven by NMT promoter lacking the first exon (Bar-30µm).

Figure 3.42g: Sub cellular localization of chlorogenic acid (Caffeine chlorogenic acid complex) (Bar-30µm).

Figure 3.42h: ***Coffea*** **electroporated with GUS gene driven by CaMV 35S promoter (Bar-30µm).**

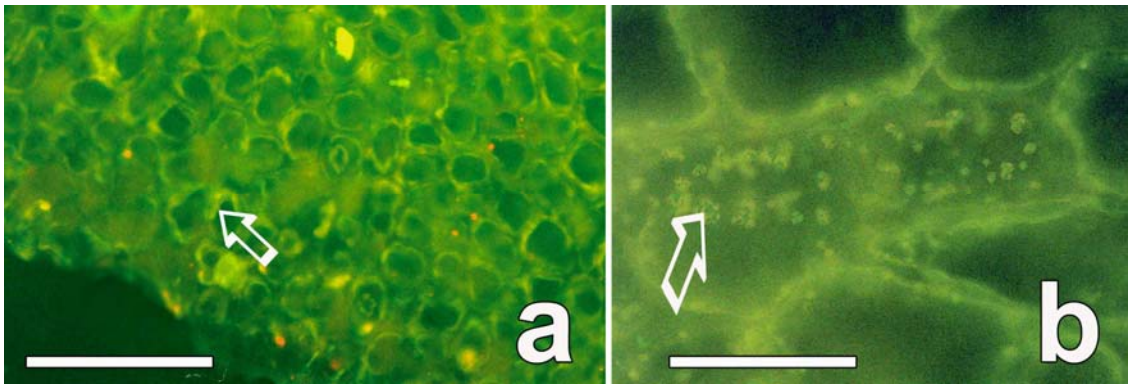


Figure 3.43a: Immunocytolocalization of NMT enzymes in endosperm tissues of *Coffea canephora* (Bar-180μm).

Figure 3.43b: Localization of NMT enzymes in a single endosperm cell (Bar-10μm)

```
 1. MEVMR-ILHMN-KGNGET-SYAKNS---
 2. MEVMR-ILHMN-KGNGET-SYAKNS---
 3. MEVMR-VLHMN-KGNGET-SYAKNST--
 4. MEVMR-VLHMN-KGNGET-SYAKNS---
 5. MELQE-VLHMN-EGEGDT-SYAKNASDN
 6. MELQE-VLHMN-GGEGEA-SYAKNSSFN
 7. MEVVE-VLHMN-GGTGDA-SYASNS---
 8. MNVEA-VLHMK-EGVGET-SYAKNST--
 9. MKVMKKLLCMNIAGDGET-SYANNSGL-
10. -------F-MNR---GEESSYAQNSQFT
```

Figure 3.44: Alignment of putative signal peptides from nine different methyl transferases.

1. *N*-methyltransferase *Coffea canephora*; **gi|66774632**
2. *N*-methyltransferase *Coffea arabica*; **gi|13365694.**
3. Jasmonic acid carboxyl methyltransferase *Capsicum annuum*; **gi|77745528.**
4. *S*-adenosyl-L-methionine:jasmonic acid carboxyl methyltransferase *Arabidopsis thaliana* **gi|13676829.**
5. Methyl transferase *Brassica juncea*; **gi|55442027.**

6. Jasmonate-*O*-methyltransferase *S*-adenosyl-L-methionine:jasmonic acid carboxyl methyltransferase Floral nectary-specific protein; **gi|56748931.**
7. *S*-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase *Stephanotis floribunda*; **gi|13235641.**
8. Putative *S*-adenosyl-L-methionine *Oryza sativa* japonica cultivar-group; **gi|54291645.**
9. Benzoate carboxyl methyltransferase *Antirrhinum majus*; **gi|54291645.**
10. *N* methyl transferase *N* terminal amino acid sequence from Tea (Kato *et al* 1999).

