MICROPROPAGATION, SOMATIC EMBRYOGENESIS AND *AGROBACTERIUM* MEDIATED TRANSFORMATION OF *COFFEA CANEPHORA* PIERRE EX FROEHNER

A THESIS
submitted to the
UNIVERSITY OF MYSORE

for the award of the degree of

DOCTOR OF PHILOSOPHY
in
BIOTECHNOLOGY

by
Mrs. E. P. Indu M. Sc.

Department of Plant Cell Biotechnology,
CENTRAL FOOD TECHNOLOGICAL RESEARCH INSTITUTE,
Mysore-570013, India.

January 2004

Humbly present at the

holy feet of

my Master Poojya Sri P. Rajagopalachari

DECLARATION

I hereby declare that this thesis entitled "**MICROPROPAGATION, SOMATIC EMBRYOGENESIS AND *AGROBACTERIUM* MEDIATED TRANSFORMATOIN OF *COFFEA CANEPHORA* PIERRE EX FROEHNER" 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 under the guidance of **Dr. G. A. RAVISHANKAR, *Scientist and Head,*** Department of Plant Cell Biotechnology, Central Food Technological Research Institute, Mysore-570020, India, during the period 1998-2004. I further declare that the results presented in this thesis have not been submitted for the award of any other degree or fellowship.

Place: Mysore **E. P. INDU**

Date:

Dr. G. A. RAVISHANKAR, Ph.D., FNASc, FAFST, FNAAS, FBS
Head & Scientist,
Department of Plant Cell Biotechnology

CERTIFICATE

This is to certify that the thesis entitled **"MICROPROPAGATION, SOMATIC EMBRYOGENESIS AND *AGROBACTERIUM* MEDIATED TRANSFORATION OF *COFFEA CANEPHORA* PIERRE EX FROEHENR"** for the award of **DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY** to the **UNIVERSITY OF MYSORE** is the result of research work carried out by her in the Department of Plant Cell Biotechnology, under my guidance during the period 1998-2004.

G. A. RAVISHANKAR

Place: Mysore **(Guide)**

Date:

ACKNOWLEDGEMENTS

When I started learning the philosophy of science I realized that, it was not just for science, I was learning the philosophy of life itself, which always has been a wonder to me. When I leave the Institute with tears in eyes and a heavy in heart I realize that I am not the same person who came five years back. The passed years made a lot of changes both in my material and spiritual life for which I am grateful to the people around me and also to *My Master*, who helped me in the endeavor. Many people helped directly or indirectly, in which I would like to pick a few names, which does not mean that others are not remembered; human heart is not just two pages of paper.

I am sincerely grateful to:

My mentor and guide, Dr. G. A. Ravishankar, Scientist & Head, Plant Cell Biotechnology Department, CFTRI, for his encouraging and untiring guidance. He is a person with so much of energy and enthusiasm, which always has been very surprise to me, and I gathered inspiration from him. He is very open minded to new ideas and gave so much of freedom of thought, which is very essential for the research activities. He respects new ideas and also people giving them importance.

Dr. V. Prakash, Director, CFTRI, for having provided me an opportunity to work in CFTRI, which is a wonderful Institute with good central instrument and library facilities as well as a calm and free working atmosphere giving the students enough freedom, independence and respect. I also would like to thank the staff of administration, of CFTRI, who were very helpful to me for the official formalities to be carried out in a smooth manner. I would like thanking M. R. Radhakantha, CoA, for allowing me stay in the quarters when it was needed, which was very helpful for the smooth progress of the work.

Dr. Arun Chandrasekhar, Scientist, Department of Food Microbiology, CFTRI, for his encouragement, inspiration and valuable suggestions for the study. I always felt free to discuss with him the problems I faced during the study and I appreciate his open mindedness.

Dr. Santa Ram A., Scientist & Head, Department of Botany, CCRI, Balehonnur, for giving valuable guidance and helping for compiling the thesis.

My loving husband who has been very supportive and encouraging. I put him in trouble with my problems many times but he expressed so much of tolerance and patience giving valuable suggestions and guidance. I bow to his broadmindedness.

Achan and Amma for the love, care and encouragement given for the study. They supported me and consoled me when I faced difficult situations during the course giving me valuable suggestions and guidance. I thank my sister and brothers for being very supportive during the study. I also would like to thank Pappa, Mummy and sister-in-laws for being very supportive and loving.

CSIR for giving me fellowship without which it would have been very difficult to proceed with the study.

The authorities of Sri Ram Krishna Vidyasala, for providing coffee plant materials.

Dr. P. Giridhar for helping me to compile the thesis, he is a very calm, contented and friendly person, with whom I always felt homely. Dr. Manoj G. Pillai, who treated me like a sister, Dr. T. Rajasekaran who showed me what is meant by systematic nature, Dr. N. Bhagyalakshmi for her kindness and suggestions which she gives at difficult times, Dr. M. S. Narayan, for being very friendly, Dr. R. Sarada, Dr. M. Mahadevaswamy and others for guiding me directly or indirectly giving me helps when required either in the study or personally. Thanks are due to Dr. C. G. Sudha and Dr. M. Madhava Naidu for valuable suggestion. I thank Srinivas, Kavitha and Madhu for helping in the official formalities of the department.

Dr. George Thomas and Dr. Lokeswary of SPIC Science Foundation, Chennai for kindly providing the construct for transformation studies and also for allowing to visit their lab which added more insight into the field of coffee research.

Vinod Kumar, for helping a lot especially during the transformation studies. I also thank, Suresh, Sarala, Aswani Sharma, Vanitha, Chidambara Murthy, Sowmya (s), Raju, Sandesh, Dayanand, Rajesh, Brinda, Gururaj, Prasad, Venkat, Vidya and Namitha for making my stay in the department memorable and happy and for the co operation they expressed during the study which helped for smooth running of the course. I also thank my seniors Dr. Harsh P. Bais, Dr. G. Sudha, Dr. Hema Kumar and M. Y. Khan for their support and guidance.

K. V. Satyanarayana and other colleagues in Arun sir's lab for being very helpful and friendly. I always felt comfortable to approach them for help.

The staff of Central Instrument Facility, especially Dr. M. A. Kumar, M. Asha and K. Anbalagan for helping during the study for using HPLC and SEM.

Dr. H. Y. Mahakuteshwar, I. Ragavan and other staff of library for being very kind and helpful.M. Vishnukumar for taking good pictures of cultures with so much of care and efficiency.

The Warden, Dr. G. Venkateswara Rao, former warden P. Haridas Rao, M. R. Rangarajan and Pradeep Bharadvaj for providing hostel accommodation and for making my stay comfortable and safe. I would like to thank mess manager Sadhu and mess workers for being very friendly and for giving homely food, which I always enjoyed.

Yashoda, Sangeetha, Sijo, Anu, Ajila and my former colleagues, Beena, Maya, Nirmala, Saby and Rekha for their valuable guidance and company. I would like to thank my dear mallu 'nanhi pyari' sisters and brothers, Chitra, Deepa, Divya, , Lincy, Rita, Shino, Saji, Sujith, Sujeeth, Jeeson and Rajani. Being with them had given me a homely atmosphere, which always would be in heart. They helped me for the critical correction of the thesis.

All the former and current, colleagues, ISMTs, M.Scs.

Mr. Prasad M.V. of Microdot Creators, Mysore for type setting the thesis. He has done the work with so much of patience and care.

Lastly, but most importantly I would like to express my sincere and heartfelt thanks to my Master for the depth of love He expresses in a very mysterious way and the care He takes in each and every moment of life, I am grateful that He chose me to know Him. I know no words are enough to express the gratitude, still……

LIST OF ABBREVIATIONS

2,4-D	2, 4-Dichlorophenoxy acetic acid
2-iP	2-isopentinyl adenine
ABA	Abscisic acid
BAP	Benzyl amino purine
CGA	Chlorogenic acid
CxR	Canephora x Robusta
DW	Dry weight
FW	Fresh weight
GFP	Green fluorescent protein
GUS	*β*-glucuronidase
HFSE	High frequency somatic embryogenesis
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
KIN	Kinetin
LS	Linsmaier & Skoog
M	Molar
mM	Milli moles
MS	Murashige and Skoog
NAA	α- Naphthalene acetic acid
TDZ	Thidiazuron
TRIA	Triacontanol
µM	Micro moles

CONTENTS

LIST OF TABLES

LIST OF FIGURES

Composition details of the plant tissue culture media adopted/developed:

CHAPTER I

General Introduction & Review of Literature

1.1. ORIGIN, SPREAD AND CLASSIFICATION OF COFFEE:

Coffee belongs to the family *Rubiaceae*. The *Rubiaceae* members are largely tropical or subtropical with nearly 400 genera and 4800-5000 species. They are usually trees or shrubs, sometimes vines and infrequently herbs. The flowers are bisexual, usually epigynous, actinomorphic, sympetalous and funnel form. The Rubiaceae members are most important as tropical crops such as Coffee (*Coffea*) and quinine (*Cinchona*). Example of ornamental is gardenia (*Gardenia*) (Langenheim and Thimann, 1982a). *Coffea* is by far the most important member of the family, economically.

1.1.1. 'Coffee':

The word 'coffee' is used to characterize not only the plant but also the stimulatory drink obtained from the roasted and ground seeds of certain species of the genus *Coffea* (Monaco *et al.*, 1997). '*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).

1.1.2. Discovery, distribution and spread of coffee:

The discovery of the coffee plant is related to Kaldi, a goatherd tending his flock in the hills around a monastry on the banks of the Red Sea who noticed that his goats started to prance excitedly about, after chewing berries from bushes growing there. A monk from the monastry observed their behavior, took some of the berries back to the monastry, roasted and brewed them and tried out the beverage on his brethren which helped them to be alert during their long prayers at night (Smith, 1985).

The genus originally had a broad distribution in the tropical and subtropical parts of Africa and Asia. 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). Coffee was known in Persia, by the 14th century and in Turkey by the 16th century (Sivetz and Foote, 1963). Following its introduction into Yemen, the cultivation of *Coffea* extended to Ceylon (1658), Indonesia (1696), and other regions with favourable crop conditions. Due to the great popularity of the beverage, its cultivation spread around the world, being introduced in Surinam in 1714, Haiti and Dominican Republic in 1715, Martinique 1723, Brazil 1727, Jamaica 1730, Peurto Rico 1755, Costa Rica 1779, Cuba 1784, Venezuela 1784, Mexico 1790, Colombia late in the 18th century, El Salvador 1852, and gradually to other countries (Monaco *et al.*, 1997).

1.1.3. Taxonomy of coffee:

While the international coffee trade is concerned with only two coffee species - *Coffea arabica* and *Coffea canephora* - botanists regard as coffee, trees of all tropical plants of the *Rubiaceae* family, which produce seed resembling coffee beans (Charrier and Berthaud, 1985).

Coffee belongs to the family *Rubiaceae*, subfamily *Cinchonoideae* and tribe *Coffeae* (Clifford *et al.*, 1989). This tribe has been (Robbrecht and Puff, 1986) restricted to two genera, *Coffea* and *Psilanthus*. All species of *Coffea* are woody, but they range from small shrubs to large trees over 10 metres tall; the leaves can be yellowish, dark green, bronze or tinged with purple. Currently three subgenera are recognized in *Coffea* (*Coffea, Psilanthopsis* and *Baracoffea*) and two in *Psilanthus* (*Paracoffea* or *Afrocoffea* and *Psilanthus*) (Leroy, 1980; Lobreau-Callen and Leroy, 1980).

Botanical explorations of coffee in tropical regions started in 16th century. Hundreds of species have been described in the herbaria but the taxonomic classification of the genus *Coffea* has become very complex and confused. The most authoritative classification is of Chevalier (1947), which is also being modified with the new discoveries of the species (Charrier and Berthaud, 1985). A de Jussieu, who studied a single plant originating from the botanic garden of Amsterdam, made the first botanical description of a coffee tree under the name *Jasminum arabicanum* in 1713. However, Linnaeus (1737a,b) classified it as a separate genus *Coffea* with the then only known species *C. arabica*. Chevalier (1947) and Leroy (1980) studied in detail the taxonomy of coffee.

1.1.3.1. Classification of coffee according to Chevalier (Chevalier, 1947) and Leroy (Leroy, 1980):

Chevalier (1947) has grouped the valid coffee species within the genus *Coffea* into the following four sections:

(1) Eucoffea (24spp.)

(2) Mascarocoffea (18spp.)

(3) Paracoffea (13 spp.)

(4) Argocoffea (11spp.)

The *Eucoffea* has been again divided into five subsections according to very diverse criteria: fruit color (*Erythrocoffea*, *Melanocoffea*), leaf thickness (*Pachycoffea*), tree height (*Nanocoffea*) and geographical distribution (*Mozambicoffea*) (Table 1.1).

Table 1.1: The grouping of the species in the subsection *Eucoffea* according to Chevalier (1947):

Section	Subsection	Species
Eucoffea	*Erythrocoffea*	*C. arabica* *C. canephora* *C. congensis*
	Pachycoffea	*C. liberica* *C. klainii* *C. oyemensis* *C.abeokutae* *C. dewevrei*
	Nanocoffea	*C. humilis* *C. brevipes* *C. togoensis*
	Melanocoffea	*C. stenophylla* *C. carissoi* *C. mayombensis*
	Mozambicoffea	*C. eugenioides* *C. schumanniana* *C. kivuensis* *C. mufindiensis* *C. zanguebariae* *C. racemosa* *C. ligustroides* *C. salvatrix*

The section *Eucoffea*, now more correctly named as *Coffea*, and *Mascarocoffea* include most of the presently known coffee species.

Coffee trees differ greatly in morphology, size and ecological adaptation, thereby leading to the description of a large number of species. Better information on the degree and distribution of genetic variation is essential for developing more efficient ways of evaluation, utilization and conservation of biodiversity (Sreenath, 2000).

Leroy (1980) using flower shape, length of the corolla tube (long or short) associated respectively with exerted anthers and long style or

inserted anthers and short style, and growth habit and type of inflorescence (monopodial with axillary flowers and sympodial with terminal flowers) indicated the relationship between species of the genus *Coffea* and those of *Psilanthus*. The classification system proposed by Leroy (1980) for the genera *Coffea* and *Psilanthus*, with indication of geographical distribution of the sub-genera, is presented in table (Table 1.2.).

Table 1.2: The classification system for the genera *Coffea* and *Psilanthus* according to Leroy (1980), with indication of species and geographical distribution:

Family: *Rubiaceae* Subfamily: *Cinchonoidea*

Genus	**Subgenus**	**No. of species**	**Localization**
Coffea L.	*Coffea*	*Ca* 85 species, including all the commercially useful species	Africa, Madagascar, Mascarene Islands
	Psilanthopsis (Chev.) Leroy	Only *P. kapakata*	Angola
	Baracoffea (Leroy) Leroy	Two or three Madagascan species and the north east African *C. rhamnifolia*	Africa, Madagascar
Psilanthus (Hook.f.)	*Paracoffea* (Miquel) Leroy	18 species	Africa, India, New Guinea, Malaysia, Torres Strait Islands
	Psilanthus (Hook.f.)	Only *P. mannii* and a poorly known species	West and Central Africa

1.1.3.2. Living collections of coffee:

A comprehensive botanical study should also include the world living collections of coffee. Emphasis in the collection of coffee germplasm was particularly on *C. arabica* because of its economic importance. Wild collections of *C. arabica* with material of the Ethiopian centre of genetic diversity are present in Jimma (Ethiopia), Turrialba (Costa Rica), Campinas (Brazil), Chinchina (Columbia), Lyamungu (Tanzania), Ruiru (Kenya), Foumbot (Cameroon), Man (Ivory Coast) and Ilaka-Est (Madagascar). There is a unique collection of species of the section *Mascarocoffea* at Kianjavato (Madagascar). The working collections of *C. arabica* and *C. canephora* maintained in India, Cameroon, Togo, Angola, and other countries contain very valuable material (Charrier and Berthaud, 1985).

1.2. ECONOMICALLY IMPORTANT SPECIES:

1.2.1 Commercial coffee:

Among more than 80 species of coffee, the most economically important are *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). *C. dewevrei* cv. Excelsa is mainly found in the Ivory Coast and Central African Republic (Krug, 1963). *C. liberica* is found almost exclusively in Malaya and Surinam, and to some extent in other countries such as Guinea, Ivory Coast, Liberia, Gabon and Angola as part of a mixed population of coffee species (Monaco *et al.*, 1997). This species is also

self-sterile and highly polymorphic (Carvalho *et al.*, 1969). In recent years, interest has been placed on species of the section *Paracoffea*, which are characterized by the production of very low caffeine (Clifford *et al.*, 1991) or caffeine-free beans. These species occur widely in the Madagascar Island (Leroy, 1962). Some of the differences between arabica and robusta coffee are listed in Table 1.3.

1.2.2 *Coffea arabica* - production and character:

C. arabica (Fig. 1.1.) is the only polyploid species so far described in this genus. Various speculations have been made about its origin, but there is still very little reliable evidence about the nature of the polyploidy involved. On the basis of the pairing observed in haploids it has been suggested that *C. arabica* could be an autotetraploid. On the other hand, the meiotic behaviour of some interspecific hybrids and the mode of inheritance of duplicate factors were used by Monaco and Carvalho (1963, 1964) to indicate that *C. arabica* is an allopolyploid or perhaps a segmental allotetraploid. The geographical distribution of *C. arabica* falls almost completely outside the range of distribution of the diploid species (Carvalho *et al.*, 1969). It produces high quality coffee with low caffeine content, but is susceptible to diseases (Ogita *et al.*, 2002). 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).

1.2.3 *Coffea canephora* - production and character:

C. canephora Pierre ex Froehner cv. Robusta (Fig. 1.2.), which contributes 25% of the total coffee production (Berthouly and Etienne, 1999), is the second most important species cultivated (Monaco *et al.*, 1997).

Table 1.3: 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/ha)	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, Available from Internet (http://www.ico.org).

Figure 1.1: *Coffea arabica* plant with berries (bar 15cm)

Figure 1.2: *Coffea canephora* plant with berries (bar 20 cm)

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). Canephora coffee was found to be a very good substitute for the arabica type which was earlier devastated completely by the coffee leaf rust disease (causal organism – *Hemileia vastatrix*) (Waller, 1987).

C. canephora is a diploid (2n=22) allogamous species that produces coffee with higher caffeine content, making poorer in quality than *C. arabica*. Its advantage, however, is resistance to diseases (Ogita *et al.*, 2002). *C. canephora* grows much larger and more vigorously than *C. arabica* and *C. liberica*, and is becoming popular particularly in the lower elevation districts of South India where *C. arabica* does not thrive well. It is hardier, more resistant to leaf disease and stem borer, and better adapted to varying climates and soils. It is a prolific bearer and produces a large number of light red berries with beans of approximately the same size and shape. 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 (The Wealth of India, 1950). *C. canephora* is highly polymorphic. *C. canephora* is used for the manufacture of instant coffee (Hatanaka *et al.*, 1995).

1.2.3.1. Origin and spread:

It has its centre of origin in Central and Western equatorial Africa and in Madagascar (Waller, 1987) and is found in Ivory Coast also. It occupies the lower humid zones. It grows at low altitudes (about 850 m) (Carneiro, 1999). *C. canephora* has a very wide geographic distribution, extending from the western to the central, tropical and subtropical regions of the African continent, from Guinea and Liberia to Sudan and the Uganda forest, with a high concentration of types in the Democratic Republic of Congo Kinshasa

(Carvalho *et al.*, 1969). The major countries cultivating robustas are listed in the following order: Ivory Coast, Angola, Uganda, Indonesia, Zaire, and Cameroon (Pan-American Coffee Bureau, 1973). Thomas (1944) reported that in Uganda it is found in more humid environments.

1.2.4. Coffee in India:

Arabica coffee is supposed to have been introduced into India sometime during 1670's by a muslim pilgrim, Bababudan, who brought seven seeds from Yemen and raised seedlings on the hills (later named Bababudan hills) near the town of Chikmagalur (Karnataka state). Coffee was gradually planted in the back-yard gardens of 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. The tropical climate, high altitude, sunny slopes, ample rainfall, soil rich in humus content and well drained sub-soil of the region are ideal for coffee cultivation (John, 1993).

A number of selections in coffee both arabica and robusta have been evolved at Central Coffee Research Institute (CCRI), Chikmagalur, India for improving yield, resistance to leaf rust, reducing fruit and bean abnormalities, exploiting the dwarf habit and maintaining good cup quality. At present more than 80% of the coffee area in India is occupied by the varieties bred at CCRI (The wealth of India, 1992). Coffee is cultivated over an area of 270,000 hectares of which arabica coffee occupied *ca.* 142,000 hectares during the year 1990-91 (Indian Coffee, 1994). In India, coffee is cultivated mainly in the southern state of Karnataka (58% of area and 72% of production),

Kerala (25% of area and 20% of production) and Tamil Nadu (9% of area and 7% of production). To a lesser extent it is also grown in non-traditional areas like Andhra Pradesh, Orissa and North Eastern states to an extent of about 30,000 hectares. India is a producer of both arabica (104,400 tones) and robusta (196,800 tones) varieties of coffee in proportion of 35:65. In India, Karnataka produces both arabica and robusta in almost equal proportion while Kerala specializes in robusta and Tamil Nadu in arabica. India produces the best robustas in the world in terms of quality (Reddy *et al.*, 2001).

1.3 COFFEE ECONOMY AND INDUSTRY:

1.3.1 Production and Exports:

At present about 7 million hectares are under coffee cultivation all over the world. Half of the total acreage of coffee is found in Latin America. The size of coffee plantation varies from 100-200 trees to large plantations with several million trees, as in Brazil and Angola (Monaco *et al.*, 1997). Among the major coffee producers in the world during the past three years, Brazil leads with approximately 40.61% of the total production, followed by Columbia (9.42%), Vietnam (8.37%), Indonesia (4.75%), India (3.84%), Mexico (3.38%), Côte d'Ivoire (2.87%), Guatemala (2.63%), Peru (2.42%) and Honduras (2.09%) (International Coffee Organization, 2003). In Table 1.4. the production of the ten major coffee producers is listed. Arabica coffee is cultivated in most of Latin America whereas robusta coffee represents 95% of Indonesian and 100% of Ivory Coast production. Costa Rica is the leading country in productivity with about 1600 kg/ha, which is almost twice that of Brazil. In India both arabica and robusta coffee are cultivated in almost equal area. The average productivity in India has increased to over 800 kg/ha in recent years (Sreenath and Naidu, 1997). Most of India's coffee export is to Western and Eastern Europe and Japan. Indian coffee is known for its quality and hence command

a premium in the European market (John, 1993). Africa has been emerging in importance as a coffee producer from 1.3% of the world market prior to World War I to 27% of the world market in 1973 (Krug, 1963; Pan American Coffee Bureau, 1973).

Table 1.4: Average of total production of coffee and average % of contribution of exports for the 10 major producers from 2000-2002

Country	**Thousands of 60 kg bags (Average % of contribution for exports)**
Brazil	38,145 (25.93)
Vietnam	12,636 (14.01)
Colombia	11,260 (11.03)
Indonesia	6,449 (5.46)
India	4,688 (4.36)
Mexico	4,338 (4.23)
Côte d'Ivoire	3,923 (4.94)
Guatemala	3,917 (4.67)
Peru	2748 (2.93)
Honduras	2,734 (2.99)
Total	113,963 (80.55)

(Source: International Coffee Organization, 2003, Available from Internet: http://www.ico.org).

The world trade in coffee is an important contributor to the income of some fifty or more 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

percentage of contribution of the first ten major coffee exporting countries is listed in Table 1.4.

The percentage of total exports from India showed a fluctuation over the years. From 1992 to 1996 there was a continuous increase, which had suddenly gone down in the next year. Again it picked up growth towards the year 2000, which again declined by 2002. In India, of a total of 230,000 tonnes of coffee produced, mostly in regions of Karnataka, about 170 thousand tones, valued over Rs. 116,220 million are exported (Vasanthi and Bhat, 2000).

1.3.2. Coffee harvesting and preparation:

Coffee berries (Fig. 1.3) can be harvested in two ways: (1) stripping the branches or (2) individual selection by hand picking. The first process is faster and less expensive but has the disadvantage of carrying many impurities e.g. green coffee, leaves, and pieces of wood. The latter mode of harvest selects only ripened berries and provides for superior preparation.

The preparation of coffee beans can be made by dry or wet process. The dry method gives the “unwashed” type of coffee whereby the berries are allowed to dry on the trees and then harvested by stripping the branches. The berries are washed, graded and dried to a water content of 12-15% on cement patios under the sun or in driers (Fig. 1.4). Then the pericarp and the silver skin are removed and subsequently the beans are classified by size, shape and color. The normal berry gives two seeds which have a convex and a flat side (Fig. 1.5). In the wet process the ripened coffee berries are harvested by hand and then the exocarp is removed. This process gives the “washed” type of coffee. “Green coffee” (Fig. 1.6) is the commercial term for coffee prior to the roasting process (Fig. 1.7). Classification and trade are performed using the green coffee.

Figure 1.3: *Coffea canephora* berries (bar 14 mm)

Figure 1.4: Dried coffee berries (*Coffea arabica*) (bar 15 mm)

Figure 1.5: Robusta and Arabica seeds

Figure 1.6: Green coffee (bar 13mm)

Figure 1.7: Roasted coffee beans (bar 13 mm)

The price of coffee depends primarily on species, arabica being more expensive than robusta coffee, and better in quality. However, among the washed and unwashed arabica, the unwashed coffee has less commercial value (Monaco *et al.*, 1997).

1.4. IMPROVEMENT OF COFFEE:

Coffee plant, especially *C.arabica,* is highly susceptible to diseases and pests, which has to be confronted seriously for the improvement of coffee. In addition to these properties improvement in quality characteristics such as uniformity in maturation, shorter maturation cycles, higher soluble solids, larger bean size and increased bean-density and texture would benefit coffee processors (Sondahl and Loh, 1988). Varying levels of caffeine content and improved aroma and taste are the qualities that directly affect coffee consumers.

1.4.1 Major problems to be targeted:

Susceptibility to biotic and abiotic stresses is the major problem to be tackled for improvement of coffee.

1.4.1.1 Biotic stress:

1.4.1.1.1. Fungal and bacterial diseases:

Fungi cause three most important diseases in coffee: coffee leaf rust (*Hemileia vastatrix*) (Fig. 1.8), coffee berry disease (*Colletotrichum coffeanum*) and brown eye spot (*Cercospora coffeicola*). A common nursery disease is damping-off, caused by *Rhizoctonia solani*, which can be occasionally observed in one-year old field plantings. Bacterial blight (*Pseudomonas syringae*) is an important bacterial disease that affects most African varieties (Medina *et al.*, 1984). Leaf rust is the most important disease in India.

Figure 1.8: Coffee leaf with rust disease

Figure 1.9: A coffee bean attacked by coffee berry borer. The other half shows a good coffee bean (bar 7 mm)

Figure 1.10: Coffee stem borer

Figure 1.11: Coffee stem attacked by coffee stem borer

1.4.1.1.2. Major pests and nematodes attacking coffee:

Major pests attacking coffee include the coffee berry borer (*Hypothenemeus hampei* Ferrari), white stem borer (*Xylotrechus quadripes*), coffee leaf miner (*Perileucoptera coffeela*), root nematodes (*Meloidogyne incognita, M. exigna, M. coffeicola* and *Pratylenchus* spp.) (Sreenath, 2000). Coffee berry borer (Fig. 1.9) has become a major pest of coffee worldwide. In New Caledonia ten years of endosulfan use has led to failure in controlling this pest due to the evolution of resistance to cyclodiene insecticides (Brun and Constant, 1997). Stem borer (Fig. 1.10), a major pest in India mainly affects arabica coffee (Fig. 11). Breeding programmes have been mostly unsuccessful in providing resistance to the coffee berry borer, stem borer or leaf miner (Sondahl and Lauritis, 1992). Resistance to leaf miner was reported in *C. stenophylla* and partial resistance in *C. racemosa, C. eugenioides, C. kapakata* and *C. dewevrei* (Medina *et al.*, 1984).

High level of nematode resistance has been found in *C. canephora, C. congensis, C. dewevrei* and *C. liberica*. Some *C. arabica* accessions from Ethiopia also seem to carry genes for nematode resistance (Medina *et al.*, 1984).

1.4.1.2 Abiotic stress:

Water stress is responsible for severe yield losses in coffee plantations. There are some arabica lines from India (BA, Sln. 9) that are tolerant to drought (Medina *et al.*, 1984). *C. racemosa* and hybrids with *C. arabica* have indicated resistance to water stress in wild diploid species (Medina *et al.*, 1984).

Frost damage in coffee plantations has caused severe losses in coffee production in Southern Brazil. If the temperature drops to -4°C, the entire plant is irreversibly affected by the death of shoot and root meristematic regions. Temperatures near freezing point cause death of

leaves and meristems, but coffee plants can recover completely within 2-3 years (Sreenath, 2000).

1.4.2. Conventional methods of improvement:

Plant breeding is a continuous process to evolve newer varieties of crop plants having improved characteristics in one or more aspect such as yield, quality, resistance to pests and diseases (mainly rust and coffee berry disease), nematode resistance, better response to fertilizer, a compact stature etc. over the existing varieties (Srinivasan, 1996; Sreenath, 2000). As for many other perennial crops, the limiting factor for development of productive coffee varieties resides in the long period demanded in a breeding programme. Usually, coffee starts to produce two to three years after the seedlings are planted in the field. The *Coffea*'s fruit development period is very lengthy and it takes 2-4 years of time for bean-to-bean generation, which make such traditional approaches costly and time consuming. Over twelve successive harvests are needed to assure the efficiency of selection and the productivity of the coffee lines (Fazuoli, 1976). Other limitations of coffee improvement by conventional breeding are the ploidy barrier (diploids *vs*. tetraploids) and autoincompatible alleles (diploid species). As a result, transfer of genetic traits from wild out bred species of the genus to the cultivated coffee species is quiet difficult. It was estimated that minimum of 24 years of continuous breeding including individual plant selection, followed by progeny evaluation, hybridization, pedigree selection and back crossing is required to release of a new variety (Sreenath, 2000).

1.4.3. Biotechnological approaches for improvement of coffee:

Biotechnological techniques developed so far have been highly useful for the improvement of coffee. The advances made 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 programmes, including genetic transformation (Carneiro, 1999).

1.4.3.1. *In vitro* vegetative multiplication:

Traditional methods of propagation are very slow and expensive and the improved seeds of superior genotypes are insufficient to satisfy farmer's demands. Micro-propagation is an effective method for multiplication. Intensive research has been carried out to develop a micropropagation technology for coffee. Micropropagation of a high-value perennial species like coffee can be an efficient method of propagating individual trees from a segregating population. Micropropagation can reduce the time taken for varietal development and preserve heterozygosity and genetic plasticity in coffee plantations (Söndahl and Lauritis, 1992). In coffee, micropropgation is possible by apical meristem and axillary bud culture and by induction of adventitious bud development.

1.4.3.1.1. Apical meristem and axillary bud culture:

The coffee plant presents one apical meristem and each leaf axil has 4-5 dormant orthotropic buds and two plagiotropic buds. The plagiotropic buds only start development from the 10^{th}-11^{th} node. The apical meristem culture and culture of dormant buds, both orthotropic and plagiotropic, give rise to plantlets which can be used as initital explants for coffee micropropagation (Carneiro, 1999). Samuel Ganesh and Sreenath (1996) reported enhanced shoot development through apical bud in *C. arabica* cv Cauvery (S. 4347) with silver nitrate (20 mg l^{-1}). Supplementing both BAP and $AgNO_3$ enhanced the sprouting percentage as well as shoot length and number of nodes per shoot. Coffee micropropagation was achieved in a liquid medium using a temporary immersion technique (Berthouly *et*

al., 1995). The advantages of this method over a semi-solid medium are: it increases the multiplication rate for micro-cuttings and it improves the quality of *in vitro* plantlets, which are ready for acclimatization.

1.4.3.1.2. Adventitious bud development:

Adventitious bud development is an alternative method of coffee micropropagation. Shoots originating in tissues located in areas other than axil or shoot tips are subjected to one phase of dedifferentiation followed by differentiation and morphogenesis (Carneiro, 1999). Plants were regenerated through adventitious shoots and somatic embryogenesis in hypocotyls tissues of *Coffea canephora* (Samuel Ganesh and Sreenath, 1999). Explants consisting of apical bud with a piece of hypocotyls cultured on MS medium supplemented with 5 mg/l BAP over a period of 150 days resulted in production of multiple adventitious shoots from hypocotyls derived calli.

1.4.3.2. Somatic embryogenesis:

In coffee, somatic embryogenesis constitutes a model system for perennial species (Söndahl and Loh, 1988) and has been widely studied and established (Herman and Haas, 1975; Hatanaka *et al.*, 1995; Muniswamy and Sreenath, 1995a; Nishibata *et al.*, 1995; Tahara *et al.*, 1995; Yasuda *et al.*, 1995; Van Boxtel and Berthouly, 1996; Samuel Ganesh and Sreenath, 1999; Fuentes *et al.*, 2000; Quiroz-Figueroa *et al.*, 2001). *In vitro* somatic embryogenesis of *C. canephora* was first reported by Staritsky (1970), who successfully induced somatic embryogenesis and plant regeneration in soft orthotropic internodes of *C. canephora* internodes.

Some of the results from different workers are summarized in Table 1.5 & 1.6.

Both the species of the *Coffea*, i. e., *C. arabica* and *C. canephora* have been given importance for embryogenesis studies. The main explant used was the leaf. Hypocotyl tissue also has been used (Samuel Ganesh & Sreenath, 1999). Both BAP and 2-iP have been used widely for the regeneration of somatic embryos but in a study conducted by Hatanaka *et al.* (1995) 2-iP was found to be the best growth regulator for the induction of somatic embryogenesis. In average two months are required for direct somatic embryogenesis from leaf explants for *C. canephora*. (Yasuda *et al.*, 1995; Hatanaka *et al.*, 1995). When embryos are induced through intermediate callusing stage the time required for embryogenesis goes up to 5 months irrespective of the variety and explants used (Muniswamy and Sreenath, 1995a; Yasuda *et al.*, 1995; Samuel Ganesh & Sreenath, 1999). More than 90% response was obtained with *C. canephora* variety in most of the studies (Muniswamy and Sreenath, 1995a; Yasuda *et al.*, 1995; Hatanaka *et al.*, 1995). Addition of other supplements like asparagine 10 mM and 10^{-12} & 10^{-10} M salicylic acid induced more number of embryos and also improved the quality of embryos in *C. arabica* (Nishibata *et al.*, 1995; Quiroz-Figueroa *et al.*, 2001). Presence of 30-60 µM $AgNO_3$ in the medium helped for 60% increase in the number of embryos formed directly from leaf explants in *C. canephora* (Fuentes *et al.*, 2000). Van Boxtel & Berthouly (1996) reported induction of embryogenic callus and regeneration of embryos from leaf explants by a two-step method in *C. canephora*. Two successive media were necessary: "the conditioning medium" containing 0.5 mg l^{-1} 2,4-D, 0.5 mg l^{-1} IBA and 2 mg l^{-1} 2-iP and "the induction medium" containing 1 mg l^{-1} 2,4-D and 4 mg l^{-1} BAP. The embryogenic callus induced was later regenerated in MS (1962) medium containing 1-4 mg l^{-1} BAP to produce high frequency somatic embryos (Van Boxtel and Berthouly, 1996). The time required for the process was found to be more (6-7 months). Under the best

conditions, one explant can yield 180±0.15 mg of friable callus giving 80,000±15,000 somatic embryos (Berthouly and Michaux-Ferriére, 1996). Earlier, Raghuramulu *et al.* (1987; 1989) has reported a similar method for induction of high frequency somatic embryos. They obtained the regeneration of plantlets through somatic embryogenesis from the soft internodal portions of orthotropic shoots of *Coffea arabica* L. and *C. canephora* Pierre. The explants were cultured in MS (1962) medium supplemented with 1 mg l^{-1} IAA and 4.3 mg l^{-1} kinetin as a basal medium for callus induction for one month. The callus formed was transferred to MS (1962) medium containing 0.5 mg l^{-1} IAA, 0.05- mg l^{-1} 2,4-D and 8.3 mg l^{-1} kinetin and incubated four three months by subculturing once in a month. The white pro-embryonic masses formed were subcultured to medium containing 4% sucrose, 6.5 mg l^{-1} BA and 2 mg l^{-1} IBA. Large quantities of globular and torpedo embryos were formed in another two months time.

Many other aspects of the somatic embryogenesis have been studied in coffee. Menéndez Yuffá *et al.* (1994) analyzed embryogenic and non-embryogenic calli from leaf sections of *C. arabica* cv, Catimor for protein patters. The patterns revealed qualitative and quantitative differences in size and charge. In non-embryogenic calli, two-dimensional analysis reveals seven distinctive polypeptides in the range of 15 to 70 kDa. Four of the polypeptides are acidic, three of 70 kDa and one of 15 kDa. In embryogenic calli there were seven characteristic polypeptides with molecular weight from 23 to 35 kDa in a broad pI from acidic to basic. Five of them are found in the neutral to acidic pI. Changes in the protein pattern appeared to correlate with histological differences in embryogenic calli and with different stages of development of somatic embryos. Tahara *et al.* (1995) studied the histological and biological events on early stage of somatic embryogenesis as well as the characters of embryogenic cells. Embryogenic callus was yellow and friable in appearance and

consisted of isodiametric spherical cells, with average 18.6 µm in diameter of protoplast. Each cell connected tightly and made small clumps. Non-embyogenic callus was translucent yellow but nearly same as embryogenic callus in appearance, cell shape and size with average 21.4 µm in diameter of protoplast. The callus consisted of small mass of cells and was dispersible. Non-embryogenic white callus was translucent white and composed of larger elongated or swollen cells with a protoplast diameter of 62.9 µm.

Table 1.5: Some of the reports on somatic embryogenesis of coffee:

Variety	Explant	Cultural conditions	Period for embryogenesis (months)	Frequency of response(%)	Ref.
C. arabica	Leaf	LS + KIN (0.1 mg l^{-1}) & 2,4-D (0.1 mg l^{-1})	2 months	-	Herman and Haas, 1975
C. arabica & *C. canephora*	Internode	Two-step method	6 months	-	Raghuramulu *et al.*, 1987; 1989
C. canephora	Leaves from *in vitro* plantlets	MS + NAA (0.1 mg l^{-1}) & BA (mg l^{-1})	5 months	93	Muniswamy and Sreenath, 1995a
C. arabica	Leaf	MS + BA (1 mg l^{-1})	5 months	76	Yasuda *et al.*, 1995
C. canephora	Leaf	MS + 2-iP (1 mg l^{-1})	1½ months	100	Yasuda *et al.*, 1995
C. arabica	Callus	MS + 2-iP (1 mg l^{-1}) + asparagine 10 mM	1 month	100	Nishibata *et al.*, 1995
C. canephora	Leaf	MS + 2-iP (1 mg l^{-1})	2 months	100	Hatanaka *et al.*, 1995
C. canephora	Leaf	Two-step procedure	6-7 months	97	Van Boxtel & Berthouly, 1996; Berthouly & Michaux-Ferriére, 1996

Table 1.6: Some of the reports on somatic embryogenesis of coffee:

Variety	Explant	Cultural conditions	Period for embryoge-Nesis	Frequency of response	Ref.
C. canephora	Apical bud with hypocotyl	MS + BAP (5 mg l^{-1})	5 months	12	Samuel Ganesh & Sreenath, 1999
C. arabica	Cell suspension	10^{-12} & 10^{-10} M salicylic acid	1½ months	Positive effect on number and quality of embryos	Quiroz-Figueroa *et al.*, 2001
C. canephora	Leaves	$AgNO_3$ 30-60 µM	2½ months	100%; 60% increase in the number of embryos	Fuentes *et al.*, 2000

Investigations were carried out to study the effect of genotypic differences among different hybrids of Hibrido de Timore (HDT) and Caturra x HDT crosses with respect to callus induction, morphology and size of callus, rate of embryogenesis, embryo germination, multiplication and plantlet regeneration (Madhava Naidu *et al.*, 1999). Maximum callusing was observed in S-4349 and S-4348, followed by S-2796 and S-2795. Embryogenesis was found to be high in S-2790 and S-2792, followed by S-2800. The rate of multiplication and conversion of somatic embryos to plantlets was high in S-2790 and S-2792 while it was low in Caturra hybrids (S-4347 to S-4350). The total time taken for plant regeneration from leaf tissue was 24 to 48 months in Caturra hybrids as against that of 16 to 20 months in HDT crosses. It appeared that where callus proliferation was limited, the chance of getting embryogenesis was more.

1.4.3.3. Embryo rescue:

The embryo represents the beginning of the new sporophytic generation of the plant and it originates from fertilized egg of zygote.

The embryos formed within the female gametophyte through the normal sexual process, can be separated from the maternal tissues at different developmental stages and cultured *in vitro* under aseptic conditions by providing artificial, nutritional and environmental conditions. The removal and culture of zygotic embryos of higher plants was one of the earliest successful techniques in plant tissue and organ culture. (Muniswamy and Sreenath, 1999a).

Embryo culture is one of the important ways of dealing with genetic variability in coffee. Immature and mature embryos of *C. arabica* and *C. canephora* have been cultured on MS medium (Sondahl and Loh, 1988). Culture of immature embryos of *C. arabica* and *C. canephora* on MS medium supplemented with various growth regulators (Sreenath *et al.*, 1989) and plant regeneration from these interspecific crosses by immature embryo culture has been reported (Sreenath *et al.*, 1992). Somatic embryogenesis and plant regeneration from cultured immature zygotic embryos of *C. canephora* has also been reported (Muniswamy *et al.*, 1993). Muniswamy and Sreenath (1995b) standardized encapsulation technique for production of synthetic seeds in coffee (*C. arabica)* and for conserving these encapsulated embryos for long-term preservation. Madhava Naidu and Sreenath (1999) showed a technique for germplasm preservation of *in vitro* cultures of zygotic embryos of *C. arabica* cv. Cauvery (Catimor). The embryos could be preserved with a little loss of viability in the presence of ABA even at the normal room temperature (25±1°C) up to two years without any transfer.

1.4.3.4. Suspension culture:

The use of liquid media enables the embryogenic tissue proliferation and mass production of somatic embryos in coffee (Van Boxtel and Berthouly, 1996). Cell suspension cultures were established from embryogenic tissues of *C. arabica* cv. Mundo Novo.

Large-scale clonal propagation was possible following embryo regeneration and complete plantlet development in these cultures (Pena, 1984). A high degree of synchronization in somatic embryo development and a high germination rate of 94.5% were achieved using liquid medium (Neuenschwander and Baumann, 1992). Etienne-Barry *et al.* (1999) proposed a method for direct sowing of germinated embryos in *C. arabica* somatic embryos mass produced in a 1-1 temporary immersion bioreactor. The effect of germination conditions on the morphology of *C. arabica* L. somatic embryos mass-produced in a temporary immersion bioreactor was studied with emphasis on direct sowing in soil. Using germinated embryos, direct sowing resulted in a highly successful conversion of embryos into plants. A culture density above 1600 embryos per 1-l bioreactor positively affected the embryo morphology by causing higher embryogenic axis elongation (4-5 mm). At this density, additional of a high concentration of sucrose (234 mM) 2 weeks before sowing prompted an increase in effective plant conversion in soil (78%) and a vigorous vegetative growth of the resulting plants. Direct sowing reduced handling time to 13% and shelving area requirements to 6.3% of the values obtained by conventional acclimatization of plants developed on gel media (Etienne-Barry *et al.*, 1999).

Other methods used for the improvement of coffee are somaclonal variation, cell-line selection, protoplast culture etc. The naturally occurring or *in vitro* –induced variability leads to somaclonal variation, which can be regenerated. Somaclonal variation provides genetic variability within existing cultivars, which helps to shorten the breeding programmes. Somaclones carry few genetic alterations, so that the genetic integrity of the commercial variety is preserved (Sreenath, 2000). Somaclonal variation could also be an important tool for selecting low caffeine varieties. Herbicide resistant, heavy metal (aluminum, manganese) tolerant cells can be selected directly

by *in vitro* selection of embryogenic coffee suspensions in the presence of increased concentrations of these metals.

Protoplasts are highly useful for plant breeding. Protoplast fusion can produce hybrids between parents, otherwise not possible by conventional crossing. Desirable traits from wild coffee species could rapidly be introduced into cultivated species. They easily take up foreign DNA, either naked or encapsulated in a carrier system. They facilitate selection of mutants (Sreenath, 2000).

1.4.3.5. Genetic transformation of coffee:

Conventional hybridization is a long and random process wherein meticulous selection is needed to screen the desirable plant types (Madhava Naidu *et al.*, 1998). A new breeding method for rapid selection of disease tolerant varieties and/or an improvement of coffee in quality is highly demanded (Ogita *et al.*, 2002). In coffee, the gene transfer technique may be worth considering as desirable genes can be transferred into the coffee plant precisely within a short span of time (Madhava Naidu *et al.*, 1998). Genetic transformation of coffee with foreign genes has been a subject of intensive research and several workers have reported the success in gene transformation and subsequent regeneration (Van Boxtel *et al.*, 1995).

There are currently many approaches to plant transformation, either by co-cultivation with *Agrobacterium* or by direct transformation. *Agrobacterium* is capable of inserting the T-DNA sequence of the Ti-plasmid into plant cells (Chilton *et al.*, 1977). The major limitations to this procedure include the host range of *Agrobacterium* and the ability to regenerate transformed plants from the transformed tissue. If a gene of interest were inserted into the T-DNA region of the Ti-plasmind of the bacterium, such a gene would be integrated into the plant genome following infection by *Agrobacterium*. Transformation with wild strains of *Agrobacterium* leads to gall

formation, which is not agronomically desirable. The hormone synthesis genes responsible for cell proliferation have been deleted in several *Agrobacterium* lines so that no galls form at the site of infection (Bevan, 1984). Instead, antibiotic resistance gene is inserted in its place to act as a selection marker for transformation.

Since coffee can be readily regenerated from leaf tissue (Yasuda *et al.*, 1985; Berthouly and Michaux-Ferrière, 1996), a leaf-disc transformation procedure is well suited for inserting genes into coffee plants (Horsch *et al.*, 1985). Somatic embryos regenerate easily into plants and may be used for genetic transformation in coffee. Alternatively, protoplasts can be induced to take up and integrate plasmid or linearized DNA sequences (Lorz *et al.*, 1985). This approach is not limited by the host range of the pathogen, but does require a capacity for protoplast regeneration. The two approaches can be combined for direct transformation of protoplasts with the use of the Ti-plasmid (Krens *et al.*, 1982).

Different methods have been adopted for the transformation of coffee (Table 1.7 & 1.8):

1.4.3.5.1. Electroporation and biolistic methods of transformation:

The first genetic transformation of coffee cells was reported (Barton *et al.*, 1991) was by protoplast electroporation. *C. arabica* suspension cultures were used to obtain protoplasts, which were transformed with a kanamycin resistant gene. Embryos were formed from the transformed cells, which were regenerated into plantlets. The regenerated embryos have been shown to contain the inserted foreign DNA, indicating that stable transformation of cells has been achieved. De Pena (1995) reported optimization of conditions for direct DNA uptake using the PEG method, and the verification of the transient expression of *gus* gene and the stable transformation using the NPTII

gene, in *C. arabica* cells. Fernandez-Da Silva and Menendez-Yuffa (2003) transformed *C. Arabica* cv. Catimor by electroporation method. The optimal conditions for electroporation were one hour of enzymatic pretreatment of torpedo shaped embryos, and electroporation at 375 V and 900 µF. The culture of electroporated tissues in liquid media with 8 m gl^{-1} BAP lead to maximal regeneration through secondary somatic embryogenesis. The secondary embryos were formed directly in the hypocotyl surface of the electroporated torpedo shaped primary somatic embryos. The secondary somatic embryos regenerated from electroporated torpedo shaped somatic embryos were positive for *gus* expression and also in the PCR analysis for the genes *gus* and *bar* (glufosinate resistance).

Van Boxtel (1994) studied the transient expression of GUS in different types of coffee tissues using the biolistic method. Further, expression studies were performed on different tissues like *in vitro* grown leaves, green house grown leaves and cell suspensions of different genotypes. Best results were obtained using *in vitro* cultured leaves of *C. arabica* and plasmids carrying the EF1a-A1 promoter of *Arabidopsis thaliana*. Out of five selective agents (chlorsulfuron, glufosinate, glyphosate, hygromycin and kanamycin), herbicide glufosinate was found to be the best for detection of stably transformed tissue. Osmotic preconditioning of cells and physical bombardment parameters including Helium pressure, gap and target distances affecting DNA delivery were evaluated by monitoring transient expression of the *uid*A gene driven by the CaMV35S promoter (Rosillo *et al.*, 2003). The highest transient GUS expression was obtained when cells were subjected to 0.5 M mannitol-sorbitol pretreatment 4 h prior to bombardment, a Helium pressure of 1550 psi, a 9-mm gap distance and 12 cm target distance as physical bombardment parameters.

Table 1.7: Some of the reports on transformation of coffee using electroporation and biolistic methods:

Variety	Explant	*Agrobacterium* strain	Reporter or Selection	Transformed tissue	Confirmation	Ref.
C.arabica	Protoplast	Plasmid with NPTII	Kanamycin	Somatic embryos & plantlets	-	Barton *et al.*, 1991
C. arabica	Proto-Plast	Direct DNA uptake-PEG	NPTII & GUS	-	GUS	De Pena, 1995
C. arabica cv. Catimor	callus, leaf sections, somatic embryos	Electroporation	Glufosinate & GUS	-	GUS; PCR-*gus & bar*	Fernandez-Da Silva & Menéndez-Yuffa, 2003
C. arabica	*In vitro* leaves	Particle bombardment-plasmids carrying EF1-A1 promoter	Glufosinate & GUS	-	GUS	Van Boxtel, 1994.
C. arabica	Suspension culture, *in vitro* leaves & endosperm	Particle Bombardment-Optimization of conditions	*gus* gene expression driven by CaMV35S*a*-tubulin & arabicin promoters	-	GUS	Rosillo *et al.*, 2003

The optimal protocol was tested with two coffee promoters: *a*-tubulin and arabicin, which presented similar activity to the CaMV35S promoter in suspension culture cells by fluorometric GUS assays. Only the CaMV35S and arabicin promoters showed histochemical activity in coffee endosperm GUS expression was lower in bombarded

tissue culture leaves than in bombarded suspension culture cells (Rosillo *et al.*, 2003).

1.4.3.5.2. *Agrobacterium* mediated transformation:

C. arabica hypocotyls were infected with wild *A. tumefaciens*. The tumors formed were able to grow in MS medium without hormones (Ocampo & Manzanera, 1991). Transformation of coffee was successfully carried out using wild type *A. rhizogenes* strain IFO 14554. Adventitious hairy-roots were formed and a small number of adventitious embryos produced from them were grown into plantlets, which have shown altered morphology. It was recognized that *rol*1 gene group in the T-DNA area of the Ri plasmid was amplified by PCR (Sugiyama *et al.*, 1995). Madhava Naidu *et al.* (1998) used leaf tissues of *C. canephora* infected with *A. tumefaciens*-LBA4404/pKIWI105 (binary vector) and pGV2260/pGSFR280 (cointegrate vector) strains for transformation. Kanamycin was used in selection medium. Transformation efficiency was higher in case of binary vector (45%) than in cointegrate vector (15%). GUS activity was determined by both histochemical and fluorometric assays. Regeneration of somatic embryos and plantlets has been achieved from transformed tissues. Embryogenic callus of *C. canephora* were infected with *A. tumefaciens* EHA101 harbouring pIG121-Hm, containing *β*-glucuronidase (GUS), hygromycin phosphotransferase (HPT) and neomycin phosphotransferase II genes. Selection of putative transgenic callus was performed by hygromycin and survived callus showed a strong GUS-positive reaction with X-gluc solution. Somatic embryos were formed from the putative transgenic calli and germinated into plantlets (Hatanaka *et al.*, 1999). Leroy *et al.* (2000) reported the transformation of *C. canephora* and *C. arabica* with a synthetic version of the *cry*1Ac gene of *Bacillus thuringiensis* to confer resistance to an important pest, the coffee leaf miner (*Perileucoptera coffeella* and other *Leucoptera* spp.).