3.10.4. Discussion

The mechanism for uptake and sequestration of caffeine in vacuole is yet to be investigated. Binding of caffeine to chlorogenic acids may allow the accumulation of these compounds in vacuoles against a concentration gradient (Ashihara and Suzuki 2004). Baumann and co-workers suggested that vacuolar compartmentation of purine alkaloids depends exclusively on the formation of complexes with chlorogenic acids (Baumann and Röhrig 1989). Results presented here are in accordance with the above observations.

Mizuno *et al* (Mizuno *et al* 2003) reported the isolation of caffeine synthase (*CCS1*) clone from coffee endosperm. However, the predicted amino acid sequences of *CCS1* are only about 40% similar to those of tea caffeine synthase (*TCS1*). It was found that the tea caffeine synthase, which is a key enzyme in catalyzing the final two steps of caffeine biosynthesis in tea, is located in chloroplasts (Ashihara and Suzuki 2004). It is also known that, the NMTs are SAM dependant and SAM synthetase was also detected exclusively in the cytosol and SAM is produced in the cytosol of tea leaves (Ashihara and Suzuki 2004). Results presented here suggest that coffee NMTs may be located in the vacuolar surface, in the cytoplasm and not in the chloroplast.

Sub-cellular localization of *Coffea arabica* methyl xanthine methyl transferase (*CaMXMT*) using the fusion protein of *CaMXMT* and GFP demonstrated its existence predominantly in the cytoplasm of onion epidermal cells. It was also predicted using the PSORT programme that the *CaMXMT* is localized in the cytoplasm (Ogawa *et al* 2001). Kolosova *et al* (2001) localized the *S*-denosyl-L-Met:benzoic acid carboxyl methyltransferase (BAMT) to the cytoplasm of the epidermal cells of the snapdragon flower. In the present study, the *CaMXMT* enzyme was located, as we have noticed for

the caffeine *N* methyl transferase in close association with the vacuole surface. Pimenta *et al* (1998) showed that though most of the Barely *O*-methyl transferase was cytoplasmic, a portion was adsorbed on the vacuole.

3.10.5. Summary

This is the first report wherein the sub-cellular targeting of NMTs in coffee was demonstrated using GUS reporter constructs driven by NMT promoter having deletion of the first exon and by immunocytolocalization. The findings of this study would be helpful in understanding the regulation of caffeine biosynthesis and also to utilize the NMT promoters to alter the expression of individual NMT genes in transgenic coffee plants in a tissue specific manner.

CHAPTER IV
Summary and Conclusion

4.1. BRIEF BACKGROUND

Coffee is an important plantation crop and used worldwide for making beverage. Commercial coffee is mainly confined to two species *C. arabica* (2n=44) and *C. canephora* (2n=22). *C. arabica* is a polyploid species and it produces high quality coffee with comparatively low caffeine content, but is susceptible to diseases. *C. canephora* Pierre ex Froehner cv. Robusta, is a diploid (2n=22) species and contributes to 25% of the total coffee production (Berthouly and Etienne, 1999). *C. canephora* coffee was found to be a very good substitute for the *C. arabica* which is highly susceptible to coffee leaf rust disease caused by *Hemileia vastatrix*. *C. canephora* produces coffee with higher caffeine content but grows more vigorously than *C. arabica* and *C. liberica.*

Coffee has been harvested from an area of more than 104 million hectare (FAO 2003). It is grown in about 80 countries across the globe of which 51 are considered to be the major producers (Anonymous 1996). Coffee production in India has reached to 275,000 tones from harvested area of 328,000 hectares in the year 2004 (FAO, 2005) Today, in India, coffee occupies an important position among the export commodities particularly in the plantation sector.

Coffee breeding by conventional methods is a long process involving several different techniques, namely, selection from wild populations followed by hybridization 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. Today coffee biotechnology has opened up new avenues in the area of quality improvement, crop management and molecular breeding.