Table 1.8: Some of the reports on transformation of coffee using *Agrobacterium*:

Variety	Explant	*Agrobacterium* strain	Reporter or Selection	Transformed tissue	Confirmation	Ref.
C. arabica	Hypocotyls of *in vitro* germinated seeds	*A.t.*-wild	Outgrowth on hypocotyl	-	-	Ocampo & Manzanera, 1991
C. arabica	Leaves	*A.r.*-wild-IFO 14554	*rol*1 gene	Hairy roots and adventitious embryos	PCR-*rol*	Sugiyama *et al.*, 1995
C. canephora	Leaf	*A.t.*-LBA4404-pKIWI105 & LBA4404-PGV2260/ PGSFR280	Kanamycin, GUS	Plantlets	GUS	Madhava Naidu *et al.*, 1998
C. canephora	Callus	*A.t.*-EHA101, pIG121-Hm with GUS, HPT&NPTII	Hygromycin, GUS, PCR	Plantlets	GUS	Hatanaka *et al.*, 1999
C. arabica & *C. canephora*	S. E*s	*A.t.*-LBA4404 with *cry*1Ac gene, GUS, *csrl-1*	GUS, chlorsulfuron	Plantlets	PCR-*cry*1Ac &*csrl-1* GUS	Leroy *et al.*, 2000

* Somatic embryos

LBA4404 strain of *A. tumefaciens* containing GUS and chlorsulfuron (herbicide) markers for selection of transformed cells was used for transformation. The transformed tissues were assayed using PCR for *cry*1Ac and *csrl-1* and also GUS. Southern blotting was used to evaluate the number of T-DNA copies integrated into the plant genome. Among the 51 events studied, 69% of the plants presented one T-DNA copy, 16% two copies and 4% of the plants had three copies integrated.

1.5. COFFEE SEED:

1.5.1. Embryo and endosperm development in coffee:

The embryogenic development runs largely parallel in both *C. canephora* and *C.arabica* and the general picture can be characterized as follows. After the pollen tubes penetrated into the embryo sac the double fertilization is effected immediately in *C. arabica*. In *C. canephora* this fusion is somewhat slower in taking place ranging from one day to a week (Leliveld, 1938; Moens, 1965) after pollination.

The fertilized embryo sac increases in volume and compress the inner integument cells but remains inactive in other species. The outer cells of the integument multiply actively, mainly by tangential divisions, giving rise to a transitorial perisperm which pushes back the swollen embryosac and eventually attains the size of a normal seed occupying the entire locule for some time (Mendes, 1941; Leliveld, 1938). This mode of development initially led Houk (1938) and Krug (1937) to misinterpret the nutritive tissue of the coffee bean as being perisperm in origin.

Inside the embryosac, the synergids and slightly later also the antipodals degenerate. The zygote stays near the micropyle is in a resting stage while the primary endosperm nucleus starts dividing approximately four weeks after successful pollination.

Generally in fertilization, when the pollen tube enters the ovary the tip of the tube breaks open and one sperm nucleus fuses with the egg to produce the zygote. The second sperm nucleus fuses with the two polar nuclei to produce a 3n nucleus, which form the endosperm. This process of double fertilization is known only among the flowering plants. The zygote develops into an embryo within the embryosac and integuments immediately surrounding the ovule develop into a seed coat. In many plants the endosperm later divide into a mass of small cells, but in some families (e.g., legumes) the endosperm becomes absorbed into cotyledons (Langenheim and Thimann, 1982b).

In coffee the endosperm originally consists of free nuclei, which develops into a cellular structure after a series of nuclear divisions occurring in rapid successions. As the number of cells increases, the endosperm takes a disc shape. The first division of the zygote takes place after 60-70 days of anthesis. The milky endosperm now rapidly increases in volume and gradually replaces the hyaline greenish perisperm. By the time the endosperm entirely fills the cavity earlier occupied by the perisperm, the embryo is well differentiated into a hypocotyl with radicle and two small cotyledons.

In the ripe seed the remains of the perisperm form the "silver skin" (Fig. 1.12), which envelops the hard endosperm. The parchment layer enveloping the seed is the endocarp (Carvalho *et al.*, 1969). These investigations showed that the coffee bean contains real endosperm, a fact supported by the other investigators (Fagerlind, 1939).

The specific form of the bean can be recognized as being folded upon itself. At the periphery there is one single layer of pavimentous epidermal cells. The major part of the bean is formed from the parenchymatous storage cells. The cells close to the epidermis are more elongated than those close to the centre. In the middle part there is a layer of mucilaginous material of variable thickness, in which embedded the embryo (Fig. 1.13; Dentan, 1985).

1.5.2. Coffee seed germination:

The protrusion of the radicle occurs in the arabica coffee within 10 days to three weeks from sowing (Fig. 1.14). In the next three weeks, while the radicle continues to grow gravitropically downwards with lateral roots appearing in tipward sequence, the hypocotyls elongates to lift the bean still covered by the parchment (Fig. 1.15). The seedling is then said to be in the *soldier* or *button* stage.

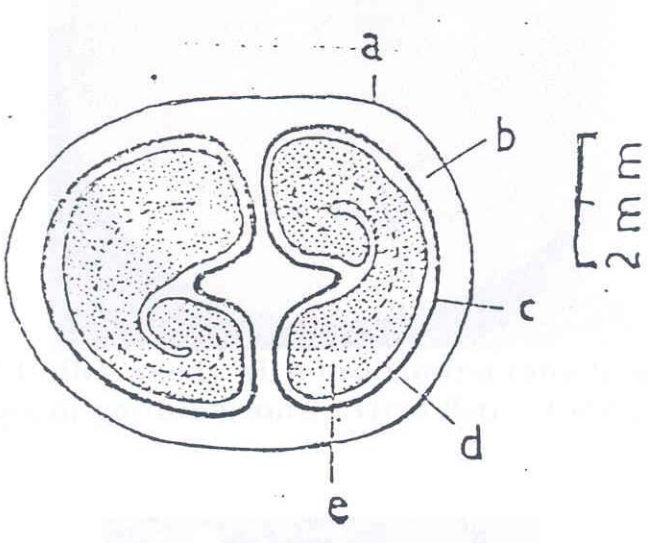


Figure 1.12: Cross section of coffee berry; a, exocarp; b, mesocarp (pulp); c, endocarp (parchment or testa); d, silver skin; e, endosperm. Adapted from Leliveld, 1938.

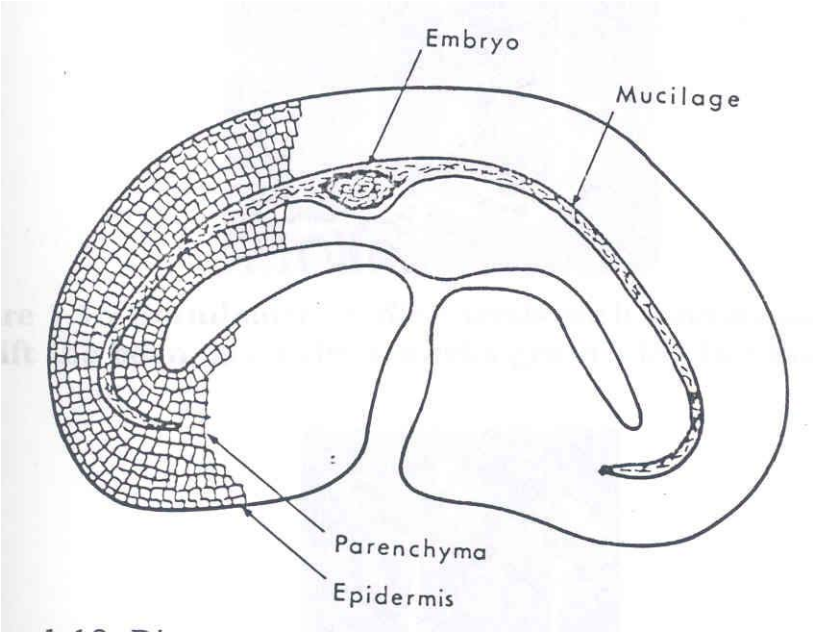


Figure 1.13: Diagrammatic transverse section of coffee bean

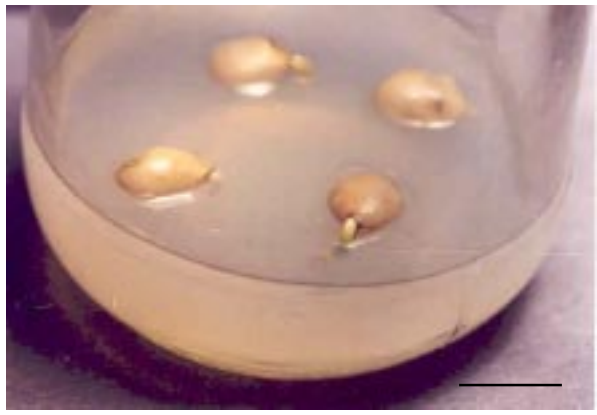


Figure 1.14: Coffee seeds with protruding radicle within ten days of germination *in vitro* (bar 13 mm)

Figure 1.15 Germinating coffee seeds with long hypocotyls, which lift the bean, after three weeks growth *in vitro* (bar 13 mm)

Figure 1.16 Fully germinated coffee seeds with expanded cotyledons after six weeks of growth *in vitro* (bar 20 mm)

Bean placement in the sowing bed with the embryo radicle up, down or horizontally may affect the extent and pattern of hook formation and the time to attain *soldier* stage (Pagacz, 1960; Vishveshwara and Raju, 1973). At this point the cotyledonary leaves begin to unfold, digesting in the course most of the endosperm; what remains of it is sloughed off as horny scales. The parchmet is slowly expelled sometime during the expansion of the cotyledonary leaves; soft parchment falls away faster than a hard one. The seedling then produces two cotyledonary leaves (Fig. 1.16), each with a dormant bud on its axil. The mature cotyledonary leaves are round-shaped with corrugated borders, and measures 20-40 mm diameter. The whole process starting from sowing lasts about eight to twelve weeks (Pagacz, 1960; Barros *et al.*, 1999).

1.5.3. Composition of the coffee seed:

Individual green beans vary in mass from below 100 mg to over 200 mg with considerable overlap between arabicas and robustas. As early as 1961, Navellier gave a mean composition of green coffee: polysacchardies (58%), lignin (2%), lipids (13%), proteins (13%), ash (4%), non-volatile acids (8%), trigonelline (1%) and caffeine (1%).

Table 1.9: A comparison of chemical composition of green arabica and robusta (Typical content in % dry mass basis). Adapted from Clifford, 1985

Component	Arabica	Robusta
Diterpenes (Kahweol)	0.7-1.1	Not detected
Caffeine	0.6-1.5	2.2-2.7
Polyphenols	6.2-7.9	7.4-11.2
Free sugars	5.3-9.3	3.7-7.1
Amino Acids	0.4-2.4	0.8-0.9
Trigonelline	~1	~1
Polysaccharide	46-59	43-54
Lipids	13-14	~10
Protein	~12	~12
Other acids	~2	~2
Ash	~4	~4
Total	91-114	86-105

1.6. COFFEE AND CAFFEINE:

1.6.1. Distribution of caffeine:

Many different species of plants contain purine alkaloids such as caffeine. Caffeine has been found in more than 60 sub-tropical plant species (Suzuki *et al.,* 1992). Caffeine-containing plants have a worldwide distribution, from *Camellia* (tea) in China, *Coffea* in Africa, *Ilex* in South America (Suzuki *et al.*, 1992) and *Paullinia cupana* in Amazon (Baumann *et al.*, 1995). Caffeine concentration varies in the tissues of these plants as well as throughout their organs. Guarana (*Paullinia cupana*) seeds may contain up to 4% caffeine (Spoladore *et al.*, 1987), while seeds of some coffee species are almost alkaloid-free (Cliffford *et al.*, 1991). Caffeine has also been found in citrus flowers (Stewart, 1985). Caffeine is the major alkaloid in coffee and variations in caffeine content are found within the same tree with the highest levels in seeds, flowers and leaves (Raju and Gopal, 1979). Variability is also observed among varieties of the same species and among different species of coffee (Mazzafera and Carvalho, 1992). The caffeine content of *C. canephora* is twice as high as *C. arabica* and comprises more than 2% and 1% dry weight, respectively (Baumann *et al.*, 1998).

Level of caffeine in young emerging leaves was studied (Frischknecht *et al.*, 1986). As long as the leaflets were fully covered by a resin layer and by two stipules, their alkaloid content varies between 1% and 3% dry wt. With leaflet emergence, the alkaloid formation increases and the variation decreases. Maximum content of 4% is reached when the leaflets are fully open. In the subsequent developmental period alkaloid content decreases (Frischknecht *et al.*, 1986). In freshly emerged leaves and young expanded leaves theobromine and caffeine were found but no theophylline was detected. The content of theobromine was slightly higher than that of caffeine in freshly emerged leaves while it was reverse in expanded

leaves. Biosynthesis of caffeine from adenine nucleotides occurs only at the very early stages of leaf development (Frischknecht *et al.*, 1986).

1.6.2. Role of caffeine in plants:

There is strong evidence that caffeine released from leaves and beans may be allelopathic (Rizvi *et al.*, 1980, 1987; Waller *et al.*, 1986). However, assays under natural conditions have not been performed to confirm this role. Estimates of caffeine release by a coffee plant were carried out in a very artificial system where branches removed from a tree were laid on a net, and after several days, the rainwater that had flowed through them was collected and analyzed (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. On the other hand, other fruit parts, which are rich in caffeine, did not release caffeine 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.

Caffeine is known to have a toxic effect on insects and fungi at concentrations found in plants. Recently 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 (CBD) (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.* In *Callosobruchus chinensis* (L.) caffeine causes nearly 100% sterility at a concentration of 1.5% (Rizvi *et al.*, 1980). The fungal toxic effect was demonstrated on a number of *Aspergillus* and

Penicillium species (Buchanan *et al.*, 1981), and on four species of *Saprolegniaceae* (Prabhuji *et al.*, 1983). In these cases, a dose dependent growth reduction was observed at concentrations between 0 and 0.4% and 0 and 1.0% respectively. Larvae of the tobacco hornworm (*Manduca sexta*) were killed when fed by a nutrient medium supplemented with 0.3% caffeine. At lower concentrations, it reduces weight gain of the larvae. The effect is mainly due to the inhibition of the phosphodiesterase activity and to the concomitant increase of the intracellular cyclic AMP (Nathanson, 1984).

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.

In tea and coffee, caffeine content is higher in young leaves and flowers than in other parts of the plant (Herndlhofer, 1933; Hamidi and Wanner, 1964; Raju and Gopal, 1979; Ashihara and Kubota, 1986). According to Frischknecht *et al.*, (1986), this might be a defense strategy. They followed the caffeine content of coffee leaves in very early stages of development, suggesting that as the leaflet emerges from the stipules that confer mechanical protection, caffeine increases as a chemical defence. As the leaf continuous its development caffeine decreases, the tissue is no longer so soft and its nutritional value decreases. In the debate concerning the significance of secondary metabolites the theory that they protect the producing plant against biotic (Swain, 1977) environment has gained increasing prominence. Based on principles of evolution, general strategies for optimal chemical defence against predation were postulated (Rhoades, 1979; McKey, 1979). According to one of these strategies plants are expected to accumulate protective secondary compounds in a tissue in direct proportion to the risk of predation of that tissue (Rhoades, 1979). Therefore, tissues with a high dietary value (seeds,

buds, young leaves) have a particularly high risk of predation (Frischknecht *et al.*, 1986). There is a genetic variability of caffeine content in fruits and leaves (Clifford *et al.*, 1989; Mazzafera and Magalhães, 1991). In cell cultures of *C. arabica* a considerable stimulation of caffeine production was achieved by the application of stress (Frischknecht and Baumann, 1985). In *C. arabica* the speed of formation of caffeine decreases by a factor of more than a hundred during leaf development (Frischknecht *et al.*, 1982).

1.6.3. Decaffeinated coffee:

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, especially with the elderly (Mazzaferra *et al.*, 1991). Over intake of caffeine is thought to induce functional diseases such as insomnia and dizziness. 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 intensively investigated (Ogita *et al.*, 2002)

The decaffeinated green coffee must contain less than 0.1% caffeine (which corresponds to about 3 mg caffeine in a cup of decaffeinated coffee) to comply with EEC regulations in the case of green beans or 0.3% in the case of coffee extracts (instant coffee) in dry matter basis (Indian Coffee, 2000).

Coffee maybe decaffeinated using

a) chemical solvents
b) supercritical gases
c) water and caffeine-free extracts (Indian Coffee, 2000)

Extraction of caffeine from seeds with organic solvents was first used to produce decaffeinated coffee. Commercial decaffeination is now a sophisticated process, and although it is not completely free of

solvent residues, there have been major improvements in the quality of the product in recent years (King, 1980). The latest methodology involves the use of supercritical fluid extraction with CO_2 to eliminate the potential health problems posed by the toxicity of the residues from solvents such as dichloromethane (Martin, 1982). However, this process is extremely expensive for a commercial scale operation (Mazzafera *et al.*, 1991).

1.6.4. Breeding for low caffeine:

In the long term, it seems more likely that the increasing demand for decaffeinated coffee would be better met by the use of *Coffea* species that produce beans containing significantly lower levels of caffeine than either *C. arabica* or *C. canephora*. Since solvent extraction is used for decaffeination, an alternative and health conscious approach would be to identify naturally occurring low caffeine lines (Srinivasan, 1996). Several breeding studies with coffee cultivars proved that the caffeine content of the seed, the so-called coffee bean, is genotypically defined in a quantitative, polygenic manner and only slightly influenced by exogenous factors.

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). Several *C. canephora* improvement programmes included intra- and inter-specific hybridization and selection of suitable genotypes aim to lower its caffeine content (Leroy and Charrier, 1989).

1.6.5. Modern biotechnological approaches for development of low/nil caffeine plants:

Biotechnology can play a vital role in large-scale multiplication of elite plant material and incorporation of novel genes for resistance to pests and diseases, drought etc. Production of plantlets through somatic embryogenesis has already been successful. The use of genetic engineering to produce caffeine-deficient *Coffea* plants may ultimately be a more practical proposition for the production of low caffeine coffee. Antisense mRNA technique can be used for blocking the synthesis of caffeine and production of naturally caffeine free coffee (Srinivasan, 1996). If this approach is to be taken, information is required on the genes and gene products controlling key steps in the biosynthesis and/or metabolism of caffeine in *Coffea*. Somaclonal variation, as a result of tissue culture, could also be an important tool for selecting low caffeine varieties. Baumann *et al.* (1998)

reported in the low-caffeine coffee mutant Laurina (*C. arabica* cv *laurina*) variation of the caffeine content from 0.36 to 1.08% with lowest values found in the tissue culture derived genotypes (somaclones) and reported a markedly lower mean caffeine content (0.56-0.61% dry wt) in the somaclones than in the parent line (0.68-0.71%).

1.6.6. Caffeine biosynthesis:

1.6.6.1. In Coffee:

Caffeine (1,3,7-trimethylxanthine) is a secondary plant product derived from purine nucleotides. Reports on the biosynthesis of caffeine and purine metabolism in coffee are fewer, compared with those in tea, and relate largely to *C. arabica* (Suzuki *et al.*, 1992; Roberts and Waller, 1979; Negishi *et al.*, 1985a, b; Baumann *et al.*, 1983; Looser *et al.*, 1974; Baumann *et al.*, 1978; Suzuki and Waller, 1984a). Caffeine biosynthesis comprises sequential methylations at *N*-7-, *N*-3- and *N*-1- of the xanthine ring catalyzed by *S*-adenosyl-L-methionine (SAM) dependent methyltransferases.

1.6.6.1.1. Source of purine and 7-methylxanthine:

The purine ring of caffeine in coffee is synthesized by the same pathway as in purine biosynthesis *de novo* as shown in arabica leaves by Anderson and Gibbs (1962). Suzuki and Waller (1984a) reported that adenine and guanine, derived from the nucleotide pool, are the primary precursors of caffeine in coffee. In *C. arabica* fruits (Suzuki and Waller, 1984a), exogenously applied adenine and guanine are effective precursors for caffeine biosynthesis; they are converted into purine nucleotides (AMP and GMP) by the reactions catalyzed by adenine phosphoribosyl transferase (Fujimori and Ashihara, 1994) and hypoxanthine-guanine phosphoribosyl transferase, as in tea leaves (Suzuki and Takahashi, 1976). Xanthine cannot serve

effectively as the direct precursor for the caffeine precursor 7-methyl xanthine and is catabolized by the conventional purine degradation pathway via uric acid in tea and *C. arabica* (Suzuki and Takahashi, 1975; Waller *et al.*, 1983; Suzuki and Waller, 1984b). Since 7-methylxanthine is not formed directly from xanthine, xanthosine is the other most promising candidates for the methylation at *N*-7 (Ogutuga and Northcote, 1970; Suzuki and Takahashi, 1975; Roberts and Waller, 1979; Negishi *et al.*, 1985b). In a study conducted by Looser *et al.* (1974) the leaf discs of *C. arabica* were infiltrated simultaneously with L-methionine- (methyl-^{14}C) and 7-methylxanthosine. Ring–labeled 7-methylxanthosine was synthesized (Baumann *et al.*, 1978) and fed to leaves of *C. arabica*. Up to 26% if the tracer was converted into caffeine within 8 days. When radiolabelled xanthosine was fed to young coffee shoots (Negishi *et al.*, 1985a) the radioactivity of the xanthosine decreased rapidly with the first 10 hr after the uptake and the radioactivity of caffeine increased continuously during the incubation period. The level of 7-methylxanthine and theobromine increased during the first 3 hr and 10 hr respectively and then decreased. Waldhausser *et al.* (1997a) purified the enzyme that methylates xanthosine at the 7' position. SDS-PAGE of a chromatofocussing-purified preparation containing only *N*-7-methyltrnasferase activity demonstrated the presence of a single labelled band of 40 kDa. In coffee and tea plants, caffeine is synthesized from xanthosine via a pathway that has three methylation steps. Mizuno *et al.* (2003a) identified and characterized the gene encoding the enzyme for the first methylation step of caffeine biosynthesis. The full-length cDNA of coffee tentative caffeine synthase 1, *CtCS1*, previously isolated by the rapid amplification of cDNA ends was translated with an *Escherichia coli* expression system and the resultant recombinant protein was purified. The protein renamed CmXRS1 has 7-methylxanthine synthase (xanthosine: *S*-

adenosyl-L-methionine methyltransferase) activity. CmXRS1 was specific for xanthosine and xanthosine 5'-monophosphate (XMP) could not be used as a substrate. The K_m value for xanthosine was 73.7 μM. CmXRS1 is homologous to coffee genes encoding enzymes for the second and third methylation steps of caffeine biosynthesis. There is very efficient conversion of xanthosine to 7-methylxanthine involving at least two enzymes: one xanhtosine methyl transferase and another a *N*-methyl-*N*^{9}-nucleoside hydrolase. Uefuji *et al.* (2003) also reported the isolation of cDNA for xanthosine methyltransferase gene, CaXMT1. CaXMT1 catalyzed the formation of of 7-methylxanthosine from xanthosine with a *Km* value of 78 μM. All these results therefore confirmed that xanthosine is the precursor of 7-methylxanthine in caffeine biosynthetic pathway. However, according to Schulthess *et al.* (1996) IMP formed from AMP and/or GMP either via *de novo* or via the purine salvage pathway, is converted to XMP, which is either hydrolyzed to xanthosine and further catabolized by purine degradation pathway, or enters the caffeine biosynthetic route by methylation to 7-methyl-XMP. This latter compound is not released but immediately metabolized to 7-methylxanthine (Schulthess *et al.*, 1996). Even after feeding with ($^{14}CH_3$)-adenine or (methyl-$^{14}CH_3$)-methionine significant amount 7-methylxanthosine could not be detected (Schulthess and Baumann, 1995). However, 7-methyl XMP was found to be inactive in *in vitro* enzymatic methylation studies in coffee (Suzuki and Takahashi, 1976). The activity of the *N*-methyl-*N*^{9}-nucleoside hydrolase has been illustrated. *C. arabica* cell free extracts made from callus cultures in which active biosynthesis of caffeine was occurring, exhibited *N*-methyl-*N*^{9}-nucleoside hydrolase enzyme activity (Waller *et al.*, 1983). The cloning of this enzyme has not yet been reported.

1.6.6.1.2. Caffeine biosynthetic pathway:

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 made from callus cultures in which active biosynthesis of caffeine was occurring exhibited *N*-methyltransferase enzyme activity (Waller *et al.*, 1983). The cell suspensions showed high activity canalizing the 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.* (1994a) reported the purification of theobromine 1-*N*-methyltransferase (STM), the enzyme responsible for the methylation of theobromine leading to caffeine formation in coffee. STM was purified from developing endosperms of immature fruits of *C. arabica*. STM is a bifunctional enzyme since it also methylated 7-methylxanthine, the immediate precursor of theobromine in the caffeine biosynthetic pathway. The specific activity of the STM with 7-methylxanthine was *ca* 55% of that determined with theobromine. The *Km* values obtained for theobromine and 7-methylxanthine were 0.196 and 0.496 respectively. STM was also purified from leaves.

Gillies (1995) improved the method of purification of *N*-methyltransferases from coffee endosperm. Purification of the enzyme by anion-exchange chromatography results in low activity yields. The loss of activity in endosperm extracts does not result from proteolysis of the enzyme since incubation with a wide range of protease

inhibitors failed to have a stabilizing effect on activity. Incorporation of 20% (v/v) glycerol in buffers during anion-exchange chromatography resulted in 54-78% yield of *N*-methyltransferase activity and a *ca.* 10-20 fold purification (Gillies, 1995). Enzyme extracts were prepared from young, emerging coffee leaflets. 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 considerable higher than the 3rd one. However, they frequently paralleled each other (Waldhausser *et al.*, 1997b; Baumann *et al.*, 1993), 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.*, 2003b), 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. Upon expression in *Escherichia coli*, one of them was found to encode a protein possessing 7-methylxanthine methyltransferase activity and was designated as CaMXMT. It consists of 378 amino acids with a relative molecular mass of 42.7 kDa and shows similarity to tea caffeine synthase (35.8%) and salicylic acid methyltransferase (34.1%). The bacterially expressed protein exhibited an optimal pH for activity ranging between 7 and 9 and methylated almost exclusively 7-methylxanthine with low activity toward paraxanthine, indicating a

strict substrate specificity regarding the 3-*N* position of the purine ring. K_m values were estimated to be 50 and 12 μM for 7-methylxanthine and *S*-adenosyl-L-methionine, respectively (Ogawa *et al.*, 2001). Mizuno *et al.* (2003b) reported isolation of a bifunctional coffee caffeine synthase (CCS1) clone from coffee endosperm by reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (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). The results 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. The proposed pathway of the final stage of caffeine biosynthesis in coffee is illustrated in Fig. 1.17.

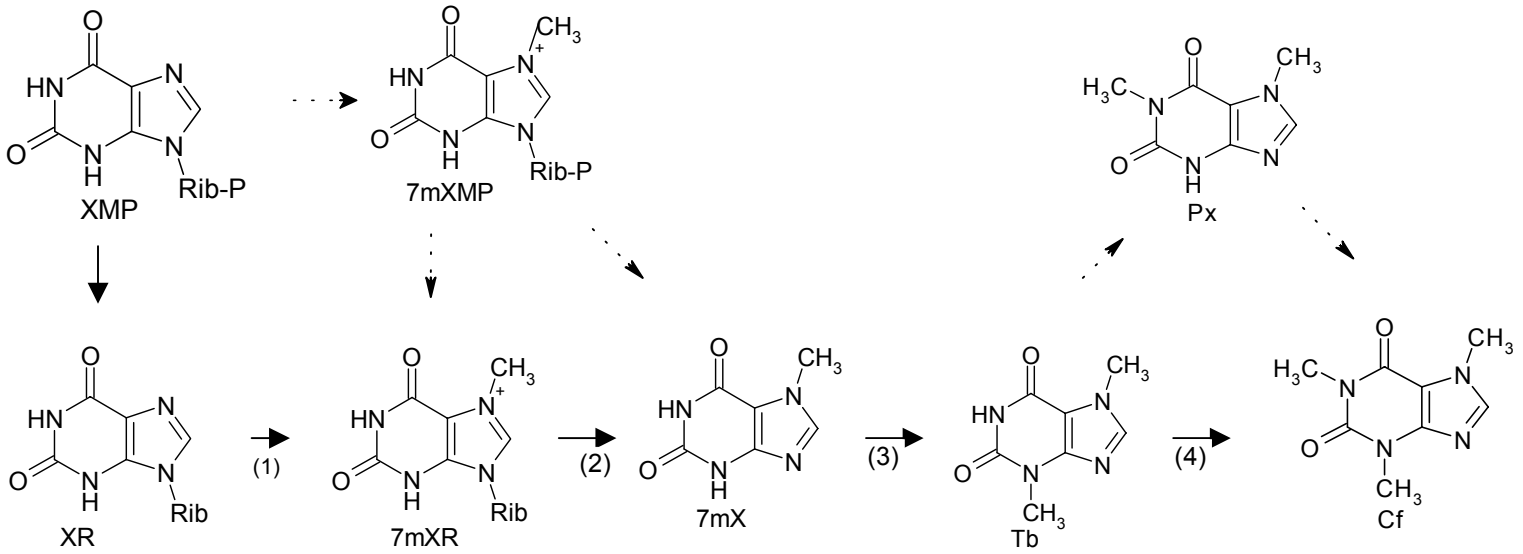


Figure 1.17 Caffeine biosynthetic pathway in coffee plants. Solid arrows indicate major routes, and broken arrows indicate minor or predicted routes. The first (1), third (3) and fourth (4) steps are *N*-methylation, and the second (2) step is Ribose removal. XMP, xanthosine monophosphate; 7mXMP, 7-methylxanthosine 5'-monophosphate; Px, paraxanthine, XR, xanthosine; 7mXR, 7-methylxanthosine; 7mX, 7-methylxanthine; Tb, theobromine; Cf, caffeine. (Uefuji et al., 2003)

1.6.6.1.3.Caffeine in *in vitro* cultures:

C. arabica cells produce caffeine and theobromine purine alkaloids in the medium (Waller *et al.*, 1983). Callus tissue culture of *C. arabica* L. cv Hibrido de Timore prepared from apical portions of orthotropic branches produced 49 to 92 times as much caffeine per unit weight of tissue as did the original explant. *C. arabica* cell free extracts made from 42-54-day-old callus cultures in which active biosynthesis of caffeine was occurring exhibited *N*-methyl-N^9-nucleoside hydrolase and *N*-methyltransferase enzyme activities (Waller *et al.*, 1983). Primary tissue culture growth was limited to 80 to 100 days. Caffeine formation paralleled tissue growth, however, as the callus grew older, less caffeine remained in the tissue and more was found in the medium. Callus showing no growth, or production of considerable polyphenolic compounds, evidenced by a light to dark brown color, produced much less caffeine than cultures of white vigorously growing callus (Waller *et al.*, 1983). The cell suspensions catalyzed the transfer of methyl groups from *S*-adenosyl-L-methionine to 7-methylxanthine and to theobromine producing theobromine and caffeine respectively. 7-methylxanthosine produced theobromine and caffeine. The same methyltransferase activities have later been detected in cell suspension (Baumann *et al.*, 1983). Suspension cultured cells of *C. arabica* have been immobilized by entrapment in calcium alginate gels to mimic natural aggregation. The production of methylxanthine alkaloid was increased up to 13-fold by the immobilization. This increased production has been ascribed to organization of the entrapped cells through physicochemical interactions between the polymer (alginate) and the plant cell wall. It has been shown that the metabolic changes induced by the immobilization are reversible (Haldimann and Brodelius, 1987). Frischknecht and Baumann (1985) reported that caffeine is a stress compound, because caffeine production was stimulated by exposure

to high light intensity and high NaCl concentration. Furuya *et al.*. (1990) reported the immobilization of *C. arabica* cells in polyurethane foam. Four phases were observed for cell growth and caffeine production i.e., immobilization, growth, caffeine production and regrowth. Caffeine production appeared to have a negative correlation with the growth of the immobilized cells. Schulthess and Baumann (1995) subjected suspension cells to various conditions such as photoperiod, adenine, ethephon or to the combination of both adenine and ethephon. When *C. arabica* cell suspension were provided with adenine and ethephon in dark, the overall purine alkaloid formation was stimulated by a factor of 4 and 7 respectively and their simultaneous application resulted in an additional increase yielding a stimulation factor of 7. In photoperiod, the caffeine formation was as compared to control in the dark, enhanced by a factor of 21 (Schulthess and Baumann, 1995).

1.6.6.2. Caffeine biosynthesis in tea:

The main pathway of caffeine biosynthesis in tea leaves begins with the conversion of xanthosine to 7-methylxanthine with *S*-adenosine-L-methionine (SAM) acting as the methyl donor (Ashihara and Crozier, 1999). Removal of the ribose moiety converts 7-methylxanthosine to 7-methylxanthine, which undergoes successive methylations, with SAM again acting as the methyl donor, to yield theobromine and then caffeine (Ashihara and Crozier, 1999; Kato *et al.*, 1999). A number of possible sources of xanthosine have been postulated. These are (i) inosine 5'-monophosphate (IMP) synthesized by *de novo* purine nucleotide biosynthesis, (ii) intracellular pools of adenine and guanine nucleotides and (iii) purine nucleotides and nucleosides produced by degradation of RNA (Suzuki *et al.*, 1992). In 2001, Koshiishi *et al.* reported a new route to caffeine that begins with the release of adenosine from *S*-adenosyl-L-homocysteine (SAH),

a product of SAM-dependent methyltransferases. In young tea leaves not only methyl group but also the purine ring of caffeine is derived from SAM and purine ring of caffeine is produced exclusively by this route. The hydrolysis of SAH also produces L-homocysteine, which will be reutilized for SAM synthesis via methionine by the so-called activated methyl cycle (Kawalleck *et al.*, 1992) or SAM cycle (Ashihara and Crozier, 1999). From the profiles of activity of related enzymes in tea extracts, it was proposed that the major route from SAM to caffeine is a SAM → SAH → adenosine → adenine → AMP → IMP → XMP → xanthosine → 7-methylxanthosine → 7-methylxanthine-→-theobromine → caffeine pathway.

1.6.7. Caffeine degradation:

1.6.7.1. In coffee:

Kalberer (1965) reported for the first time 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). 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 are 3-methylxanthine, allantoin, allantoic acid, urea and an unknown compound (Suzuki and Waller, 1984a,b; Crozier *et al.*, 1995). The conversion of caffeine to theophylline is the rate limiting step in purine alkaloid catabolism and provides a ready explanation for the high concentrations of endogenous caffeine that are found in *C. arabica* leaves. Although theobromine is converted primarily to caffeine, a small portion of the theobromine pool appears to be degraded to xanthine by a caffeine-independent pathway. In addition to being broken down to CO_2, via the purine catabolism pathway, xanthine is metabolized to 7-methylxanthine (Crozier *et al.*,

1995). The pathway for the degradation of caffeine in coffee is illustrated in Fig. 1.18.

1.6.7.2. Microbial degradation of caffeine:

Microbial degradation has not been considered when caffeine is discussed as an allelochemical, but 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 to organic solvent extraction for the decaffeination of beverages and would remove health concerns associated with solvent residues. Such a system would also prove useful in the detoxification of by-products of the coffee industry as, at present, coffee pulp despite high levels of carbohydrates and proteins is rendered useless as an animal feed because of its high caffeine content (Sideso *et al.*, 2001). 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 *et al.* (1999) using C^{14}-labelled caffeine has indicated that *Pseudomonas putida* demethylates caffeine sequentially by removing methyl groups rather than simultaneously demethylating. Cell free extracts (CFE) from *Pseudomonas putida,* cultured on caffeine as sole carbon and/or nitrogen source contained proteins, which demethylate caffeine. HPLC analysis of caffeine degradation by *P. putida* showed that caffeine is converted to uric acid via 3,7-dimethylxanthine, 7-methylxathine and xanthine. The use of whole cells in the decaffeination procedure would not be acceptable because the cells have to be removed at a later stage, which may affect the taste of the final product for the commercial preparation of beverages (Sideso *et al.*, 2001). The pathway for the degradation of caffeine by *Pseudomonas putida* C1 is illustrated in Fig. 1.19.

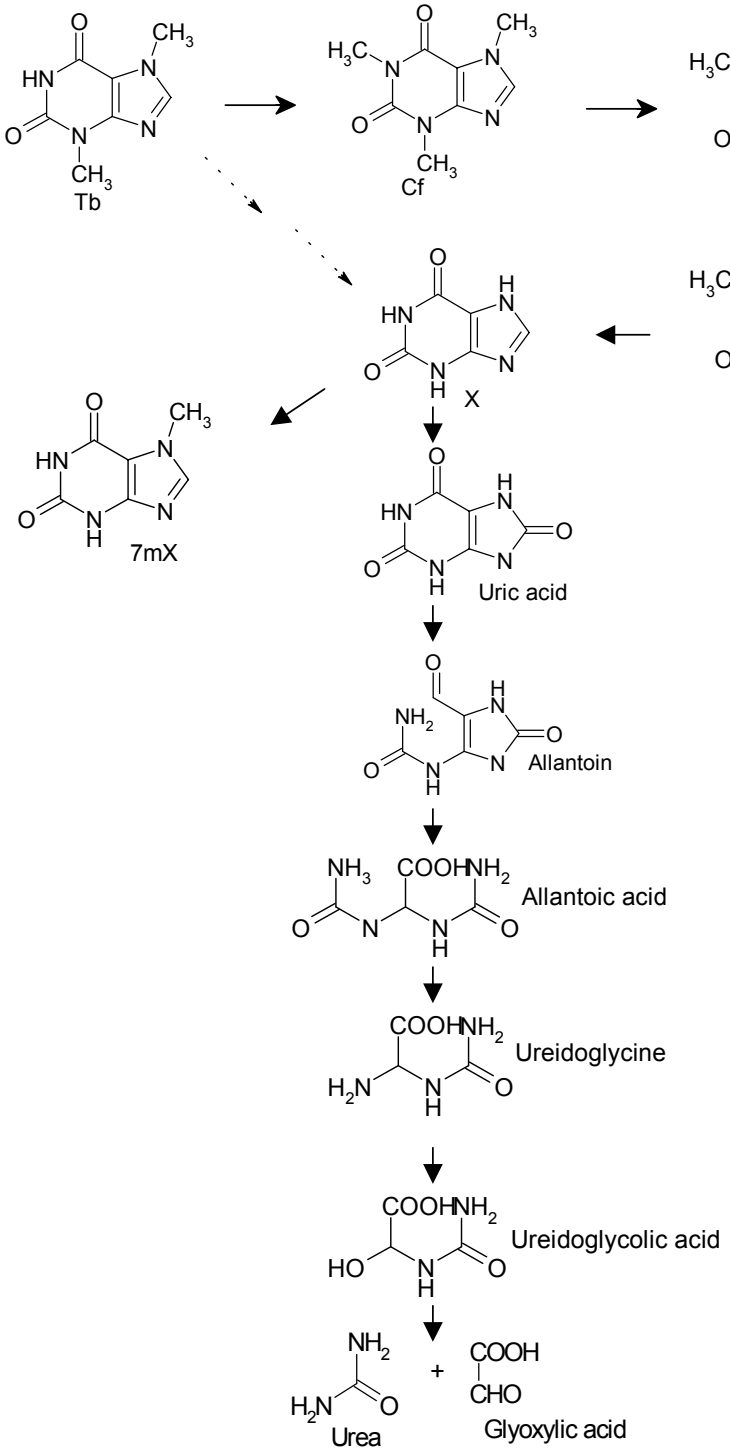


Figure 1.18: Purine alkaloid catabolism pathways operating in leaves of *C. arabica*. Solid arrows indicate major routes and broken arrows indicate minor routes. Tb, theobromine; Cf, caffeine; Tl, theophylline; 3mX, 3-methylxanthine; X, xanthine; 7mX, 7-methylxanthine (Crozier *et al.*., 1995).

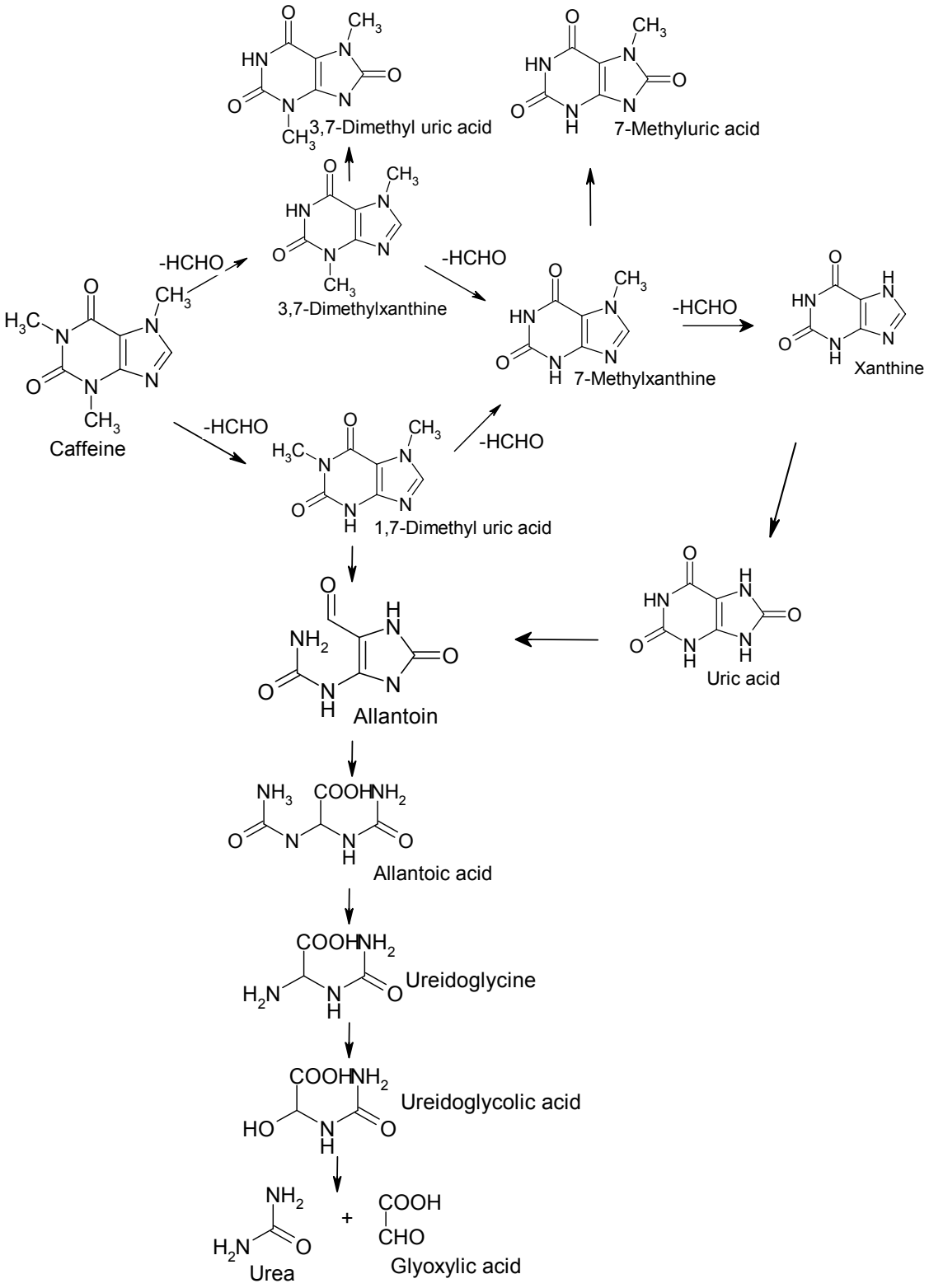


Figure 1.19: Pathways of caffeine degradation by *Pseudomonas putida* C1. Adapted from Blecher and Lingens, 1977.

1.6.8. Caffeine and chlorogenic acid:

In addition to caffeine coffee has a lot of polyphenols (Clifford, 1985). Quinic acid occurs free and in esterified form. The esters, known as chlorogenic acids (the 5-*O*-caffeoyl ester of quinic acid, 5-CGA), form a quantitatively important fraction of green and roasted coffee beans, soluble coffee powders and coffee brews. The CGA may be subdivided into groups usually of three isomers each on the basis of the number and identity of the acylating residues: (a) the caffeoylquinic acids (CQA); (b) the dicaffeoylquinic acids (diCQA); (c) the feruloylquinic acids (FQA); (d) the *p*-coumaroylquinic acids (CoQA) and (e) several caffeoylferuloylquinic acids (CFQA) (Clifford, 1985). Horman and Viani (1971) using NMR spectroscopy concluded that in aqueous media the caffeine-potassium chlorogenate was best described as 'hydrophobically bound π- molecular complex' which is most likely the precursor of the well-described crystalline caffeine-potassium chlorogenate 1:1 complex (Martin *et al.*, 1987). Chlorogenic acid functions as part of the oxidase system in plants (Hanson and Zucker, 1963).

The seeds of *C. arabica* and *C. canephora* have been analyzed and are known to contain relatively large, but distinguishable amounts of chlorogenic acid and caffeine (Clifford, 1985). The seeds of all *Coffea* species analyzed showed obvious parallels in their levels of purine alkaloids (PuAs) and chlorogenic acids (CGAs) (Carelli *et al.*, 1974). Baumann and Röhrig, (1989) reported that compared to standard conditions (dark regime) the cell lines kept under the photoperiod produce considerably more of both caffeine and CGA in *C. arabica* suspension cultures. Clifford *et al.* (1989) reported the possibility of chlorogenic acid and caffeine content as possible taxonomic criteria in *Coffea* and *Psilanthus*.

The free CGA and also the complex with caffeine are stored intracellularly in the central vacuole. Free caffeine is found both

intra- and extracellularly because of its dual hydrophilic and lipophiic character (Gundlach *et al.*, 1992). Increasing CGA concentration led to enhanced cellular compartmentation of caffeine in suspension cultures of coffee (Waldhausser and Baumann, 1996). Caffeine and related PuAs *per se* freely penetrate cell-, tissue- and organ related barriers (Waldhausser and Baumann, 1996).

In contrast to CGA, which is exclusively located within the cells, caffeine is released into the medium in *C. arabica* suspension cultures (Baumann and Röhrig, 1989). Caffeine is accumulated intracellularly to a certain extent and this is correlated with the CGA concentration in the cells (Baumann and Röhrig, 1989). Vital staining with methylene blue indicated that CGA is compartmented predominantly in vacuole (Baumann Röhrig, 1989).

From washout experiments with the coffee leaf discs it is known that only a fraction of caffeine can be removed. It was assumed that the residual caffeine is intracellularly complexed by chlorogenic acid forming a 1:1 complex not only in crystals (Martin *et al.*, 1987) but also in aqueous solution with CGA and related methyl xanthines (Hormann and Viani, 1971; Baumann and Röhrig, 1989).

From the literature survey some lacunae in the knowledge base has been observed, which are:

1. There are more knowledge on arabica than that on canephora
2. There is very little work on caffeine production during somatic embryogenesis. This data is particularly important when somatic embryos are going to be used for transformation of coffee for regulation of caffeine biosynthesis.
3. There are two studies on the use of A*grobacterium rhizogenes* for transformation and a comparison of the transformation events with *A. rhizogenes* and *A.tumefaciens* is lacking.
4. Transformation of Indian varieties is not achieved

Based on this the major objectives of the research work has been formulated.

1.7. MAJOR OBJECTIVES OF THE PRESENT STUDY:

1. To evolve a rapid somatic embryogenesis and micropropagation system to propagate coffee variety and for the improvement of the crop:

The main objective of the study was to establish an effective protocol for somatic embryogenesis in *C. canephora* varieties and to standardize the media requirements for various genotypes for somatic embryo formation. Enhancement of somatic embryogenesis response using triacontanol and thidiazuron has been investigated. Studies on micropropagation, zygotic embryo culture and efficient *in vitro* coffee seed germination were also conducted.

2. Development of a suitable transformation protocol for coffee through *Agrobacterium* strains.

In addition to these major objectives, studies were conducted on the caffeine analysis in different parts of the coffee plant, seedlings, and hybrids with major emphasis on seeds and *in vitro* cultures.

CHAPTER II

Somatic Embryogenesis & Micropropagation

2.1 INTRODUCTION

Tissue culture has been successfully employed for rapid multiplication in a number of tree species like *Coffea* (Söndahl and Sharp, 1977; Hatanaka *et al.*, 1995; Van Boxtel and Berthouly, 1996). Various plant organs and tissues can be used as a source of explants for the initiation of embryogenic cultures. Although coffee is a perennial, somatic embryos can be induced at high frequency. Thus coffee is an ideal *in vitro* model system for improvement via tissue culture as well as genetic transformation (Söndahl and Loh, 1988). In coffee, micropropgation is possible by apical meristem and axillary bud culture, and induction of adventitious bud development.

2.1.1. High frequency somatic embryogenesis in coffee:

High frequency somatic embryogenesis (HFSE) in coffee was first reported by Söndahl and Sharp (1977, 1979). They reported that small number of somatic embryos were produced from leaf explants of *C. arabica* L. 'Bourbon' which was referred to as low-frequency somatic embryogenesis; this was followed by the production of somatic embryos on top of the brown tissue, which was called high-frequency somatic embryogenesis. According to these authors, HFSE is characterized by the abundant appearance of friable, highly embryogenic callus.

They obtained such HFSE-callus after a two-step procedure: a primary culture on auxin-containing medium called the "the conditioning medium" for callus induction followed by a second culture on auxin-free medium named "the induction medium" for embryo induction. Successful induction of high frequency embryogenic callus was found to be dependent on the 2,4-D and kinetin concentrations in conditioning medium. A histological study on these high frequency embryogenic calluses (Söndahl *et al.*, 1979) suggested that embryos induced in high quantities were obtained from

proliferation of “proembryo mother cells” and previously formed somatic embryos.

Nakamura *et al.* (1992) obtained friable high frequency somatic embryogenic calluses from different genotypes of coffee using a two step culture method similar to that described by Söndahl and Sharp (1977, 1979). The specific nature of HFSE-callus permits its use in liquid culture systems (Söndahl *et al.*, 1985; Zamarripa *et al.*, 1991). Because it rapidly generates into large amounts of uniform plant material, the procedure is of great value in accelerating classical coffee breeding methods: e.g. in the rapid multiplication of interspecific hybrid varieties of *C. arabica* that combine multiple resistance and hybrid vigor. The application of bioreactors for large-scale multiplication of coffee plants has further advanced these developments (Ducos *et al.*, 1993; Noriega and Söndahl, 1993).

Optimal conditions for callus induction and high frequency of somatic embryogenesis (HFSE) were established for *C. canephora* (Berthouly and Michaux-Ferriere, 1996), Arabusta and *C. congusta* (Van Boxtel and Berthouly, 1996). Cytological origin of this type of callus and of regenerated somatic embryos was also studied by Berthouly and Michaux-Ferriere (1996). High rates of multiplication were obtained by culturing the inoculum at a density of 10 g l^{-1} in MS modified medium. The maintenance of the embryogenic potential was achieved by culturing aggregates of 250-1000 mm in diameter and at a density of 5 g l^{-1}. Under these conditions, the embryogenic potential of *C. canephora* was maintained for 2 years, producing 1,20,000 embryos per gram inoculum after 8-10 weeks in regeneration liquid medium (Van Boxtel and Berthouly, 1996).

Reproducible regeneration system is a pre-requisite for development of plants having specific qualities like disease resistance, pest resistance and special nutritional characteristics through *in vitro* culture technology either through genetic transformation or via

selection and propagation. Genetic modifications using cellular and molecular approaches offer new possibilities for specific changes without modifying the genetic basis of established cultivars (Fuentes *et al.*, 2000). For transformation and genetic improvement programs, it is desirable to have a fast and efficient protocol by which to produce coffee somatic embryos (Quiroz-Figueroa *et al.*, 2001). Somatic embryogenesis has been widely used for propagation and genetic modification of higher plants. Due to its multiplication potential the process offers a mean to propagate large number of elite clones and transgenic plants over a short period of time (Fuentes *et al.*, 2000; Ammirato and Styer, 1985).

Somatic embryogenesis in coffee is very different from somatic embryogenesis in other species. In carrot, the process takes only a few days (Krikorian and Smith, 1991) while the best results in coffee are obtained after several weeks (Quiroz-Figueroa *et al.*, 2001).

2.1.2. Effect of triacontanol (TRIA) and thidiazuron (TDZ) on somatic embryogenesis:

Triacontanol (TRIA), a long 30-carbon primary alcohol, is a naturally occurring plant growth promoter (Reis *et al.*, 1977; Ries and Houtz, 1983) and has been shown in some instances to increase the growth and yield of plants *in vivo* as well as in *vitro* (Ries and Wert, 1977; Ries *et al.*, 1983; Mamat *et al.*, 1983; Hashim and Lundergan, 1985; Stautemyer and Cook, 1987; Ma *et al.*, 1990; Tantos *et al.*, 1999). However, effects of TRIA often have been inconsistent in some plants (Hoagland 1980; Eriksen *et al.*, 1982). The reason for this inconsistency has been attributed to the formulation and or inhibition by traces of aliphatic hydrocarbons and phthalate-esters (Ries *et al.*, 1977; Jones *et al.*, 1979; Laughlin *et al.*, 1983; Reis *et al.*, 1983; Ries *et al.*, 1984). From our study as well as from the reported procedures it is experienced that somatic embryogenesis in coffee generally

requires 3-7 months (Hatanaka *et al.*, 1995; Van Boxtel and Berthouly, 1996; Fuentes *et al.*, 2000). Moreover, problems such as morphological abnormalities, asynchronous development and difficulty in extending embryo development beyond its torpedo stage are common (Staritsky and Van Hassel, 1980; Ganesh and Sreenath, 1999). As triacontanol is known for its growth promoting nature under *in vitro* conditions for various morphogenetic responses (Tantos *et al.*, 1999; Reddy *et al.*, 2002) it was thought to study its influence if any on somatic embryogenesis in *in vitro* leaf and hypocotyl segments along with secondary embryos formation.