Caffeine has been the subject of extensive research. Caffeine is an important constituent of coffee and the one responsible for the stimulating effect of the coffee drink. It is present in the bean chiefly as potassium caffeine chlorogenate (The Wealth of India, 1950). The deleterious effects of coffee and caffeine are well documented (Table 1.4, 1.5). However possible reported adverse effects include withdrawal symptoms, coronary heart disease (Kawachi *et al* 1994), fibrocystic breast disease (Garattini 1993, Curatolo and Robertson 1983), several types of cancer, (La Vecchia 1993), and reproductive health effects (Hinds *et al* 1996). This led coffee industry to provide an artificially de-caffeinated coffee. Therefore health conscious consumers welcome the developments

towards naturally decaffeinated coffee. This would be important for *C. canephora*, for which the caffeine content on a dry matter basis varies from 1.2% to 3.5% (Charrier and Berthaud 1975). Caffeine is lacking in most Malagasy species (Charrier and Berthaud 1975) and in the East-African species *C. pseudozanguebariae.* Unfortunately, caffeine-free species are low yielding and give a beverage of poor tasting quality. *C. pseudozanguebariae* yields a highly bitter coffee beverage, which could be due to a specific heteroside, diterpene, found in large amounts in *C. pseudozanguebariae* beans. Silverolla and co-workers (2004) discovered three caffeine free plants obtained from Ethiopia. These plants found to possess Theobromine but not caffeine indicating that the plants might be deficient in enzyme caffeine synthase.

The developments *in vitro* micro propagation techniques such as somatic embryogenesis, scale-up of somatic embryogenesis using bio-reactors, 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 (Reviewed by Carneiro 1999) supported the transgenic research. A number of protocols have been developed for various genotypes of *Coffea*. Since the embryogenesis process in coffee is genotype specific, it is very essential to develop regeneration protocol through embryogenesis in varieties or cultivars used in the present study.

The success of transgenic research in coffee has opened up new avenues for quality improvements. A number of transformation systems have been reported (Reviewed by Carneiro 1999) for arabica and robusta coffee, however only a few reports demonstrated the recovery of whole plant with stable integration of DNA. A major hindrance to genetic transformation of coffee is its slow rate of regeneration *in vitro*.

Since solvent extraction is used for decaffeination, an alternative and health conscious approach would be to identify naturally occurring low caffeine lines (Srinivasan 1996). Although low caffeine material is currently available, for a variety of reasons, none is suitable for commercial exploitation (Mazzafera *et al* 1991). But inferior quality beans and low productivity of trees of this species is a major hindrance for commercial coffee production. Attempts to transfer the caffeine-free trait from *Mascarocoffea* to *C. arabica* and *C. canephora* have been unsuccessful because of the infertility of the hybrid progeny (Charrier, 1978). Therefore there is a need to develop alternative methods such as gene

silencing approaches in order to develop low caffeine coffee without compromising with the cup quality.

In Coffee plants, caffeine is synthesized through three independent methylation steps from xanthosine (Ogawa *et al* 2001, Uefuji *et al* 2003). The main caffeine biosynthesis route is illustrated below.