Additionally the effect of TDZ on somatic embryogenesis is also attempted. 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 study the effect of TRIA and TDZ on somatic embryogenesis of *C. canephora* (S-274) is also discussed.

2.1.3. Embryo culture:

The embryo represents the beginning of the new sporophytic generation of the plant and it originates from fertilized egg of zygote. The embryos formed within the female gametophyte through the normal sexual process can be separated from the maternal tissues at different developmental stages and cultured *in vitro* under aseptic conditions by providing artificial nutritional and environmental conditions.

The removal and culture of zygotic embryos of higher plants was one of the earliest successful techniques in plant tissue and organ culture. Zygotic embryo culture is the aseptic isolation and growth of sexually produced embryos *in vitro* with the objective of obtaining

viable plants (Muniswamy and Sreenath, 1999a). Hannig (1904) first cultured the mature embryos of two cruciferous genera *Raphanus* and *Cochlearia* using aseptic techniques.

2.1.3.1. Applications of zygotic embryo culture:

1. Rescuing incompatible hybrid crosses:

The most useful and popular application of zygotic embryo culture is rescuing incompatible hybrid crosses. In breeding programmes, interspecific and intergeneric crosses are frequently carried out to transfer valuable genes from wild species to cultivated species. Incompatibility is often encountered in such crosses and results in shriveled seeds containing aborted embryos (Muniswamy and Sreenath, 1999a).

2. Propagation of elite plants:

Regenerative capacity is essential in non-traditional genetic manipulation and some times in breeding process. Because of the juvenile nature with high regenerative potential, embryos are excellent materials for *in vitro* propagations (Muniswamy and Sreenath, 1999a).

3. *In vitro* preservation of germplasm:

It is useful for long-term preservation of embryos of coffee germplasm (Muniswamy and Sreenath, 1999a).

2.1.4. Micropropagation:

Micropropagation is an effective method for multiplication. It can reduce the time taken for varietal development and preserve heterozygosity and genetic plasticity in coffee plantations (Sondahl and Lauritis, 1992). In coffee, micropropagation is possible by apical meristem, axillary bud culture and induction of adventitious bud development

The main objective of the study was to establish an effective protocol for the somatic embryogenesis in *C. canephora* varieties and

to investigate the media requirements for various genotypes for somatic embryo formation. Towards this, the study concentrated to find out an effective method for the production of embryogenic callus and regeneration of somatic embryos from the tender hypocotyls of germinated coffee seedlings. Three different selection of *C. canephora* was used for the study to see the difference in response. The effect of triacontanol and thidiazuron on the enhancement of somatic embryogenesis response has been investigated. Some studies on zygotic embryo culture have been conducted. Studies on establishment of nodal cultures for micropropagation have been conducted. The influence of silver nitrate on the apical bud culture of coffee has also been studied.

2.2. MATERIALS AND METHODS:

2.2.1. Seed germination and explant preparation:

2.2.1.1. Source:

Certified ash coated seeds of three varieties of *C. canephora* viz., 274, CxR and Old Robusta were obtained from Central Coffee Research Institute, Coffee Research Station, Chikmagalur District, Karnataka, India. Ash coating is done to maintain the moisture content in the seeds as well to prevent the microbial growth on the seeds. Seeds were sown in germination beds to raise seedlings. Germinated seedlings with unopened cotyledons and hypocotyls length of 5-6cms were obtained in one and a half months.

2.2.1.2. Sterilization:

Hypocotyls were washed in tap water to remove the traces of soil and dipped in 70% alcohol for 30 seconds, and after removing alcohol by thorough washing in tap water surface sterilized with 0.1% (w/v) $HgCl_2$ solution for 8-10 minutes followed by 5-6 rinses in sterile

distilled water. The cut ends were removed and 0.5-0.75 cm long explants were excised and collected in Petri dishes containing sterile water. Phenolics exudation was minimal from these explants.

2.2.2. Media and culture conditions:

Two media were tried for the induction of embryogenic callus. The hypocotyl explants were placed both on callus development medium (Van Boxtel and Berthouly, 1996) and modified callus development medium.

2.2.2.1. Callus formation in callus development medium:

Callus development medium (Van Boxtel and Berthouly, 1996; Table 2.2), contained half strength Murashige and Skoog (1962) basal salts (Table 2.1), 3% sucrose, 100 mg l^{-1} casein hydrolysate, 400 mg l^{-1} malt extract, 10 mg l^{-1} thiamine-HCl, nicotinic acid, pyridoxine-HCl, glycine, each 1 mg l^{-1}, 100 mg l^{-1} myo-inositol, 50 mg l^{-1} cysteine-HCl, 9.8 µM 2-iP, 2.2 µM 2,4-D and 4.9 µM IBA. The medium was gelled with 0.8% (w/v) tissue culture grade agar (Himedia, India) in 200ml glass jars, having 40ml of the medium. The jars were closed with polypropylene caps. The pH was adjusted to 5.6 prior to autoclaving at 121°C, 104 kPa for 20min. The cultures were incubated at 25±2°C with a 16 h photoperiod under fluorescent light under low PPF (5 µmol m^{-2} s^{-1}) for the first 55 days and later on subcultured to embryo induction medium (Table 2.3; Van Boxtel and Berthouly, 1996) and cultured at an irradiance of 25 µmol m^{-2} s^{-1}.

2.2.2.2. Embryo induction:

Hypocotyl segments were placed on callus induction medium for the first 55 days. After this, the induced callus along with the hypocotyl explants were subcultured on to the embryo induction medium containing 4.5 µM 2,4-D and 17.6 µM BA (Van Boxtel and

Berthouly, 1996) (Table 2.3). The explants were kept in this medium for two and a half months.

2.2.2.3. Regeneration of somatic embryos:

The embryogenic callus obtained from the embryo induction medium was transferred to regeneration media (Van Boxtel and Berthouly, 1996) (Table 2.3) containing five different combinations of growth regulators i.e. 4.4, 8.8, 13.2 µM BAP (namely Ra, Rb and Rc respectively), 2.2 µM BAP with 2.4 µM 2-iP, and 6.6 µM BAP with 7.3 µM 2-iP (Rd and Re respectively). Auxins were avoided. The callus was kept in the respective media for five months by subculturing once in 45 days. They were cultured at low inoculum density (1 g callus per litre) in 20 ml of the medium in culture tubes under dark.

2.2.2.4. Callus induction in modified callus development medium:

In this procedure the callus development medium (Table 2.2), was modified in the composition of growth regulator. Modified callus development medium had the same composition but it contained full strength MS salts and used 4.9 µM 2-iP as the sole growth regulator. The cultures were incubated at 25±2°C with a 16-h photoperiod under fluorescent light at an irradiance of 25 µmol m^{-2} s^{-1}. The culture conditions remained the same in rest of the experiments if otherwise mentioned. The cultures were kept in the same medium by subculturing once in 45 days for callus induction and embryo formation. Adventitious shoots were started forming in this medium in one month and globular embryos were formed from previously formed embryogenic callus in five months time. The frequency of hypocotyls producing callus, embryos and shoots were scored at intervals.

2.2.2.5. Embryo germination and rooting:

The torpedo shaped embryos formed in the modified callus development medium were transferred to both solid and liquid embryo

germination media (Table 2.3) containing 1.1 µM BAP and 2.5 µM IAA as growth regulators (Van Boxtel and Berthouly, 1996) for germination.

Table 2.1: Composition of Murashige and Skoog (1962) and Heller (1953) media♦:

	Murashige and Skoog (MS)	**Heller (H)***
Macroelements:		
$CaCl_2.7H_2O$	440	-
$CaCl_2$	-	56.62
KCl	-	750.00
KH_2PO_4	170	-
KNO3	1900	-
$MgSO_4$	-	121.56
$MgSO_4.7H_2O$	370	-
$NaNO_3$	-	600.00
NaH_2PO_4	-	108.70
NH_4NO_3	1650	-
Microelements:		
$AlCl_3.6H_2O$	-	0.054
$CoCl_2.6H_2O$	0.025	-
$CuSO_4.5H_2O$	0.025	0.03
$FeCl_3.6H_2O$	-	1.00
$FeSO_4.7H_2O$	27.85	-
Na_2 EDTA	37.35	-
H_3 BO_3	6.20	6.20
KI	0.83	0.01
$MnSO_4.H_2O$	-	0.08
$MnSO_4.4H_2O$	22.3	-
$Na_2MoO_4.2H_2O$	0.25	-
$NiCl_2.6H_2O$	-	0.03
$ZnSO_4.7H_2O$	8.60	1.00
Myo-inositol	100.00	100.00
Vitamins:		
Glycine	2.00	-
Nicotinic acid	0.50	-
Pyridoxine HCl	0.50	-
Thiamine HCl	0.10	-

♦ Quantities expressed in mg l^{-1}; Murashige T. and Skoog F. (1962); *****Heller R. (1953)

Table 2.2: Composition of callus development medium (C) and modified callus development medium (mC)#:

	Callus development medium♦	**Modified callus development medium**
Macrominerals	MS*-Half strength	MS-Half strength
Microminerals	MS-Half strength	MS-Half strength
Niacin	1	1
Pyridoxine-HCl	1	1
Thiamine-HCl	10	10
Glycine	1	1
Myo-inositiol	100	100
Cysteine-HCl	50	50
Casein hydrolysate	100	100
Malt extract	400	400
2,4-D	2.2 µM	-
IBA	4.9 µM	-
2-iP	9.8 µM	4.9 µM
Sucrose	30,000	30,000
Agar	8,000	8,000
PH	5.6	5.6

Quantities expressed in mg l^{-1} if otherwise mentioned
*Murashige and Skoog (1962; See Table 2)
♦ Van Boxtel and Berthouly (1996)

Plantlets formed were later transferred to half strength MS medium without growth regulators for further growth. When plantlets were 3-4 leaf pairs, they were taken for hardening.

For rooting of hypocotyls derived plantlets that were formed in modified callus development medium, the cut ends of plantlets were dipped in sterile IBA (246 µM) and NAA (268 µM) solution mixture prepared in distilled water just before inoculation on half strength MS (Murashige and Skoog, 1962) solid medium.

Table 2.3: Composition of embryo induction (E), regeneration (R) and embryo germination (EG) media#:

	E♦	**R♦**	**EG (or A)♦**
Macrominerals	MS*-Half strength	MS-Half strength	MS-Half strength
Microminerals	MS-Half strength	MS-Half strength	MS-Half strength
Niacin	-	1	-
Pyridoxine-HCl	-	1	3.2
Thiamine-HCl	20	10	8
Glycine	20	2	-
Myo inositiol	200	200	100
Cysteine-HCl	40	-	-
Adenine sulphate	60	40	-
Casein hydrolysate	200	400	-
Malt extract	800	400	-
2,4-D	4.5 µM	-	-
IAA	-	-	2.5 µM
2-iP	-	2.9 & 7.3 µM	-
BA	17.6 µM	4.4 –13.2 µM	1.1 µM
Sucrose	30,000	40,000	20,000
Agar	8000	8000	8000
PH	5.6	5.6	5.6

Quantities expressed in mg l^{-1} if otherwise mentioned
♦ Van Boxtel and Berthouly, 1996
*Murashige and Skoog, 1962

2.2.3. Scanning Electron Microscopy:

Embryos of different stages were subjected to scanning electron microscopy. The samples were fixed in 50% ethanol, 5% acetic acid and 3.7% formaldehyde in phosphate buffer (pH, 7.0; 50 mM), for 4 hours at 4°C and dried through an alcohol series (60%-absolute alcohol, 30 minutes each).

Embryos were mounted on stubs, gold coated (Polaron SEM coating system) and then viewed using a LEO 435 VP scanning electron microscope (LEO Electron Microscope Ltd, Cambridge) at a magnification of 29-72 X.

2.2.4. Influence of triacontanol (TRIA) on somatic embryogenesis:

2.2.4.1. Stock solution of TRIA:

Triacontanol [$CH_3(CH_2)_{28}$ CH_2OH] was obtained from Sigma-Aldrich Chemicals, USA. A stock solution of TRIA was prepared by dissolving 1 mg of TRIA in 0.75 ml of $CHCl_3$ containing 1 drop of Tween 20 and diluting with distilled water to a final volume of 200 ml (Tantos *et al.*, 1999).

2.2.4.2. Source of explant and explant preparation:

The experiments were conducted with *C. canephora* variety S-274 that were selected from 2-year-old plants growing under green house conditions. Callusing, subsequent embryogenesis and regeneration were achieved from leaf explants using the protocol described earlier in the study (modified callus development medium, Table 2.2). Regenerated plantlets with two cotyledonary leaves were cultured on half-strength MS medium (Murashige and Skoog, 1962). Regenerated plantlets were used as explants for subsequent experiments with TRIA.

2.2.4.3. Culture media and incubation conditions:

The medium devised for primary and secondary somatic embryogenesis in this study contained half-strength MS salts, thiamine HCl at 8.0 mg l^{-1}, inositol at 100 mg l^{-1}, pyridoxine HCl at 3.2 mg l^{-1}, 2% sucrose supplemented with 2.5 μM IAA and 1.1 μM BAP (Embryo germination medium, Table 2.3) and hereafter referred to medium A. Media B and E were without IAA, but were supplemented with triacontanol at concentrations of 4.55 and 11.38 μM,

respectively. Media C and D contained 2.5 μM IAA and were supplemented with TRIA at concentration of 4.55 and 11.38 μM, respectively. All the media were adjusted to pH 5.6, gelled with 0.8% (w/v) tissue culture grade agar (Himedia, India) and autoclaved at 120ºC temperature and 104 kPa for 20 min. The explants were cultured in dark at 27^{0}C in 90x15 mm petri dishes containing 20 ml medium.

2.2.4.4. Embryo induction:

The first and second leaves of regenerated plants were cut into 1 cm^2 pieces and placed with upper surface touching the medium. Similarly, 1 cm long *in vitro* stem segments were cut and used as explants. After 45 days growth embryogenic callus was obtained from leaf and stem segments of regenerated plants. Moreover, small globular primary embryos developed from the surface of the explants. Embryogenic callus (weighing 250 mg) or small globular primary embryos (a clump of 4 embryos approx. 50-60 mg) were used for proliferation to produce somatic embryos and for secondary embryogenesis on media containing IAA, BA and either with or without TRIA as indicated above.

Each petri plate contained five explants and 10 replicate plates were maintained. Observations for embryogenesis were made after 45 days incubation. The experiment was repeated twice. For generating secondary embryos from primary embryos, 50 clumps of small, round globular primary embryos (each clump containing ~ 4 embryos) were used. To test the embryogenic nature of yellow friable callus, 10 replicate plates, each containing five 250 mg callus clumps were used for obtaining embryogenesis.

2.2.5. Thidiazuron (TDZ) induced somatic embryogenesis:

2.2.5.1. Plant material:

The cotyledons and hypocotyls of *C. canephora* (S-274) were used for the experiment. Similarly leaf and stalk portion of *in vitro* regenerated plantlets were used as explants for repetitive embryogenesis experiments. These *in vitro* sterile plantlets were obtained through inducing embryogenic callus, subsequently somatic embryos and plantlets from hypocotyls explants by using the protocol followed in the study earlier. Surface sterilization of hypocotyls and cotyledonary leaf explants were performed as explained above (see 2.2.1.2.). Explants were cut in 10 mm square from cotyledonary leaf blade with a scalpel excluding mid vein and margins. Hypocotyl and stalk explants were cut into ten mm segments and collected in petridishes containing 0.025% cysteine-HCl. The explants were cultured in 100x20 mm disposable petridishes containing approximately 25 ml of the medium. Leaf explants were cultured with their adaxial side in contact with the medium.

2.2.5.2. Media and culture conditions:

Medium a comprises MS basal medium, 2% sucrose (w/v) supplemented with 2.27–9.08 µM TDZ, medium b comprises MS basal + 3% sucrose (w/v) + 2.27–9.08 µM TDZ, medium c is MS basal + 10% (v/v) coconut water (CW) + 2% (w/v) sucrose + 2.27–9.08 µM TDZ and medium d contains 2% (w/v) sucrose + 10% (v/v) CW. Controls were maintained on medium (e) comprising MS basal and 2% sucrose. All the media were gelled with 0.8% agar (w/v). The pH of all the media was adjusted to 5.7 and was autoclaved at 120ºC temperature and 104 kPa for 20 min. All the cultures were incubated in dark at 25±1ºC for 45 days.

Five cotyledonary leaves (from each leaf 10 replicates), ten hypocotyls (from each hypocotyl 5 replicates), ten leaf explants (from

each leaf four replicates) and ten stalk explants (from each stalk 2 replicates) and were used for the experiment. The experiment was repeated twice. In results only the optimal TDZ concentration treated data was provided (Tables 2.19-2.22).

2.2.6. Zygotic embryo culture:

Unripened green *C. canephora* (S-274) fruits were collected from coffee growing areas in Mysore. They were washed in tap water and surface sterilized with 0.2% (w/v) $HgCl_2$ for 20 min and again washed in sterile water. The fruits were dissected and embryos were removed aseptically with forceps, scalpel and needle and collected in 0.1 percent L-cysteine-HCl in petriplates. Embryos were extracted from mature seeds also, which were sterilized in the same manner.

Three media were used for culture viz., MS basal medium (Table 2.1), MS salts supplemented with Morel and Wetmore vitamins (Morel and Wetmore, 1951) (Bertrand-Desbrunais *et al.*, 1991,1992; embryo culture medium-1, Table 2.4) and Heller's medium (Heller, 1953; Table 2.1) with other supplements (embryo culture medium-2, Table 2.4).

All the media were supplemented with 3% sucrose and pH was adjusted to 5.7±1. Media were solidified with 0.8% (w/v) agar and autoclaved at 121°C, 104 kPa for 20min. Embryos were inoculated directly on to the media. The cultures were incubated at 16 h photoperiod at temperature 25±2°C. The shoots obtained were further cultured in MS solid medium supplemented with BAP (4.4 µM).

2.2.7. Micropropagation:

Nodal explants of *C. canephora* (S-274) were used for the experiment. They were collected from field growing plants grown in coffee growing areas in Mysore, Karnataka. Leaves and stipules were removed and 2 cm long nodal explants were cut from the orthotropic branches of the plant.

Table 2.4: Composition of Embryo Culture Media (ECM-, 1 & 2):

	ECM-1♣	**ECM-2**
Macrominerals	MS*	H▪- Half strength
Microminerals	MS	H- Half strength
Niacin	1	1
Pyridoxine-HCl	1	0.8
Thiamine-HCl	1	1
Ca-pantothenate	1	0.05
Biotin	0.01	-
Glycine	2	3
Myo inositiol	100	200
Cysteine-HCl	-	2
Adenine sulphate	-	1
BA	0.88 µM	-
Sucrose	30,000	20,000
Agar	8,000	8,000
PH	5.7	5.7

♣ Bertrand-Desbunais *et al.* 1991; 1992
*Murashige and Skoog, 1962 (Table 2.1)
▪Heller, 1953 (Table 2.1)

They were washed thoroughly in tap water and dipped in 70% alcohol for 20-30 seconds and again washed in tap water and sterilized. The different methods followed for sterilization and the effects are summarized in table 2.24. The explants were used for the establishment of nodal cultures for micropropagation.

2.2.7.1. Effect of silver nitrate on shoot growth:

Seeds of *C. canephora* (S-274) from fresh fruits were surface-sterilized with 1% NaOCl (sodium hypochlorite, v/v) for 20 min. and washed three times with sterile distilled water. A second sterilization was done with 0.1% (w/v) $HgCl_2$ solution for 5 min and washed three times with sterile distilled water. Embryos were separated under aseptic conditions and inoculated onto the following media. Full

strength MS medium supplemented with 3.8 µM abscisic acid (ABA) and 3% sucrose was used. After 30 days the embryos that matured were further cultured on medium devoid of ABA as suggested by Muniswamy and Sreenath (1999a). Medium was adjusted to pH 5.6 and gelled with 0.8% agar. The culture was incubated in the dark at 25ºC for 2 months. When the shoots that developed had tips 1-1.5 cm long, they were inoculated directly on to 40 ml growth medium comprising MS components supplemented with 8.8 µM BAP and 2.5 µM IAA and 3% sucrose. Some of the explants were used as controls and cultured continuously for 45 days. The other explants were inoculated after 3 days onto the same growth medium fortified with 5-40 µM silver nitrate. Ten explants were used in each treatment. The cultures were incubated at 25ºC with 16:8h photoperiod at a high intensity of 25 µmol m^{-2} s^{-1} for 45 days. The shoot length, number of nodes, number of leaves and leaf area were recorded and total chlorophyll content was determined by the method of Jayaraman (1996).

2.3 RESULTS AND DISCUSSION:

2.3.1. Efficiency of sterilization:

The alcohol treatment was found to be very effective to prevent contamination with a drastic reduction (only 5.5% compared to 83.5% in the CxR variety). Percentage of browning was also very less, which shows that alcohol washing doesn't affect the survivability of the tissue and in general callusing response was found to be good. Initially cysteine-HCl solution (1%) was used while dissecting the hypocotyls explants to avoid browning; however, it was found that the explants not treated with cysteine-HCL also exhibited similar response up on culture as compared to non-treated ones. Only less than ten percentages of explants turned brown in all the cultures.

Explant preparation is an important step for successful application of micropropagation and this stage is considered the remedy for overcoming the problem of microbial contamination. 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). Standardization of surface disinfection of explant in the so-called preparative stage (stage-0) is considered to be the most important step for successful establishment of *in vitro* culture. Selection of suitable disinfectant, its optimum concentration and treatment duration are the prerequisite for effective disinfection of explants in any plant species. The disinfection protocol varies for different plant species and between the explants of the same plant. In general, $HgCl_2$ is considered as a more potent sterilant and it has been widely used to disinfect explants of plantation crops like tea (Rajasekaran and Raman, 1993), cashew (D'Silva and D'Souza, 1993) rubber (Seneviratne and Wijesekara, 1996) etc. In our study, dipping the explants in the alcohol solution for 30 seconds before surface sterilization with 0.1% $HgCl_2$ solution was found to be effectively suppressing the contamination without harming the explants causing browning.

2.3.2. Embryo formation and regeneration by two-step method:

2.3.2.1. Morphological developments of the explants in callus development medium and genotypic effects:

The earliest sign of callus formation had started within two weeks in callus development medium 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. Some explants produced spongy callus, which did not form the yellow embryogenic callus in the embryo induction medium, and this type of tissue proliferated in higher rate than the hard callus (Figure 2.1). Callus formation was observed in

91-93% of the explants of different varieties (Table 2.5). The amount of callus formed varied. S-274 produced the highest amount (152 mg per explant in average) followed by CxR (121 mg) and Old Robusta (68 mg). Old Robusta responded with the highest rate of callusing but least amount of callus formation (Table 2.5).

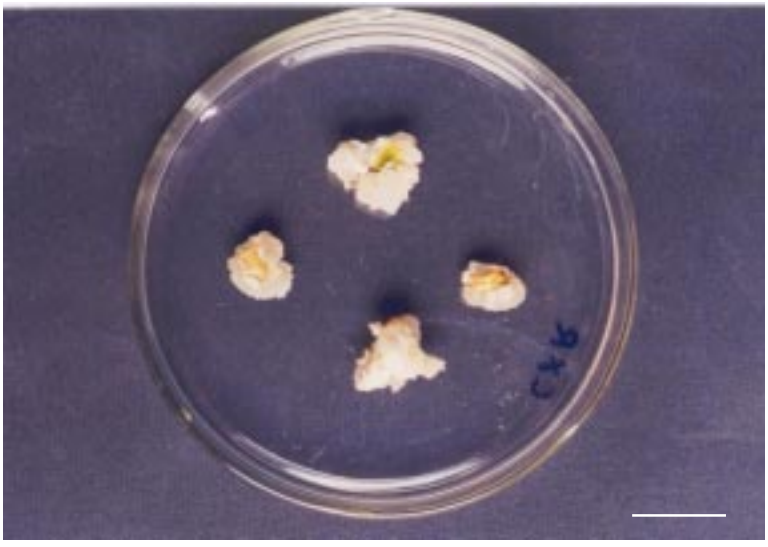
Table 2.5: Morphological developments of the explants in callus development (C) and embryo induction (E) media and genotypic effects:

Variety	Medium	Incubation time (days)	Callusing (%)
CxR	C	55	91.2
CxR	E	55	84.5
CxR	E	75	91.8
Old Robusta	C	55	94.2
Old Robusta	E	55	73.1
Old Robusta	E	75	85.1
274	C	55	93.5
274	E	55	47.3
274	E	75	70.1

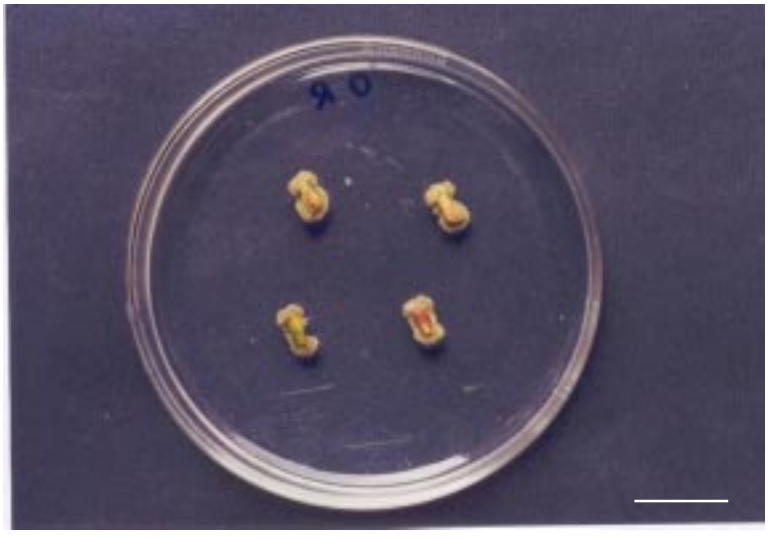
2.3.2.2. Response in embryo induction medium:

After transferring to the embryo induction medium containing half strength MS salts with 4.5 µM 2,4-D and 17.6 µM BA, the calli turned brown and yellow friable callus was formed on the surface of these calli (Fig. 2.2). The time taken for the formation of embryogenic callus was higher in the embryo induction medium (60-75 days) compared to callus development medium (30days). 70-91% of the explants formed callus after two and a half months. The colour of the callus was different in this medium and produced yellow friable callus. CxR variety showed the highest percentage of response (91%) and maintained almost the same percentage in both callus development and embryo induction media as against the S-274, which responded well in the callus development medium (93%; Table 2.5). More than one gram of callus was formed per explant in some explants.

(a)



(b)



(c)

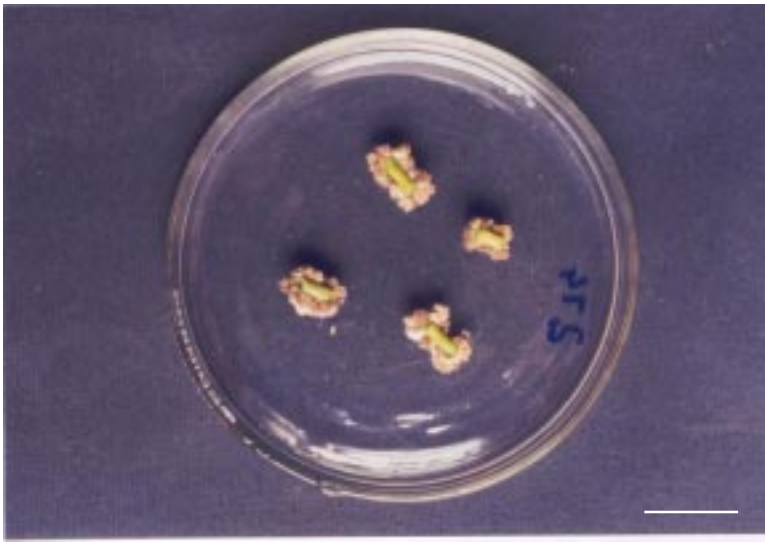


Figure 2.1: Callus formation from the hypocotyls explants in callus development medium after 55 days of culture a. CxR b.OldRobusta c. S-274. Spongy nonembryogenic callus may be seen in a. (bar 12mm)

(a)

(b)

(c)

Figure 2.2: Yellow friable callus forming on the primary callus in embryo induction medium after 60 days of culture (bar 9 mm)
a. CxR b. Old Robusta c. S-274

2.3.2.3. Embryo regeneration:

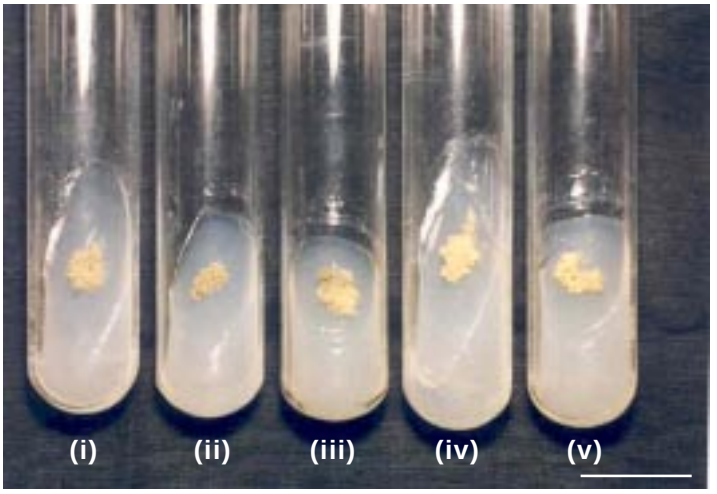
The embryogenic tissue formed in the embryo induction medium was transferred to regeneration media containing half strength MS salts with five different combinations of growth regulators i.e. 4.4, 8.8 and 13.2 µM BAP; 2.2µM BAP and 2.4µM 2-iP and 6.6µM BAP and 7.3µM 2-iP, for embryo formation (Table 2.6). Around 100 mg tissue was inoculated in each tube. Callus proliferation was observed after transferring to different media but in some, the rate was restricted (Fig. 2.3). It should be noted that the embryo formation was associated with the less proliferating callus (Table 2.6).

Table 2.6: Response of embryogenic callus of different varieties of *C. canephora* to various combinations of growth regulators in regeneration medium:

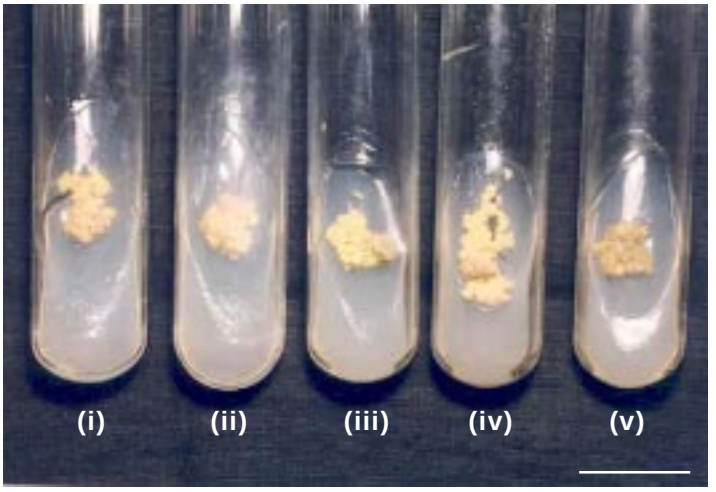
Growth regulator	Variety	Callus response (3 Months)	% of Embryo formation (5 Months)
BAP 4.4 µM	C x R	++	55.5
	Old. R.	+++	0
	274	+++	0
BAP 8.8 µM	C x R	+	30
	Old. R.	+++	11.1
	274	++	0
BAP 13.2 µM	C x R	++	22.2
	Old.R.	+++	0
	274	+	22.2 (only proembryogenic callus)
BAP 1.1 µM, 2-iP 2.9 µM	C x R	+++	18.1
	Old. R.	+++	12.5
	274	+++	0
BAP 6.6 µM, 2-iP 7.3µM	C x R	+++	11.1
	Old.R.	++	88.8
	274	+++	0

+++ Extensive (>1 gm/culture), ++ Moderate (0.5-1 gm), + Slight (<0.5 gm)

(a)



(b)



(c)

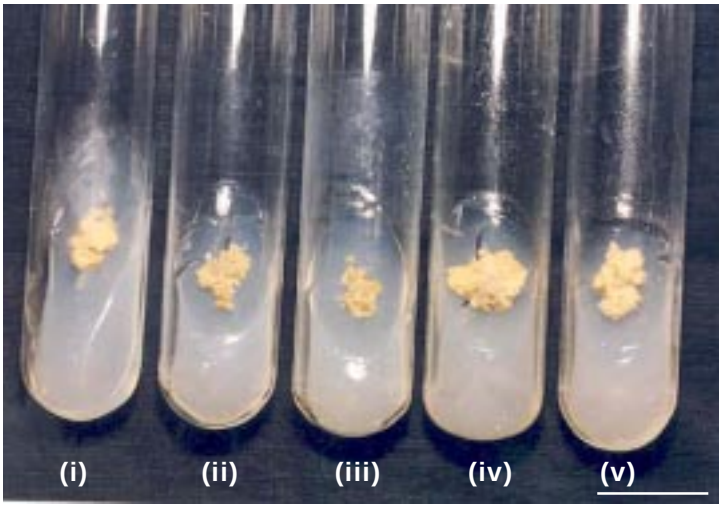


Figure 2.3: Mode of callus proliferation in different growth regulator levels in regeneration media after three months of culture. (i), 4.4 µM BAP; (ii), 8.8 µM BAP; (iii), 13.2 µM BAP; (iv), 2.2 µM BAP and 2.4 µM 2-iP ; (v), 6.6 µM BAP and 7.3 µM 2-iP. Less proliferation leads to faster embryo formation (bar 25 mm)

a. CxR b. Old Robusta c. S-274

C x R formed embryos in all the media tested but the rate was different. Comparatively better response was observed in 4.4 µM BAP (55.5%) and 8.8 µM BAP (30%) (Table 2.6). Even if more percentage of response was found in 4.4 µM BAP, the number of embryos formed was less in this medium initially (2.5±0.5 per 100 mg, after six months) (Table 2.7). Embryos were found to be producing more in 8.8 µM BAP medium (88.5±38.3 embryos in 100 mg). After seven and a half months, CxR produced torpedo embryos in 8.8 µM BAP (Fig. 2.4) and in 4.4 µM BAP, more number of embryos were produced (45±28.0) while Old Robusta produced only globular embryos (Table 2.8). S-274 did not produce any embryos but continued to form proembryogenic callus (Table 2.7).

Table 2.7: Somatic embryo formation in different growth regulators after six months. Cultures kept in the respective media by subculturing once in 45 days:

Variety	**Growth regulator**	**% of Embryo formation (No.*)**	**Pro. Emb. Callus Formation (%)**
CxR	BAP 4.4 µM	11.1(2.5±0.5)	16.6
CxR	BAP 8.8 µM	38.8(88.5±38.3)	27.7
Old. R.	BAP 6.6 µM, 2-iP 7.3 µM	22.2(8.7±3.2)	5.5
274	BAP 13.2 µM	-	38.8

* Embryos formed in 100 mg tissue

Table 2.8: Somatic embryo formation after seven and a half months in different growth regulators. Cultures kept in the respective media by subculturing once in 45 days:

Variety	**Medium**	**Frequency of response (%)**	**Globular embryos***	**Torpedo embryos***
CxR	$^1/_2$MS+ BAP 4.4 µM	33.3	45±28.0	-
CxR	$^1/_2$MS+ BAP 8.8 µM	66.6	81.1±49.7	7.6±5.51
Old. R.	$^1/_2$MS+ BAP 6.6 µM, 2-iP 7.3 µM	22.2	84±37.6	-

- Embryos formed in 100 mg tissue

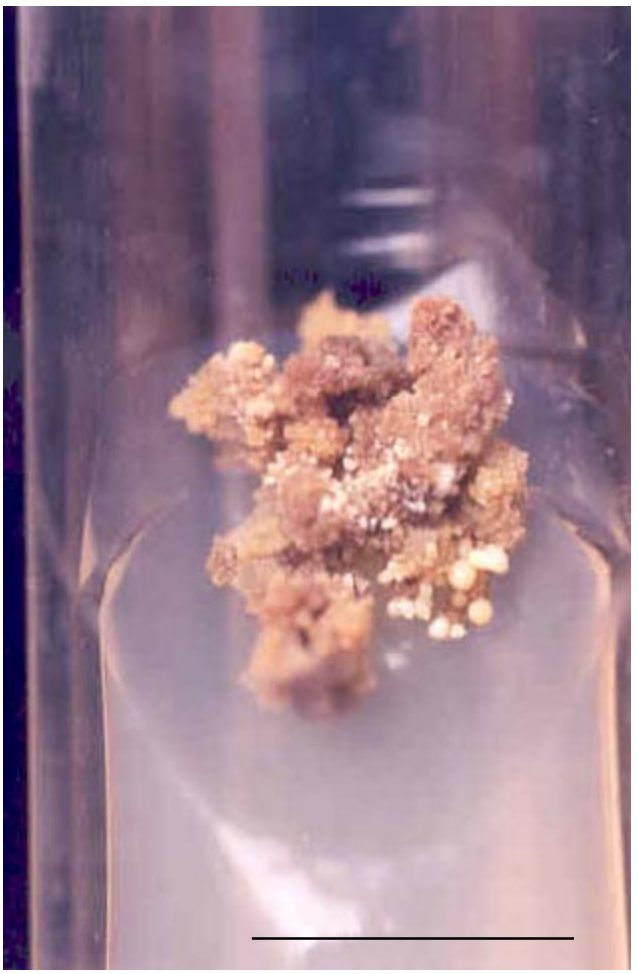


Figure 2.4: Somatic embryo formation in *C. canephora* (CxR) after seven and a half months in 8.8 µM BAP. Callus has been kept in the medium by subculturing once in 45 days (bar 12 mm)

Old Robusta responded with embryo formation in 8.8 µM BAP; 1.1 µM BAP, 2.9 µM 2-iP and 6.6 µM BAP, 7.3 µM 2-iP media with better response in the last (88.8%) (Table 2.6). It could be concluded that the presence of 2-iP is a requirement for inducing embryo formation in this variety. An optimum level of BAP is required for embryo formation and additional presence of 2-iP induces further formation of embryos. In modified callus development medium where 2-iP was used as the sole growth regulator, friable proembryogenic callus was observed in all the explants and the highest rate of embryo formation was observed in this variety

In the case of S-274 variety, even if proliferation rate was less in some media (8.8 and 13.2 µM BAP), no embryo formation could be seen (Table 2.6). Only pro-embryogenic callus was observed in 13.2 µM BAP. However, even the prolonged incubation in the same medium did not give any embryos (Table 2.7).

On increasing the incubation time in respective media, the frequency as well as the number of embryos formed was found to be increasing and embryo development was observed. The Old robusta, which produced only 8.7±3.2 embryos per 100 mg tissue initially, started producing 84±37.6 embryos upon further incubation in the same medium for another 45 days (Table 2.7 & 2.8).

2.3.3. Embryo formation and regeneration by single-step method:

2.3.3.1. Response of hypocotyl explants in the modified callus development medium:

2.3.3.1.1. Adventitious shoot formation:

Around 31- 32% of the explants of all the varieties produced shoots (Table 2.9; Fig. 2.5). Shoots formed directly from the cut end without any callus phase and were emerging either from the epidermal or vascular region (Fig. 2.6). These shoots were later separated and

cultured in $^1/_2$MS + BAP (4.4 µM) for further growth (Fig. 2.7). Hypocotyl explants responded with rooting with 39-50% efficiency.

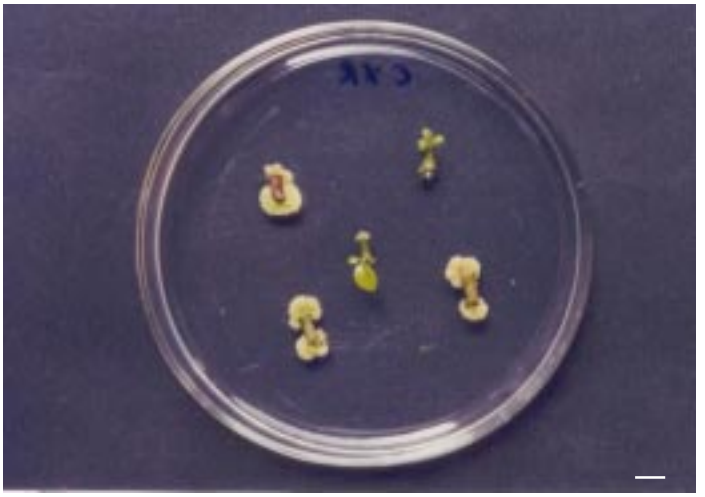
Roots also emerged from the cut ends of hypocotyls. Even if callus was there, roots were found to grow. They appeared as cream coloured projections with root hairs (Fig. 2.5c).

In the present study, we used hypocotyl explants mainly because hypocotyl tissues are very easy to handle and young enough for callus proliferation and embryo formation. From the results, it was shown that they are very effective explants for embryo formation (Table 2.5; Table 2.9). Moreover, hypocotyl explants also responded to the formation of shoots (Table 2.9; Fig. 2.5), which gives additional advantage of using them for organogenesis. Tender hypocotyls can be successfully used for *Agrobacterium* mediated transformation because of these properties. Sugiyama *et al.* (1995) reported transformation of hypocotyl explants using wild *Agrobacterium rhizogenes* strains.

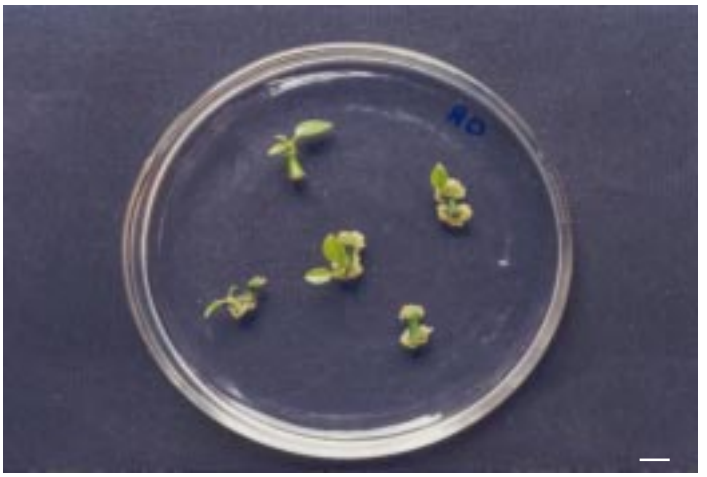
Table 2.9: Response of hypocotyls explants of different varieties of *C. canephora* in the modified callus development medium:

Variety	Incubation time (months)	% Explants producing shoots	Friable callusing (%)	% Explants with embryos
CxR	4	31.2	51.6	-
CxR	5		73.5	16
CxR	6		82	16
Old. R.	4	32.4	100	-
Old. R.	5		100	21.4
Old. R.	6		100	30.8
S-274	4	32.1	28.2	-
S-274	5		33.3	17.1
S-274	6		81.8	28.6

(a)



(b)



(c)

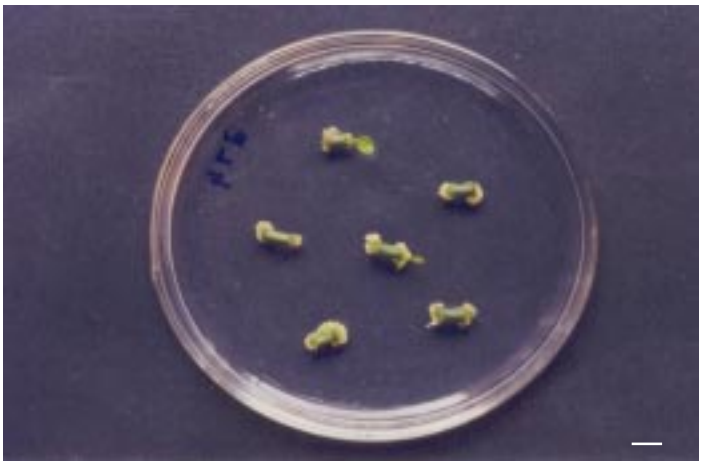


Figure 2.5: Initial callusing and shoot formation in the hypocotyls explants in modified callus development medium after one and a half months. Callus appeared to be slightly green or cream coloured. Root formation also can be seen in c; (bar 10 mm)
a. CxR; b. Old Robusta; c. S-274

Figure 2.6: Shoot emergence and yellow friable embryogenic callus formation from the hypocotyls after four months in modified callus development medium (CxR). Shoots emerged directly from the cut end without any callus phase (bar 12 mm)

(a)

(b)

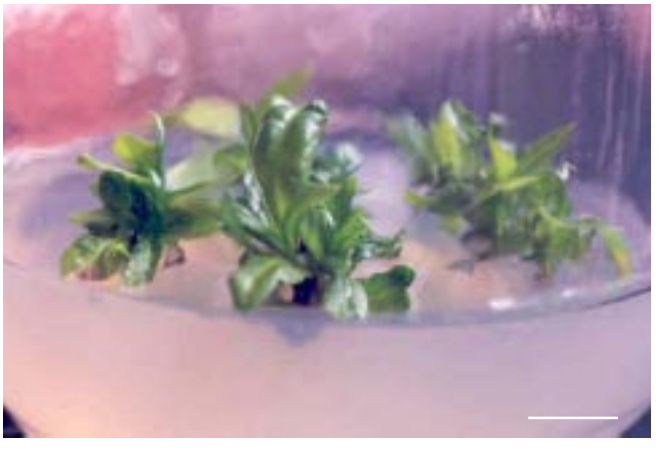


Figure 2.7: Hypocotyl derived shoots of Old Robusta after (a) two months and (b) four months of growth in $^{1}/_{2}$MS + BAP (4.4 µM) (bar 12 - 15 mm)

Reports on plant regeneration from hypocotyl tissues are well documented in several plant species. In general, hypocotyl tissues are reported to have more morphogenic potency than other tissues (Lippman and Lippman, 1984). This has been evidenced in legumes (Lippman and Lippman, 1984). The high morphogenic potency of hypocotyls tissue has been exploited for regeneration of plants either through induction of adventitious shoots or somatic embryos (Lazzeri *et al.*, 1987). This possibility has been extended for regeneration of several woody species (Loh and Rao, 1989). In coffee, Nyanje *et al.* (1993) have induced high frequency adventitious shoots from cultured hypocotyl tissues of *C. arabica* L. In 1999, Samuel Ganesh and Sreenath reported the simultaneous regeneration of plants through high frequency adventitious shoots and somatic embryogenesis from hypocotyl tissues of *C. canephora*.

2.3.3.1.2. Callusing and embryo formation from shoot ends:

When the hypocotyls derived shoots were incubated in $^1/_2$MS+ BAP (4.4 µM) medium, callus formation was observed from the cut ends inserted into the medium. These calli were highly embryogenic which formed embryos in three to four months time (Table 2.10; Fig.2.8)

Table 2.10: Callusing and embryo formation from shoot ends inoculated in $^1/_2$MS+ BAP (4.4 µM):

Variety	**% of shoots with embryogenic callus**	**% of callus with embryos**
Old Robusta	83.3	50
C x R	23.5	75
274	14.2	-

Figure 2.8: Callus and embryo formation in 3-4 months from the shoot ends inoculated in $^{1}/_{2}$MS+ BAP (4.4 µM) medium. (bar 10 mm

a. CxR **b. Old Robusta**

2.3.3.1.3. Initial callus formation from hypocotyls:

After 3-4 weeks a slight proliferation of cells observed along the length and also at the cut ends and small globular structures appeared. The callus was slightly green to cream coloured (Fig 2.5a, b, c). They might be clusters of buds (Fig 2.9). A few globular embryos were to be seen in one-two month's time. The callusing nature is entirely different from those in callus development medium in which additional auxins were provided (Fig. 2.1). The proliferation of cells was more in callus development medium (Fig. 2.1) and the callus in modified callus development medium appear to be harder (Fig. 2.5) than those in the former medium (Fig.2.1).

In some explants when only one cut end touched the medium, the contacted portion produced cream coloured bunch of cells in greater amount and the projecting end found to be green with only less proliferation (Fig 2.5b).

Similar observation was recorded by Hatanaka *et al.* (1995) who observed that when half of the leaf disc was immersed vertically in the medium, embryoids were formed only at the cut edges of the discs that were in contact with the medium. When it was fully inserted in the medium parallelly, callusing was observed in the whole length mainly in the portion touching the medium, which shows that close contact with the medium is required for callus formation (Hatanaka *et al.*, 1995). Hatanaka *et al.* (1991) reported that cytokinins are absorbed from cut edges in contact with medium, and not through the leaf epidermis and that absorbed cytokinins, remains at the cut edges and not transported into the leaf tissues. Attached to one explant a germinated embryo with shoot and root could be seen and in another 2-3 torpedo shaped embryos were found. Even if the callus clusters looked embryogenic they did not develop further except forming a few embryos.

2.3.3.1.4. High frequency somatic embryogenesis (HFSE):

Later yellow friable embryogenic callus started forming by 4th month on these previously formed callus clusters (Fig. 2.6). Upon subculturing to the same medium proliferation of callus was observed. The embryogenic callus might have formed by the proliferation of "proembryo mother cells" (Söndahl *et al.*, 1979) formed initially. In another one month, embryos also were observed with a rate of 16%, 21.42% and 17.14% in CxR, Old Robusta and S-274 respectively (Table 2.9; Fig. 2.10). Yasuda *et al.* (1995) reported that yellow friable callus appeared from few points on the edges of the leaf explants of *C. arabica* after 4 months of culture and somatic embryos formed 4 weeks after initiation of callus.

Old Robusta and S-274 gave almost similar percentage of somatic embryogenesis response (maximum of 30.8% and 28.6% respectively in six months) while CxR showed comparatively less (16%) (Table 2.9). All the varieties viz., CxR, Old Robusta and S-274 responded well to the medium with embryogenic callus formation of 82%, 100% and 81.8% respectively by six months time. CxR and Old Robusta showed high frequency of somatic embryo formation on further culturing in the same medium (Table 2.11; Fig. 2.11; Fig. 2.12; Fig. 2.13). The yellow embryogenic calli formed turned brown and produced white globular embryos on them. Browning of the tissues, caused by an excessive accumulation of phenolic compounds and which starts when the tissue stops proliferation, is necessary for the somatic embryogenesis process in coffee (Quiroz-Figueroa *et al.*, 2001). Similar observations have been reported by other authors (de Garcia and Menendez, 1987; Neuenschwander and Baumann, 1992; Van Boxtel and Berthouly, 1996; Menendez-Yuffa and de Garcia, 1997). Quiroz-Figueroa *et al.* (2001) suggested the possibility of these phenolic compounds acting as signals to induce the differentiation process. An alternative explanation is the possibility that, due to the

chelating properties of these compounds, some inhibitors present in the embryogenic cultures are inactivated (Quiroz-Figueroa *et al.*, 2001). Even though S-274 variety showed moderate rate of response in % of explants with embryo formation (28.6%, Table 2.9), the number of embryos produced was comparatively less (10.2±3.4 per 100 mg, Table 2.11).

Table 2.11: Response of embryogenic callus in modified callus development medium on further incubation (2 months):

Variety	**Globular embryos***	**Torpedo embryos***
CXR	25.8±5.7	10.7±6.1
Old. R.	30.4±8.4	24.8±6.8
274	10.2±3.4	3.8±1.3

* The number of embryos formed in 100mg of tissue

Two general types of somatic embryogenesis may occur in coffee, direct embryogenesis, in which embryos originate directly from tissues in 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. In this study, even if embryoid structures were formed in some hypocotyls in one to one and a half months (Fig. 2.9) they did not develop further, but turned brown upon subculturing. Later, embryogenic callus was observed on these tissues (Fig. 2.6), which would have formed, by the proliferation of these embryoids.