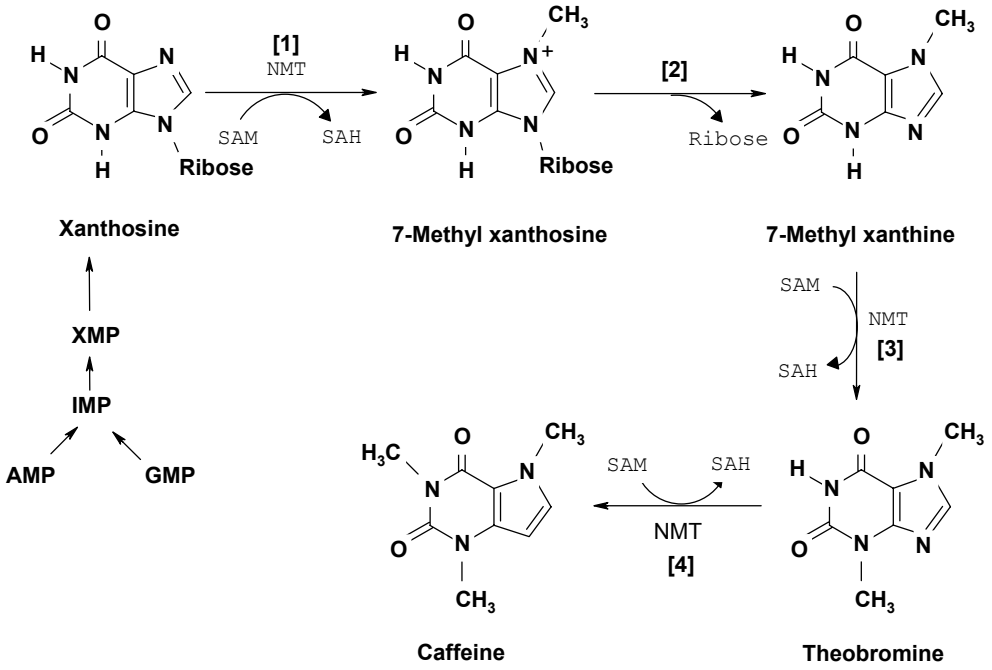


Fig 4.1. The major route of caffeine biosynthetic pathway in coffee plants.

[1] xanthosine *N*-methyltransferase, [2] *N*-methylxanthine nucleosidase [3] theobromine synthase and / or caffeine synthase [4] caffeine synthase (Ashihara and Suzuki 2004).

Three SAM-mediated methylations of xanthosine results in the formation of caffeine. The cloning of the promoter for the gene involved in caffeine biosynthetic pathway opens 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 (RdDM) (Mette *et al* 2000).

Considering all these aspects, the present study was aimed at development of efficient regeneration system through somatic embryogenesis, secondary embryogenesis, genetic transformation system using *Agrobacterium tumefaciens* as well as

Agrobacterium rhizogenes and generation of low caffeine transgenic plants through post transcriptional gene silencing in *Coffea canephora.*

4.2. OBJECTIVES OF THE STUDY:

6. Development of somatic embryogenesis protocols in *Coffea* sp.
7. Optimization of *A. rhizogenes* mediated genetic transformation system using a model system.
8. Development of genetic transformation system for *Coffea* sp.
9. Expression of desired antisense genes for *N*-methyl transferases to block caffeine production with a view to improve processing characteristics and value addition.
10. Development of electroporation mediated gene delivery systems in *Coffea* somatic embryos and endosperm tissues.

4.3. SUMMARY OF RESULTS

4.3.1. Establishment of *in vitro* cultures

In vitro seed germination, callus induction, embryogenesis and secondary embryogenesis was optimized. The seeds were germinated in 30-40 days in ¼ MS basal medium and vitamins with 2% sucrose; 40 mgl^{-1} cysteine HCl. Callus induction was maximum in medium comprising 2,4-D, IBA and 2 iP. The callus was further transferred to callus multiplication medium comprising 1 mgl^{-1} 2,4-D, 4 mgl^{-1} BA resulted in rapid multiplication of callus. Two kinds of callus were obtained in the callus multiplication medium i.e., embryogenic callus (EC) and non-embryogenic callus (NEC). Globular somatic embryos were obtained (85% embryogenesis) from embryogenic calli in medium comprising IAA 0.5$mgl^{-1}$and BA 0.25mgl^{-1}. Embryogenic callus was produced in medium comprising BA alone at 2mgl^{-1} (Table 3.4). These globular embryos matured and turned to torpedo stage when cultured on ½ strength MS medium with B5 vitamins for 45-60 days. In conclusion, somatic embryogenesis was achieved in *Coffea* sp. Embryogenic and non-embryogenic callus was noticed. Further studies were planned to identify the role of polyamines and calcium in determination of embryogenic nature in *Coffea* sp.

4.3.2. Influence of polyamines on secondary embryogenesis.

The biochemical basis for the embryogenic or non-embryogenic nature for these calluses was assessed. Since polyamines known to regulate morphogenesis the endogenous levels of polyamines were estimated. The embryogenic callus contained 11 fold more spermine and 3.3 fold higher spermidine when compared to NEC. Somatic embryogenesis was obtained in medium comprising 0.5 mgL^{-1} IAA and 0.25 mgL^{-1} BAP. These globular embryos matured and turned to torpedo stage. The torpedo embryos were able to undergo repeated cycles of secondary embryogenesis when cultured on the medium comprising 0.5 mgL^{-1} IAA and 0.25 mgL^{-1} BAP. Medium containing 9.08 µM TDZ supported direct somatic embryogenesis in *C. arabica* and *C. canephora.* The higher sucrose concentration (with 3% sucrose) reduced somatic embryogenesis in *C. canephora.* Experiments were also carried out to study the influence of exogenous incorporation of polyamines in embryo cultures. Further studies were carried out by incorporating the ethylene action inhibitor silver nitrate, since polyamines and the ethylene pathway are closely linked. Incorporation of 40-60µM silver nitrate an ethylene action inhibitor enhanced the embryogenesis and secondary embryogenesis.

3.3.3. TDZ induced direct embryogenesis in *C. arabica* and *C. canephora*

Background and objective.

Direct somatic embryogenesis was successfully achieved for the first time using TDZ as a growth hormone in *C. arabica* and *C. canephora*. Healthy regenerates were obtained using TDZ as a sole growth hormone. This study was highly useful in further work on genetic transformation of *Coffea canephora*.

3.4.4. Somatic embryogenesis under the influence of silver nitrate.

Highly efficient somatic embryogenesis and secondary embryogenesis was achieved under the influence of an ethylene action inhibitor silver nitrate. Silver nitrate 40-60µM promoted somatic embryogenesis in *C. arabica* and *C. canephora*. The results indicate that regulation of ethylene perception pathways leads to enhancement in embryogenesis responses. The results were found to be useful in transformation of *Coffea canephora*.

4.3.5. Involvement of calcium in somatic embryogenesis.

Calcium was found to play a crucial role in somatic embryogenesis in *Coffea canephora*. Maximum embryogenesis and secondary embryogenesis was observed at 5mM and 1mM exogenous calcium respectively. Depletion of calcium resulted in formation of non-embryogenic callus. Protein analysis revealed the presence of CDPK (58kda) isoforms only in embryogenic callus and somatic embryos. The 58kDa ccCDPK isoform found to possess kinase activity. The results demonstrated the involvement of calcium and possible involvement of calcium mediated signaling through ccCDPKs during embryogenesis and secondary embryogenesis.

4.3.6. Optimization of *A. rhizogenes* mediated genetic transformation in *Nicotiana tabacum*: assessment of physical and chemical treatments.

Agrobacterium rhizogenes mediated transformation efficiency was assessed under the influence of sonication, calcium treatment, acetosyringone and macerating enzymes in suitable combinations in *Nicotiana tabacum* as a model system. Manual wounding resulted in 21% transformation frequency. Where as sonication resulted in 2.2 fold increase, followed by sonication with $CaCl_2$ treatment resulted in 2.5 fold increase and sonication with acetosyringone treatment resulted in 4.1 fold increase in transformation frequency. However, sonication with macerating enzyme treatment resulted in 1.5 to 5.25-fold decrease in transformation frequency. Micro wounding through sonication followed by acetosyringone treatment enhanced transformation frequency substantially. The results of this study was taken into consideration during standardization of transformation protocol in *Coffea canephora* and found to be highly useful.