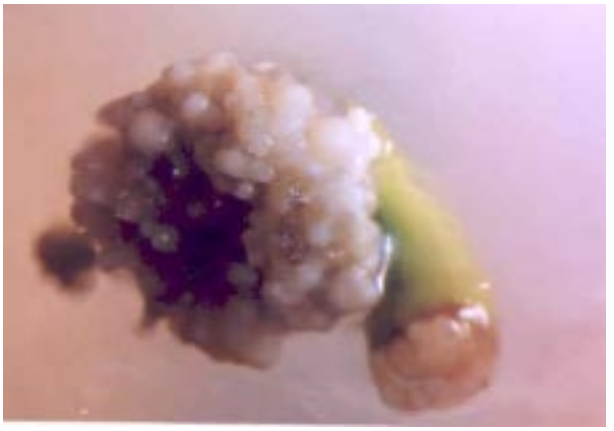


Figure 2.9: Microscopic view of formation of callus mass on hypocotyls cut ends during the initial 1-2 months in modified callus development medium (50 x)

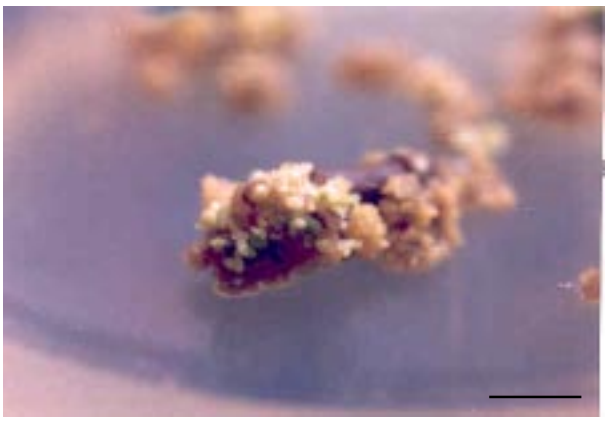


Figure 2.10: Embryos formed from hypocotyls derived callus of S-274 in modified callus development medium after five months (bar 5 mm)

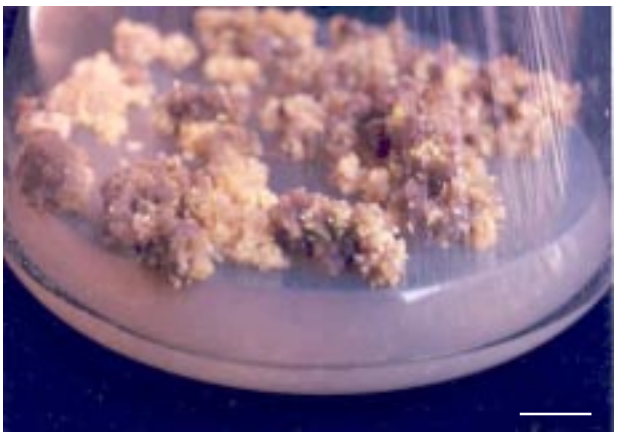
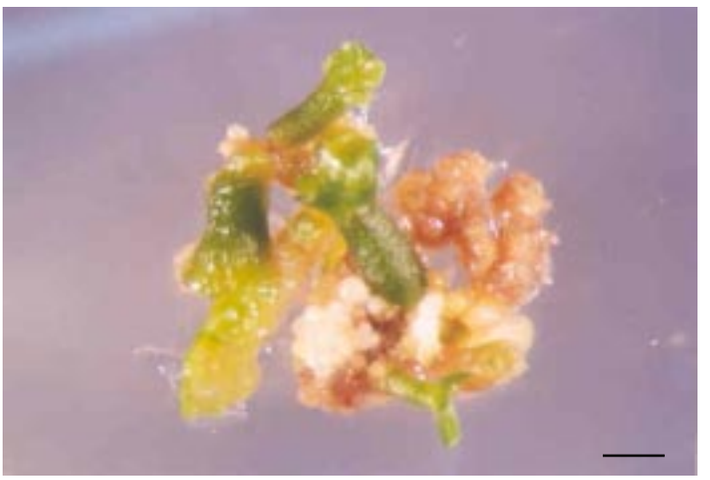


Figure 2.11: High frequency somatic embryo formation in CxR (a) and Old Robusta (b) in modified callus development medium in two months (bar 5 mm)

(a)

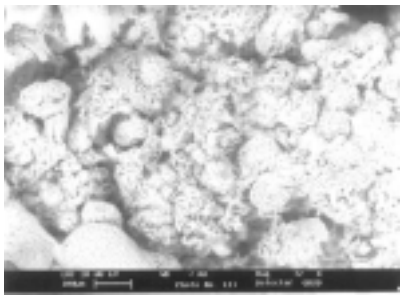


(b)

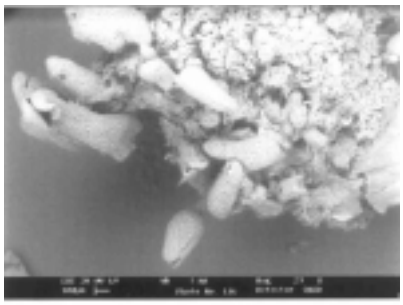
Figure 2.12: a. Microscopic view of the developing embryos formed in modified callus development medium, after two months of culture. Globular and torpedo embryos with opening leaves could be seen (CxR) (bar 2 mm)

b. Torpedo shaped embryos and plantlets formed on the callus in modified callus development medium in dark after four months of culture (CxR) (bar 10 mm)

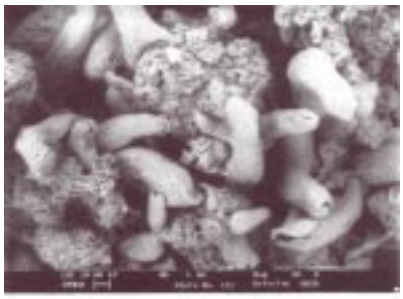
(a)



(b)



(c)



(d)

(e)

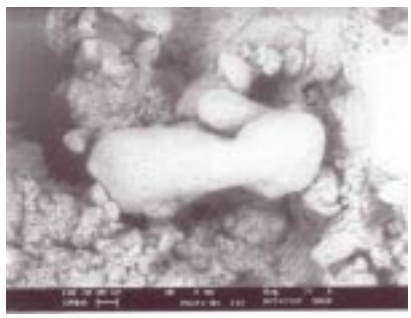


Figure 2.13: Scanning electon micrograph of different stages of developing embryos.
a. globular embryos (72 X) (?); b., c. and d. torpedo shaped embryos (29 X, 30 X and 65 X respectively); e. plantlet (37 X)

A histological study on high frequency embryogenic callus (Söndahl *et al.*, 1979) suggested that embryos induced in high quantities were obtained from proliferation of "proembryo mother cells" and previously formed somatic embryos.

Hatanaka *et al.* (1991) reported that direct somatic embryogenesis, which is induced in *C. canephora* leaf explants when cytokinins were used as the growth regulators was characterized by the absence of callus or embryogenic tissue proliferation and somatic embryos formed from cut edges of cultured leaf explants in contact with cytokinin of the medium. In the present study (Table 2.9) we used 2-isopentenyl adenine (2-iP) as the sole growth regulator in the modified callus development medium. But, direct embryogenesis was not observed except producing very few embryos. Hypocotyls tissue seems to have some inherent levels of auxins which helps for proliferation and which is sufficient for multiplication. In the work done in *C. canephora* Hatanaka *et al* (1995) have reported that auxins inhibited somatic embryogenesis in proportion to its concentration. They also demonstrated that all explants cultured with 5 µM cytokinin formed embryoids and almost all of them developed to plantlets, and that cytokinins play an important role in somatic embryogenesis in coffee (Hatanaka *et al.*, 1991). Söndahl and Sharp (1977) reported that the production of embryogenic tissue in *C. canephora* in indirect somatic embryogenesis is triggered by the combination of auxins and cytokinins in the induction medium. So it is possible that the amount of endogenous auxin in the explant was sufficient to provide an adequate auxin:cytokinin ratio for inducing a low proliferation of embryogenic tissue.

2.3.3.2. Germination of embryo:

When the somatic embryos reached torpedo stage (Fig. 2.12a, b; Fig. 2.13b, c, d), they were transferred to both solid (Fig. 2.14a) and liquid (Fig. 14b) embryo germination (EG) medium (Table 2.12). The germination rate was 67-81% among different varieties

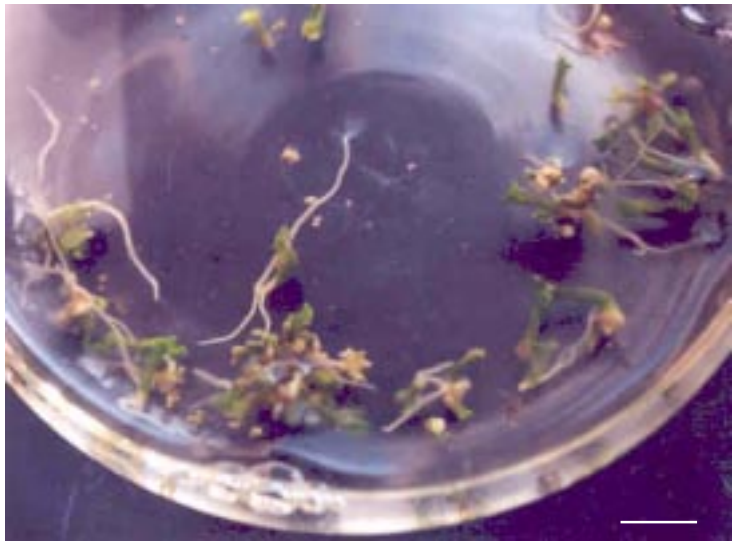
Solid medium (67.36%) was found to be more effective than liquid medium (35.08%) for development of somatic embryos with well-developed shoot and root polarity. In the solid medium, the embryos were bigger and produced larger leaves than in liquid medium. However root growth was faster in liquid medium. Plantlets were produced in liquid medium after 3-4 weeks while in solid medium plantlets were fully developed after 6 weeks.

Table 2.12: Development of embryos (%) in one month in solid and liquid embryo germination media*:

Variety	**Solid**	**Liquid**
CXR	67.4	35.1
Old. R.	81.6	-
274	72.7	-

*Medium- $^{1}/_{2}$MS+ BAP 1.1 µM, IAA 2.5 µM

(a)



(b)

Figure 2.14: a. Germinating embryos in liquid embryo germination medium after one and a half months (CxR) (bar 9mm)

b. Germinating embryos in solid medium embryo germination medium after one and a half months (CxR) (bar 5 mm)

2.3.3.3. Secondary embryogenesis:

Somatic embryos gave rise to secondary embryos and produced callus upon prolonged incubation in the embryo germination medium, which subsequently regenerated to produce embryos at high frequency (Table 2.13; Fig. 2.15, 2.16). It was formed either directly (Fig. 2.15) or through callus (Fig. 2.16). The embryo germination medium has been observed to help in the production of secondary embryos from primary ones and hence can be used for embryo multiplication. Secondary embryos are produced either from basal portions (Fig. 2.17) or from surface of somatic embryos during long-term cultures. These embryos germinate and develop as normal plantlets similarly to primary somatic embryos (Sondahl and Loh, 1988). The special feature of embryos is highly helpful for *Agrobactrium* mediated transformation. Fernandez-Da Silva and Menendez-Yuffa (2003) reported transient *gus* and *bar* gene expression in secondary somatic embryos.

The secondary embryos were formed directly in the hypocotyls surface of electroporated torpedo-shaped primary somatic embryos in liquid medium containing 8 mg l^{-1} BA. The production of secondary somatic embryos was significantly greater than the production of primary embryos, therefore, this is an excellent method to propagate the products of genetic transformation (Fernandez-Da Silva and Menendez-Yuffa, 2003). The variety CxR responded very well with 90±43.0 embryos in 100mg tissue as against 78±35.7 and 17.7±10.8 embryos for Old Robusta and S-274 respectively (Table 2.13).

Table 2.13: Secondary embryo formation in embryo germination medium from cultured primary somatic embryos (45 days)*:

Variety	Globular embryos	Torpedo embryos
CXR	90±43.0	6.3±2.5
Old. R.	78±35.7	-
274	17.7±10.8	-

*The number of embryos formed in 100mg of tissue

Figure 2.15: Microscopic view of secondary embryo formation on the plantlets after two months in embryo germination medium (CxR). Globular embryos can be seen (50 x)

Figure 2.16: Embryo formation in somatic embryo derived callus in embryo germination medium after two months of culture (CxR) (bar 12 mm)

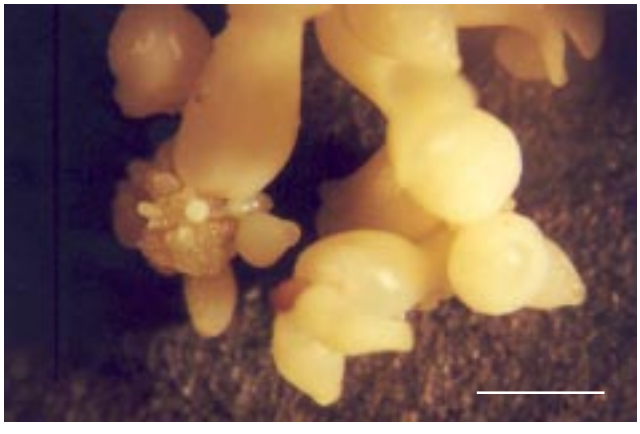


Figure 2.17: Secondary somatic embryo formation from the basal portion of primary embryo in embryo germination medium after two months (CxR) (bar 2 mm)

In another observation, 237±10.2 numbers of embryos were formed when the wounded embryos of CxR variety were incubated in $^1/_2$MS + 2.2 µM BAP and 5 µM IAA. The medium can be successfully used for the regeneration of *Agrobacterium* mediated transformed cells.

2.3.3.4. Rooting and hardening:

When the plantlets were with 3-4 pairs of leaves (Fig.2.18), they were transferred to $^1/_2$MS medium for further development. 77.77% of the CxR variety and 50% of Old Robusta plantlets were grown into fully matured hardening-ready coffee plants after two and a half months of incubation in $^1/_2$MS medium (Fig. 2.19).

Hypocotyl derived shoots were also rooted in IBA (246 µM) and NAA (268 µM) mixture, which was found to be very effective for root formation (Table 2.14; Fig. 2.20). Even if the solution seems to be highly concentrated, just dipping in the solution was found to be effective for healthy and stout root formation, which enhanced the shoot growth also. The plants were hardened in the green house (Fig. 2.21).

Table 2.14: Rooting of hypocotyl derived shoots*:

Variety	Root formation (%)	Number of roots/explant	Length of roots (cms)
C x R	27.77	1.8±0.4	2.11±0.96
Old Robusta	25	1.6±0.5	2±0.63
274	44.44	3±1.2	1.6±0.58

* Data recorded after 45 days

Figure 2.18: Coffee (CxR) plantlets derived from somatic embryos. The plantlets were cultured in embryo germination medium for two months (bar 11 mm)

(a)

(b)

Figure 2.19: Somatic embryo derived plants, which were grown in $^{1}/_{2}$MS medium for two months. They are ready for hardening; a. CxR; b. Old Robusta (bar 11 mm)

(a)

(b)

(c)

Figure 2.20: Hypocotyl derived shoots with root formation after two months in $^{1}/_{2}$MS medium. Root induction was done by dipping the shoots in IBA (246µM) and NAA (268µM) solution mixture prepared in water and later inoculated in $^{1}/_{2}$MS solid medium (bar 19 mm)
a. CxR b. Old Robusta c. S-274

(a)

(b)

(c)

Figure 2.21: Hardened coffee plants
a. CxR (2 months) (bar 30 mm) ;
b. S-274 (4 months) (bar 30 mm);
c. S-274 (6 months) (bar 50 mm)

C. canephora is strictly allogamous and so each seedling represents a different genotype. The hypocotyl derived callus can be used for the susceptibility studies of fungal pathogens. Hypocotyl derived calli of genotypes and segregating populations of *C. arabica* differing in susceptibility to *Colletotrichum kahawae*, were used to produce resistant cells suspensions and protoplasts, which were exposed to partially purified culture filtrates (PPCFs) prepared from the pathogen (Nyange *et al.*, 1995). The initial screening methods help for the further breeding by reducing the time required for progeny evaluation. Nyanje *et al.* (1997) reported for the first time the applicability of embryogenic cell suspension cultures established from hypocotyl-derived callus for obtaining totipotent protoplast and for the regeneration of resistant plantlets. The callus derived from hypocotyls may offer a suitable source of material for *in vitro* studies of coffee berry disease resistance (Nyange *et al.,* 1993, 1995). Our study presents an effective method of production of embryogenic callus as well as regeneration of plantlets from individual germinated seedlings (Table 2.5; Table 2.6; Table 2.9; Fig.2.1; Fig.2.2; Fig. 2.4; Fig.2.6; Fig.2.10). The shoots emerging from the hypocotyls could be additional explant source for *in vitro* studies, which are true to type to the mother plant because they formed without any intermediate callus phase (Table 2.9; Fig. 2.5; Fig.2.6).

The induction of somatic embryos is very sensitive to culture conditions such as the composition of the medium, the physical environment of the culture, and the genotype and the explant source (Fuentes *et al.*, 2000). In this study we tried cytokinin (modified callus development medium) as well as the combination of auxins and cytokinin (callus development medium and embryo induction medium) with the intention of getting faster and more number of embryos. Generally, auxins are necessary for the onset of the growth of callus and for the induction of somatic embryos (Ammirato, 1983). But in *Coffea* cytokinin is very important and auxins have inhibitory effects on somatic embryogenesis (Yasuda *et al.*, 1985; Hatanaka *et al.*, 1991). 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). The results showed that only cytokinin (single-step method, in modified callus development medium) is sufficient for the induction of high frequency embryogenic callus from hypocotyls explants, which can be multiplied later by addition of auxins. And this method was comparatively faster (5 months) than the two-step method of induction of embryogenic callus and embryos. The callus obtained through the second method takes more time for regeneration (9-10 months; Table: 2.5, 2.6, 2.7). It could be because of the presence of 2,4-D in the embryo induction medium, which leads to proliferation of cells in the regeneration medium, which further delays the embryo formation.

So it would be preferable to avoid the incorporation of auxins in the embryo induction medium for faster embryo formation and once the embryogenic callus starts forming, they can be multiplied by adding auxins. We also found that the presence of higher amount of tissue leads only to further multiplication of tissues, but less amount of tissue always led to embryo formation (Table 2.6).

The protocols followed for the somatic embryogenesis from hypocotyl explants in the present study has been summarized in the following page.

In the method I, embryogenic callus was produced by incubating the hypocotyls explants in two different media. For the first 55 days the explants were kept in callus development medium for the initial proliferation of callus. Then they were transferred to embryo induction medium for the formation of embryogenic callus. The explants were kept in the medium for another 70-75 days. In the second method, only modified callus development medium was used for embryogenic callus induction. In both methods, almost same time has been taken for the embryogenic callus induction (4 months). But in the method II, when the embryogenic callus was transferred to regeneration media, it took minimum five months for the somatic embryos to be formed. However, as in the method II, when the embryogenic callus was incubated in the callus development medium further, embryos could be induced in one month. Prolonged incubation in the same medium produced more number of

embryos. Secondary somatic embryos also could be induced from the primary ones. Thus callus development medium (single-step procedure) gives faster embryogenesis response. A comparison of the best response of each variety in callus development medium has been summarized in table 2.15.

Method I Hypocotyl explants

↓

Callus development medium (55 days) –
primary callus has been formed

↓

Somatic embryo induction medium (75 days) – yellow embryogenic callus formed on the primary callus

↓

Somatic embryo regeneration media (seven and a half months) – somatic embryos regenerated from the embryogenic callus

Method II Hypocotyl explants

↓

Modified callus development medium (4 months) – direct shoot and embryogenic callus formation

↓

Somatic embryo formation in the same medium (5 months)

↓

Prolonged incubation (two months) – more embryos were produced

↓

Somatic embryo germination (two months) & secondary embryo formation (two months) in embryo germination medium

↓

Development of plantlets in ½ MS medium (two months)

↓

Hardening of the coffee plants

Table 2.15: Protocol for the best somatic embryogenesis response in hypocotyls explants in different varieties of *C. canephora* as obtained from the study:

CxR	**Old Robusta**	**S-274**
Hypocotyl ↓ Modified callus development medium(4 months) - embryogenic callus formation - **52%** ↓ Embryo formation (one month) - **16%** ↓ Prolonged incubation (two months) - **35** embryos per 100mg callus ↓ Embryo development in embryo germination medium - **67%** ↓ Development of plantlets in ½MS - **77%** ↓ Hardening	Hypocotyl ↓ Modified callus development medium(4 months) - embryogenic callus formation - **100%** ↓ Embryo formation (one month - **21%** ↓ Prolonged incubation (two months) - **54** embryos per 100mg callus ↓ Embryo development in embryo germination medium - **81%** ↓ Development of plantlets in ½MS- **50%** ↓ Hardening	Hypocotyl ↓ Modified callus development medium(4 months) - embryogenic callus formation - **28%** ↓ Embryo formation (one month) - **17%** ↓ Prolonged incubation (two months) - **13** embryos per 100mg callus ↓ Embryo development in embryo germination medium - **72%** ↓ Development of plantlets in ½MS- **50%** ↓ Hardening

2.3.4. Influence of triacontanol on somatic embryogenesis:

Embryo germination medium containing BA (1.1 μM) and IAA (2.5 μM) was primarily used for somatic embryogenesis. On this medium low level of small, round globular embryos along with some cotyledonary stage somatic embryos were obtained directly from *in vitro* stem segments of regenerated plants and leaf explants after a period of 45 days culture (Tables 2.16-2.19). BA, IAA or TRIA alone did not induced somatic embryogenesis. Triacontanol at both 4.55 and 11.38 μM (Medium B-E) induced direct somatic embryogenesis from both leaf and *in vitro* stem explants (Table 2.16; Table 2.17; Fig. 2.22). TRIA at a concentration of 11.38 μM with 1.1 μM BAP supported a maximum of 58.6 ± 10.3 embryos per leaf explant on medium E (Table 2.16). A maximum number of 68 ± 14.2 embryos was produced from *in vitro* stem segments on medium E (Table 2.17) containing TRIA (11.38 μM) + BA (1.1 μM). When small, green globular primary embryos were inoculated, maximum secondary embryogenesis was noticed from explants on medium E only (Table 2.18), whereas, the lowest levels of secondary embryogenesis occurred in the absence of TRIA on medium A (Table 2.18). TRIA also increased somatic embryo formation from embryogenic callus (Table 2.19). Maximum embryo formation (35± 6.2) was observed again on medium E. The direct development of embryos formed into plants was scanty or absent in medium with only TRIA and BA. The most significant feature of this study was that it was possible to get high levels of direct somatic embryogenesis in 2 months time. Most of the reports (Sondahl and Sharp, 1977; Dublin, 1981; Van Boxtel and Berthouly, 1996; Pierson *et al.*, 1983; Hatanaka *et al.*, 1995; Fuentes *et al.*, 2000) about the somatic embryogenesis in both *C. arabica* and *C. canephora* have been via a callus phase. The results obtained in this study are unique amongst *Coffea* species as direct somatic embryogenesis induction was achieved with TRIA

According to Yasuda *et al.* (1985), 4-6 months time is required for somatic embryogenesis in *C. canephora.* Similarly, 2 to 6 months time is required for somatic embryogenesis on improved medium by Hatanaka *et al.* (1991). Direct and indirect plant regeneration in *C. canephora* through somatic embryo formation reported by Ganesh and Sreenath (1999) required 7-8 months time to get 11-13 embryos per explant. Even the high frequency somatic embryogenesis through callusing reported by Van Boxtel and Berthouly (1996) required 6-8 months. As genetic transformation of coffee with foreign genes has been a subject of intensive research (Hatanaka *et al.*, 1999), an efficient regeneration system through somatic embryogenesis illustrated in this study would further facilitate the application of *Agrobacterium* mediated transformation.

Table 2.16: Influence of triacontanol on direct somatic embryogenesis fron *in vitro* leaf explants of *C. canephora* (S-274) in dark after 45 days:

Treatment	**Hormone (μM) (BA + IAA + TRIA)**	**% of explants producing embryos**	**Number of embryos per explant**
A	1.1 + 2.85 + 0	80	12.97± 2.5
B	1.1 + 0 + 4.55	90	45.68± 4.9
C	1.1 + 2.85 + 4.55	76	20.0 ±5.6
D	1.1 + 2.85 + 11.38	100	46.47 ±5.5
E	1.1 + 0 + 11.38	100	58.59 ±10.3

Table 2.17: Influence of triacontanol on direct somatic embryogenesis from *in vitro* hypocotyl explants of *C. canephora* (S-274) in dark after 45 days:

Treatment	**Hormone (μM) (BA + IAA + TRIA)**	**% of explants producing embryos**	**Number of embryos per explant**
A	1.1 + 2.85 + 0	20	2.0± 0.47
B	1.1 + 0 + 4.55	74	41.0± 10.5
C	1.1 + 2.85 + 4.55	64	25.0 ± 2.5
D	1.1 + 2.85 + 11.38	76	53.3 ±13.2
E	1.1 + 0 + 11.38	72	68.0± 14.2

Table 2.18: Influence of triacontanol on secondary embryogenesis from primary embryos of *C. canephora* (S-274):

Treatment	**Hormone (μM) (BA + IAA + TRIA)**	**% of primary embryos* per inoculum producing secondary embryos**	**Number of secondary embryos per inoculum of primary embryos**
A	1.1 + 2.85 + 0	64	9.96± 5.43
B	1.1 + 0 + 4.55	80	17.55±4.74
C	1.1 + 2.85 + 4.55	75	13.55± 1.74
D	1.1 + 2.85 + 11.38	88	14.59 ±2.89
E	1.1 + 0 + 11.38	96	18.0± 1.20

* small globular light yellow-green embryos

Table 2.19: Influence of triacontanol on embryogenesis from embryogenic callus of *C. canephora* (S-274):

Treatment	Hormone (μM) (BA + IAA + TRIA)	% of primary embryos* per inoculum producing secondary embryos	Number of secondary embryos per inoculum of embryogenic
A	1.1 + 2.85 + 0	32	10.0± 1.2
B	1.1 + 0 + 4.55	28	28.0± 2.0
C	1.1 + 2.85 + 4.55	28	25.0 ±1.8
D	1.1 + 2.85 + 11.38	48	32.0± 4.8
E	1.1 + 0 + 11.38	60	35.0± 6.25

*The callus is yellow friable in nature

2.3.5. Thidiazuron (TDZ) induced somatic embryogenesis:

Initially 80–90 % of the leaf and hypocotyl explants remained green when cultured in dark on MS medium with or without TDZ. No deterioration was observed within 20 days. Initially the embryos appeared as white small globular masses, which germinated and passed through successive developmental stages. The effects of TDZ on the frequency of embryos and callus formation per explant is described in tables 2.20-2.23.