4.3.7. *A. rhizogenes* mediated genetic transformation studies on *Coffea canephora.*

After developing efficient regeneration protocols, transformation studies were carried out using *A. rhizogenes* strain A4. Hairy roots were initiated from *A. rhizogenes* co-cultivated explants. These experiments demonstrated the susceptibility of *Coffea canephora* to *A. rhizogenes* mediated transformation. Subsequently the explants were co cultivated with *A. rhizogenes* harboring a binary vector pCAMBIA 1301 and transgenic plants were obtained. The putative transformants were subjected to PCR and Southern blot to confirm

the transgenic nature. Secondary embryos and plantlets growing on 10 mg l^{-1} hygromycin selection medium were subjected to GUS assay and confirmed the expression of transgenes in the T_0 lines. Strong expression of intron *uid* A gene was obtained in transgenic plants. Different T-DNA integration patterns were observed in transgenic plants. In general the transgenic plants devoid of *rol* genes were normal in appearance without any abnormalities associated with the *rol* genes. Sixteen months old plantlets were tested for the presence of the *rol* A gene from Ri-T-DNA and the *hpt* II gene from the binary vector T-DNA. PCR, using gene specific primers for hygromycin phosphotransferase resulted in amplification of 479bp fragment of the *hpt* II gene in all five hygromycin resistant plants. However, 60% of these plantlets were negative for presence of the *rol* A gene. These results clearly indicate the independent integration of T-DNA from the pCAMBIA 1301 binary vector and the Ri native plasmid from *A. rhizogenes*. The present study indicates that, during transformation process co-transfer of T-DNA from both Ri plasmid and binary vector are not obligatory. Southern analysis further confirmed the transgenic nature and stable integration of T-DNA in hygromycin resistant plantlets. The presence of several fragments of variable size in some lines indicates insertion of multiple copies of the T-DNA into the plant genome. In conclusion, this study resulted in the development of a protocol for genetic transformation of *Coffea canephora*. *Agrobacterium rhizogenes* can deliver transgenes present in T-DNA of binary vectors designed for use in *A. tumefaciens*. Optimisation of hormonal regimes and culture conditions can help to avoid an intervening hairy root phase in the regeneration of plants transformed with *A. rhizogenes*.

4.3.8. Transformation of coffee for down regulation of caffeine biosynthesis.

In subsequent experiments only *A. tumefaciens* was used to transform *Coffea* with constructs for posttranscriptional gene silencing (PTGS). The binary vector pCAMBIA 1301 was used as the primary vector and co-suppression (pSAT 201), antisense (pSAT 202), and inverse repeat (pSAT 222) constructs containing homologous sequence of N methyl transferase was used for transformation of coffee. Transgenic secondary embryos and plantlets were produced under hygromycin selection. Strong expression of intron *uid* A gene was obtained in transgenic plants. The transgenic plantlets were confirmed by

PCR for the presence of *hpt* II gene and for decreased caffeine content in the plantlets. Transformation using pSAT 222 construct was found to be most efficient and the caffeine content was less than 0.025%DW. In some plants transformed with pSAT 222, caffeine was not detected. Coffee transformed with antisense construct also showed less than 0.1% DW caffeine. These data clearly demonstrated reduction in caffeine levels. However the extent of silencing varied in independent transformants. RT-PCR analysis indicated concomitant reduction in *N*-methyl transferase transcript levels in transgenic plantlets. Transgenic plants with low caffeine were achieved and ready for greenhouse transfer.

4.3.9. Optimization of electroporation mediated gene delivery: Analysis of *N* methyl transferase and 11s globulin promoters in coffee.

The efficiency of DNA uptake of linear or circular plasmids at 275, 500 and 750 V/cm field strengths was estimated, 48 h after electroporation, by analyzing transient expression of the *uid A* gene in the cells. GUS activity was observed in somatic embryos electroporated at 500V/cm. Gus assays demonstrated that the cell wall degradation using enzyme treatment was absolutely required for delivery of plasmid vectors. The transformation frequency varied between 0.4-4.8%. The results clearly demonstrated that, the NMT promoter and 11S globulin promoter could drive the expression of reporter genes in somatic embryos of *Coffea canephora.*

The electroporated somatic embryos were further cultured on secondary embryogenic medium containing 5 to 20 mgl^{-1} hygromycin. Transgenic secondary embryos were produced after a period of 4 months. The stable expression of *uid A* reporter gene was observed in transgenic secondary embryo. It was evident from the present experiments that, the isolated promoter is able to drive expression of *uid* A gene in endosperm tissues also. GUS expression was observed in seed endosperm electroporated with GUS gene driven by 11S Globulin promoter and NMT promoter.

4.3.10. Localization of *N* methyl transferase of caffeine biosynthetic pathway through localization of GUS reporter gene driven by NMT promoter in coffee endosperm.

Electroporation of coffee endosperm was carried out with constructs containing the *uid* A gene driven by NMT promoter bearing the 1st exon and without 1st exon. In tissues electroporated with constructs containing the 1st exon sequence, activity of GUS was targeted to the external surface of the vacuole. Deletion of the 1st exon resulted in a diffused expression of *uid* A gene in the cytoplasm, not localized to the areas around the vacuole. Staining for chlorogenic acid (CGA) revealed localization of CGA-caffeine complex in the vacuole. The NMTs of coffee were traced using antibodies raised against coffee NMTs and found to be localized surrounding the vacuole as seen with GUS expression driven by NMT promoter containing the first exon. *In silico* analysis of the 1st exon sequence of the coffee NMT revealed homology to that of methyl transferase from many different plant species.

4.4. CONCLUSIONS

- Somatic embryogenesis, secondary embryogenesis has been achieved successfully and regenerated plantlets have been successfully transferred to green house.
- Unraveled the involvement of polyamines and calcium in somatic embryogenesis.
- Identified isoforms of CDPKs that may be involved in calcium mediated responses in plant morphogenesis.
- Hairy roots were induced from coffee by *A. rhizogenes* mediated transformation of *Coffea canephora*.
- Transformation of coffee was achieved by *A. rhizogenes* harboring Ri plasmid and binary vector pCAMBIA 1301. Differential T-DNA integration patterns were observed. Integration of T-DNA from the binary vector was observed in all the transgenic plants. However, the integration of Ri T-DNA was not observed in all the tested transgenic plants.
- Silencing of caffeine biosynthesis was achieved by transformation of coffee with *A. tumefacience* harboring antisense and inverse repeat constructs designed based on the *N* methyl transferase (NMT) genes involved in caffeine biosynthesis.

- NMT and 11S globulin promoters were analyzed in coffee and the results confirmed the ability of these promoters to drive transgene expression in coffee embryos and endosperm.
- Results of the study indicate that the first exon may be encoding for a signal peptide, which targets NMTs to the vacuolar surface.

4.5. Future lines of work

The extension of studies carried out in this thesis could be in the following lines.

- The study gives an insight to the *in vitro* morphogenetic behavior of coffee and biochemical basis for the embryogenic and non-embryogenic nature of the callus cell lines, involvement of calcium and polyamines in plant morphogenesis. The results may be useful for further studies on determination of Ca^{2+} signatures and identification of novel Ca^{2+}dependent protein kinases involved in morphogenesis.
- The study demonstrated the regeneration of normal transgenic plants from *A. rhizogenes* mediated transformation system. The transgenic plants, which contain Ri T-DNA, may be adopted for breeding purpose in order to eliminate the Ri T-DNA, which possibly segregates in subsequent generations.
- Efficient silencing of caffeine synthesis was demonstrated here. This has opened up new dimensions. Seed specific silencing of NMT gene could be achieved by driving the antisense NMT fragment with 11S globulin promoter. The NMT 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.
- Further advancement in this direction may lead to the development of marker free low caffeine transgenic plants. This may be more attractive to the health conscious consumers who prefer caffeine free coffee from transgenic plants having coffee promoter and free of marker genes.

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