In *C. canephora* (S-274) variety 60% of *in vitro* leaf explants (Table 2.20, Fig. 2.23) and 72% *in vitro* stalk of regenerated plants (Table 2.21, Fig. 2.24) produced direct somatic embryogenesis from its margins and surface respectively (180.1±3.0 and 102.8±6.8 embryos) on medium a. The medium a containing 9.08 μM TDZ supported direct somatic embyogenesis from leaf explants compared to medium c where 10% coconut water was incorporated (Table 2.20). Though coconut water was tried to see its influence on somatic embryogenesis, it was found to be insignificant in this study as there was no response for embryogenesis.

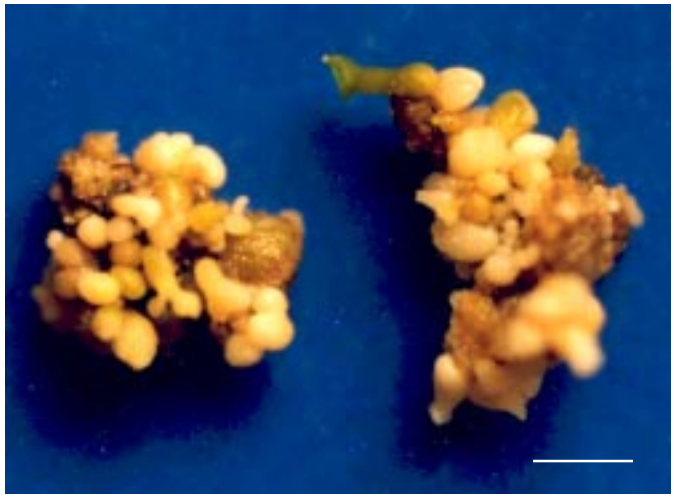


Figure 2.22: Direct somatic embryogenesis from *in vitro* stem segments of regenerated plants of *C. canephora* on medium containing 11.38 µM TRIA (triacontanol) and 1.1 µM BA (bar 10mm)

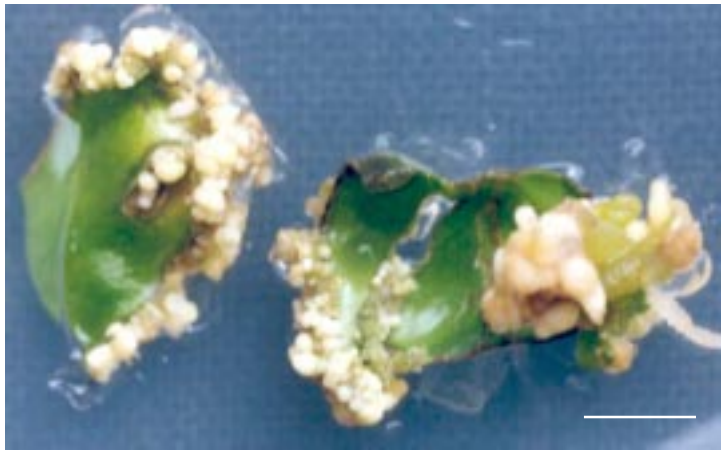


Figure 2.23: Direct somatic embryogenesis from leaf explants of *C. canephora* 274 variety on MS with 2% sucrose and 9.08 µM thidiazuron (bar 10 mm)

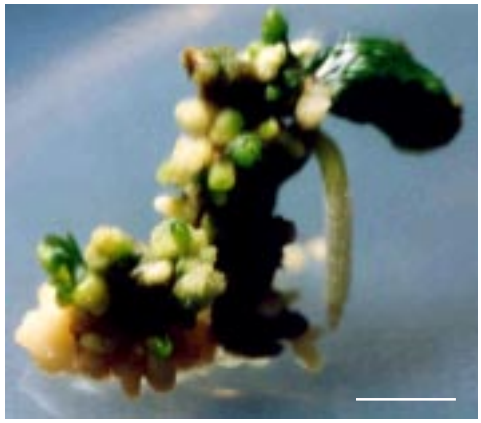


Figure 2.24: Direct somatic embryogenesis from *in vitro* stalk explants of *C. canephora* 274 variety on MS with 2% sucrose and 9.08 µM thidiazuron (bar 10 mm)

Only 22% *in vivo* cotyledonary leaf explants produced embryos (16.5 ± 1.51) on medium a (Table 2.22). Callusing was not noticed in *in vitro* leaf and stalk explants in contrast to cotyledonary leaf explants wherein, 50% explants with marginal callusing was noticed (Table 2.22). The higher sucrose concentration in medium b (with 3% sucrose) reduced somatic embryogenesis in *in vitro* leaf and stalk explants, wherein, only 20 and 60% explants produced 36.0 ± 0.66 and 40.5 ± 1.65 embryos per explant respectively (Table 2.20, 2.21). Almost 100% *in vitro* leaf explants produced light yellow friable callus on medium c (Table 2.20) containing 2% sucrose. *In vitro* stalk explant and cotyledonary leaf explants produced light yellow friable callus (Table 2.21, 2.22) which were embryogenic. The response of hypocotyls explants was almost similar to cotyledonary explants and not much significant difference was found in the percentage of explants responded for somatic embryogenesis (Table 2.23). The number of embryos produced (36.5±1.4) was more in hypocotyls explants

Table 2.20: Effect of TDZ on somatic embryogenesis from *in vitro* leaf explants of *C. canephora* (S-274):

Medium#	**Supplements**			**Explants respo-nded (%)**	**No. of embryos per explant**	**% of explants producing callus**
	Sucrose %(w/v)	TDZ (µM)	CW (v/v)			
a	2	9.08	0	60	180.1 ± 3.0	-
b	3	9.08	0	20	36.0 ± 0.6	-
c	2	9.08	10	-	-	100*
d	2	0	10	-	-	10**
e	2	-	-	-	-	-

* light yellow friable callus, ** white non friable callus, incubation= 45 days; # = MS medium, E=control, CW=coconut water

Table 2.21: Effect of TDZ on somatic embryogenesis from *in vitro* stalk explants of regenerated plants of *C. canephora* (S-274):

Medium#	**Supplements**			**Explants respo-nded (%)**	**No. of embryos per explant**	**% of explants produc-ing callus**
	Sucrose %(w/v)	TDZ (µM)	CW (v/v)			
a	2	9.08	0	72	102.8 ± 6.8	-
b	3	9.08	0	60	40.5 ± 1.6	-
c	2	9.08	10	-	-	100*
d	2	0	10	-	-	-
e	2	-	-	-	-	-

- light yellow friable callus, incubation= 45 days; # = MS medium, E=control, CW=coconut water,

Table 2.22: Effect of TDZ on somatic embryogenesis from *in vivo* cotyledonary leaf explants of *C. canephora* (S-274):

Medium#	**Supplements**			**Explants respo-nded (%)**	**No. of embryos per explant**	**% of explants producing callus**
	Sucrose %(w/v)	TDZ (µM)	CW (v/v)			
a	2	9.08	0	22	16.5 ± 1.5	50 *
b	3	9.08	0	20	20.2 ± 0.6	20 *
c	2	9.08	10	-	-	30 **
d	2	0	10	-	-	25 *
e	2	-	-	-	-	-

* light yellow friable callus, ** white non friable callus,

incubation= 45 days; # = MS medium, E=control, CW=coconut water

Table 2.23: Effect of TDZ somatic embryogenesis from *in vivo* hypocotyl explants of *C. canephora* (S-274):

Medium#	**Supplements**			**Explants respo-nded (%)**	**No. of embryos per explant**	**% of explants producing callus**
	Sucrose %(w/v)	TDZ (µM)	CW (v/v)			
a	2	9.08	0	20	36.5 ± 1.4	40 *
b	3	9.08	0	18	26.0 ± 0.4	18 *
c	2	9.08	10	-	-	28 **
d	2	0	10	-	-	26 *
e	2	-	-	-	-	-

* light yellow friable callus, ** white non friable callus,

incubation= 45 days; # = MS medium, E=control, CW=coconut water

This study provides a model for investigating the mechanisms underlying the process of somatic embryogenesis in coffee. The most interesting aspect of the study is the rapid induction of direct somatic embryogenesis in *C. canephora* on medium supplemented with TDZ. TDZ was first used for the mechanized harvesting of cotton balls and more recently incorporated into tissue culture media as a means of inducing regeneration. 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). The utilization of TDZ in tissue culture of dicotyledonous plants for the direct differentiation of somatic embryos is gaining importance (Gill and Saxena, 1992; Iantcheva *et al.*, 1999). In general TDZ at very low levels induces direct somatic embryogenesis in geranium (Quereshi and Saxena, 1992) alfalfa (Iantcheva *et al.*, 1999), water melon (Compton and Gray, 1992) and muskmelon (Gray *et al.*, 1992). The association between TDZ-induced responses and endogenous plant growth regulators of dicotyledonous plants has been documented by means of the quantification of their profiles. TDZ

promotes the synthesis & accumulation of purines (Capelle *et al.*, 1983) and also alters cytokinin metabolism (Mok *et al.*, 1982). Since TDZ is involved in cytokinin metabolism, we decided to explore its effect on direct somatic embryogenesis in *Coffea*, and the results of present study proved this.

In all the treatments the initial appearance of small white globular embryos appears to be affected by the stage of maturity of the leaves especially this was true with *in vivo* explants (data not shown). When 30 days old cotyledonary leaf explants of CxR and 274 varieties used, the response was moderate to good compared to 2-3 months old first and second leaves of seedlings (data not shown). Clumps of the leaf derived primary embyogenic nodule mass proliferated to produce more embryos when subcultured on to the medium containing half strength MS salts and B5 vitamins supplemented with 0.91 µM TDZ (data not shown). Trials were conducted to determine the optimal TDZ dosage for embryo formation by inoculating nodular clumps (5x5 mm) onto half strength MS basal medium containing different levels (0.045- 4.54 µM) of TDZ. The use of TDZ at 0.91 µM most favored embryo proliferation.

The significance of this study is rapid repetitive somatic embryogenesis from regenerated plantlets by using TDZ. Direct somatic embryogenesis has been obtained from explants of *in vitro* regenerated plantlets derived through indirect embryogenesis route. Use of other hormonal regimes results in callusing from cut portions (margins) and eventually used for indirect somatic embryogenesis to form plantlets (Nishibata *et al.*, 1995). In high frequency somatic embryogenesis callus intermediates were involved and takes long duration for the embryo induction (Hatanaka *et al.*, 1991, 1995; Etienne-Barry *et al.*, 1999; Fuentes *et al.*, 2000). Callus derived somatic embryos known to possess somaclonal variations in regenerated lines. In the present study healthy regenerates were obtained from leaf, hypocotyls and stalk explants on MS salts

containing medium supplemented with TDZ, through morphogenetic routes including direct somatic embryogenesis.

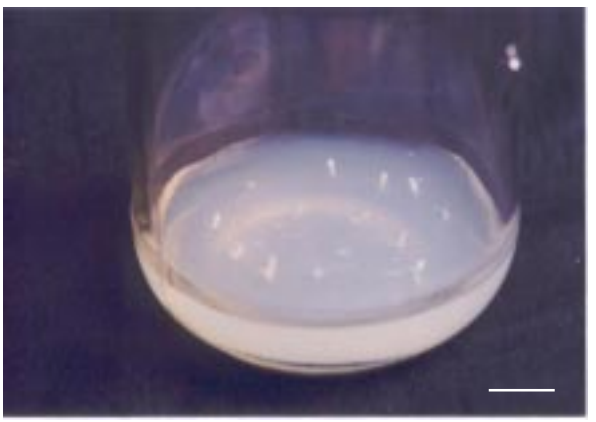
2.3.6. Zygotic embryo culture:

The zygotic embryos were inoculated (Fig. 2.25a) in MS basal medium (Table 2.1), MS medium supplemented with Morel and Wetmore vitamins (Morel and Wetmore, 1951) (embryo culture medium-1; Table 2.4; Bertrand-Desbrunais *et al.*, 1991; 1992) and Heller's medium (Heller, 1953; Table 2.1) with other supplements (embryo culture medium-2; Table 2.4). Heller's medium has been used by Muniswamy and Sreenath (1995b) for zygotic embryo culture. In all the media, response was good with 80-90% survival. 2-2.5 cm long plantlets could be obtained in 60-75 days with proper rooting and shoot growth. Later, roots were removed and the shoots were propagated in MS medium containing BAP (4.4 μM) as the growth regulator (Figure 2.25a, b). Callus proliferation was observed from the shoot ends in MS medium containing 2-iP (4.9 μM), which produced embryos on further incubation in the same medium (Fig. 2.26). The embryos also developed into fully-grown plantlets. Contamination is the major problem while using explants from the natural environmental conditions and zygotic embryo derived plantlets are excellent source of aseptic plant material for the embryogenesis in coffee.

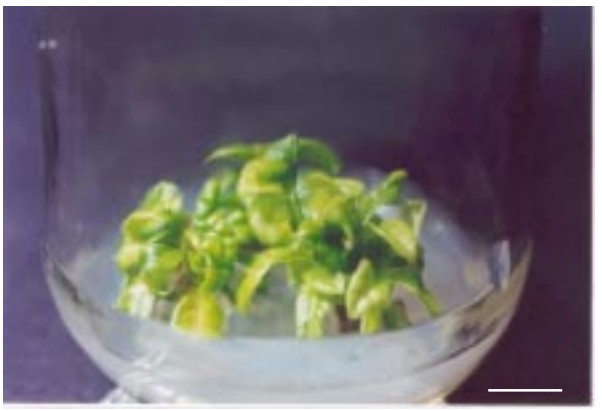
2.3.7. Micropropagation:

Shoot cultures can be established from shoot or meristem tips of axenic seedling, and will produce axillary shoots which can be subcultured in a conventional manner on MS (1962) medium or modified MS medium. Cultures can also be initiated by incubating nodal sections of orthotropic shoots from seedlings or mature plants. But explants from greenhouse and field-grown plants can be heavily contaminated and may also turn brown when first isolated, so that a high proportion may be lost. We also faced this problem many times.

(a)



(b)



(c)

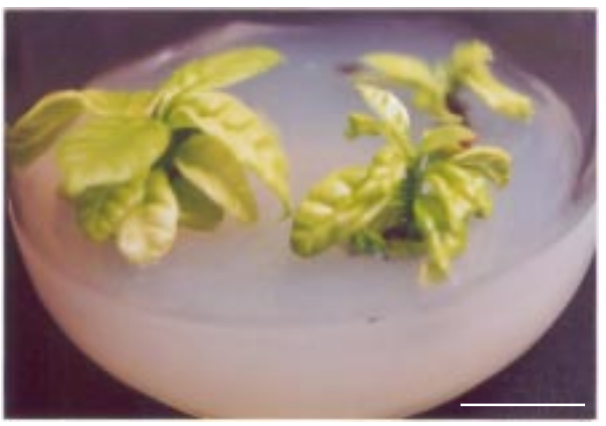


Figure 2.25: a. Zygotic embryos cultured in $^{1}/_{2}$MS medium b. Shoots formed from embryos after four months of culture in $^{1}/_{2}$MS medium containing BAP (4.4 µM) c. Shoots formed from embryos after six months of culture in $^{1}/_{2}$MS medium containing BAP (4.4 µM) (bar 1 cm)

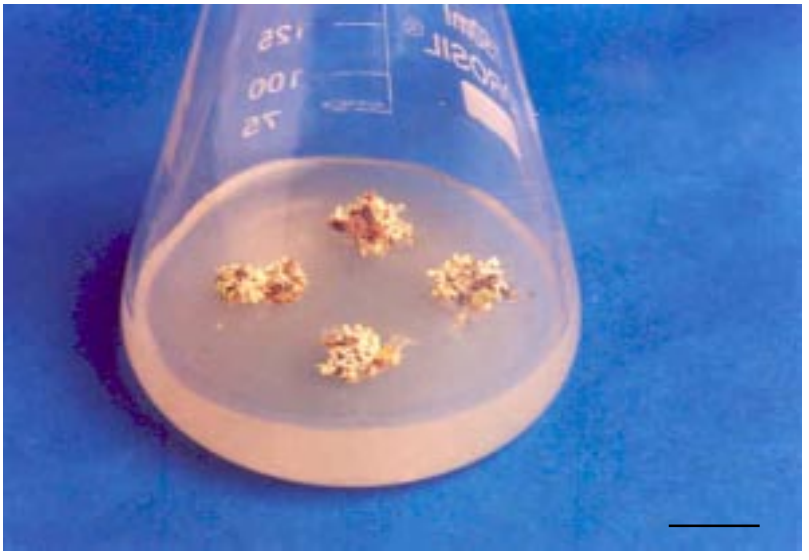


Figure 2.26: Embryo formation from the shoot end derived callus in $^1/_2$MS medium containing 2-iP (4.9 µM) (Shoots were obtained from zygotic embryos). Callus and embryos were formed when the shoots were kept without subculturing in $^1/_2$MS medium containing 2-iP (4.9 µM) for 3-4 months (bar 12 mm)

From the results (Table 2.24), it should be concluded that while fungal contamination was prevented effectively by adding bavistin in the medium, bacterial contamination could not be controlled. However, some explants remained contamination free, but they were died due to browning. Method 6 (Table 2.24) was found to be very effective to prevent the browning in the medium. Therefore, the use of this medium with the use of less amount of mercuric chloride for sterilization should be sufficient to maintain the tissue fresh. Frequent subculture to fresh medium can prevent the bacterial overgrowth and once the axillary buds starts sprouting, keeping the explants in antibiotic medium can completely suppress the bacteria.

Table 2.24: Different protocols tried for the sterilization and initial culture for the nodal explants (S-274) for the establishment of preliminary cultures for micropropagation:

Method	Sterilization	Medium	Comments
1	0.3% $HgCl_2$ for 20', kept in 0.5% Cy-HCl while cutting	MS + BAP (4.4 µM)	Severe browning and fungal contamination, all died within a week
2	0.1% $HgCl_2$ for 10', cefotaxim (100 m g l^{-1}) in the sterilization solution, dipping in antioxidant solution* while cutting.	MS + BAP (4.4 µM) + bavistin (80 m g l^{-1})	Browning started even before inoculation
3	-	Transfer of bacterial contaminated explants of the above method to antibiotic medium (cefotaxim, 100mg/l and augmentin 50 m g l^{-1})	The explants which had retained green colour also turned brown in two days
4	0.1% $HgCl_2$ for 8',	MS+BAP (4.4 µM)+ bavistin (80mg l^{-1}) and two combi-nations of antioxidants concentrations*	Browning could be prevented effectively in higher antioxidant medium, bacterial contamination persisted
5	0.1% $HgCl_2$ for 8'	MS (liquid) + BAP (4.4 µM) + bavistin (80 m g l^{-1}) and higher amount of anioxidants* and PVP (1 g l^{-1}) and antibiotics•	The medium itself turned severe blackish brown making the explants dark brown in one day
6	0.1% $HgCl_2$ for 10'	MS (liquid) + BAP (4.4 µM) + bavistin (80 m g l^{-1}) and higher amount of anioxidants* and PVP (1 g l^{-1}) but without antibiotics	No browning in the medium (positive response) but the explants had turned brown slowly, could be due to higher concentration of $HgCl_2$

*Antioxidant solution- Ascorbic acid (1%), Citric acid- (0.25%) and Cysteine-HCl- (0.25%); * Ascorbic acid (1% and 100 m g l^{-1}), citric acid (0.25% and 100 m g l^{-1}), Cy-HCl (500 m g l^{-1} and 100 m g l^{-1}); • Cefotaxim (200 m g l^{-1}), Augmentin (100 m g l^{-1}), Streptomycin (100 m g l^{-1}), Tetracyclin (100 m g l^{-1}).

2.3.7.1. Effect of silver nitrate on *in vitro* shoot growth:

Silver nitrate enhanced growth of the coffee shoots (Fig. 2.27). The greatest shoot length and more number of leaves were observed in 10 µM silver nitrate (Table 2.25), but 5 and 10 µM induced more leaf area. The seedling-based shoot tips were not exposed to silver nitrate for the first 72 h because it seemed likely that ethylene may be required for growth during this period, in view of observations by Cho and Kasha (1989, 1992) with barley. Ganesh and Sreenath (1996) found that 20 mg l^{-1} silver nitrate is required to get arabica shoots 3 cm long only after 70-80 days on a medium containing 8.8 µM BA. In the present study much lower levels of silver nitrate with IAA (2.5 µM) and BA (8.8 µM BA) gave long shoots with more nodes and leaves in only 45 days.

Table 2.25: Effect of silver nitrate on *C. canephora* (S-274) shoot growth:

$AgNO_3$ (µM)	Shoot length (cm)	Number of leaves	Number of nodes	Leaf area (cm^2)	Total chlorophyll (mg/g FW)
Control	2.0 ± 0.24	2.4±0.18	1.4±0.10	0.40±0.24	4.03±0.17
5	2.2±0.24	7.6±0.80	3.8±0.40	1.15±0.08	4.08±0.33
10	3.3±0.40	16.8±4.66	7.8±1.16	1.15±0.76	5.18±0.12
20	2.6±0.20	13.2±1.60	6.6±0.8	0.99±0.56	4.86±0.19
30	2.6±0.20	13.0±1.4	6.4±0.75	0.85±0.5	4.64±0.27
40	2.4±0.20	8.0±0.98	4.0±0.85	0.41±0.27	3.18±0.18

FW = fresh weight

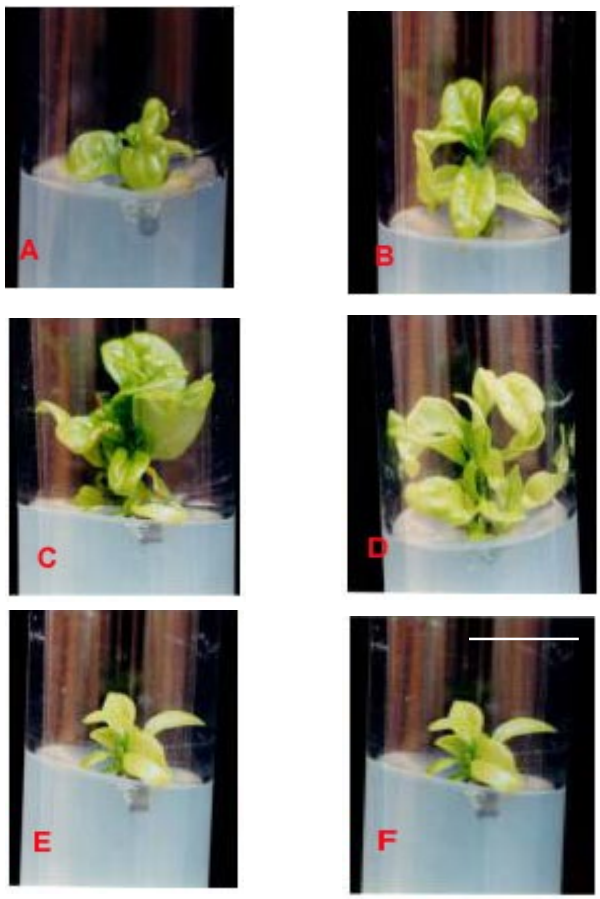


Figure 2.27: Effect of silver nitrate on *in vitro* coffee shoot growth (S-274). a. Control; b. 5 µM; c. 10 µM; d. 20 µM; e. 30 µM f. 40 µM silver nitrate (bar 12 mm)

2.4 CONCLUSION:

Modified callus development medium gives faster embryogenesis response and different varieties showed variation in response. CxR and Old Robusta varieties responded well to the media. Embryos could be multiplied by secondary embryogenesis which is an important property helping for using them for genetic transformation via *Agrobacterium*. It was also found that high rate of callus proliferation and frequent subculture prevents embryo development. Prolonged incubation in the same medium increases embryo production Hypocotyl tissues were found to be highly amenable for somatic embryogenesis. Use of hypocotyl explants gives additional advantage for organogenesis.

A highly reproducible method for regeneration of *C. canephora* plants via direct somatic embryogenesis method from *in vitro* leaf and *in vitro* stem segments of regenerated plants was developed. Embryogenesis was influenced by the presence of triacontanol (TRIA) in the medium. TRIA incorporated at 4.55 and 11.38 μM in half strength MS basal medium containing 1.1 μM BAP and 2.5 μM IAA induced direct somatic embryogenesis. A maximum of 59.2 ± 12.8 somatic embryos per culture were induced from *in vitro* leaf explants of *C. canephora* (S-274). TRIA also induced embryo formation from *in vitro* stem segments and callus tissues along with multiplication of primary embryos into secondary embryos.

The rapid direct and repetitive somatic embryogenesis response of *C. canephora* genotypes was tested on Murashige and Skoog medium (MS) containing thidiazuron (9.08 μM). Segments taken from cotyledonary leaves, hypocotyls, first leaves and stalks of regenerated plantlets produced clusters of somatic embryos directly from cut portions of explants on TDZ (9.08 μM) containing medium within a period of two months. These direct somatic embryogenesis from leaf

and hypocotyl explants in *Coffea* sp. is a strong evidence of their cell totipotency.

Zygotic embryos were cultured with 80-90% survival in different media. Silver nitrate enhanced growth of the coffee shoots. The rapid somatic embryo induction protocol would be useful for the mass propagation, direct regeneration and in genetic transformation of selected elite lines. The results are useful for studies related to coffee crop improvement through genetic transformation using somatic embryos and for mass multiplication.

CHAPTER III

Agrobacterium Mediated Genetic Transformation

3.1. INTRODUCTION:

Coffee is an extremely important agricultural crop. Various avenues of biotechnology have been explored for genetic improvement of coffee. Coffee improvement through biotechnology could include micropropagation, embryo rescue, anther culture, somaclonal variation, cell line selection, protoplast fusion, *in vitro* conservation, DNA markers and genetic transformation.

The first transformation of coffee cells reported (Barton *et al.*, 1991) was by protoplast electroporation. Genetic transformation with *Agrobacterium* sp. has also been reported (Feng *et al.*, 1992; Freire *et al.*, 1994). The regeneration of transgenic coffee trees was first obtained by transformation of somatic embryos via *Agrobacterium tumefaciens* (Leroy *et al.*, 1997). Somatic embryos are excellent explants for genetic transformation due to their vigorous nature and ability of regeneration.

The main objective of the study was to develop a suitable transformation protocol for coffee through *Agrobacterium* strains. There are two reports on the use of *Agrobacterium rhizogenes* for transformation of *Coffea* sp. and demonstrated the susceptibility of *Coffea* sp. for *A. rhizogenes* mediated transformation (Sugiyama *et al.*, 1995; Leroy *et al.*, 1997). In the present study for the first time binary vectors were used in *A. rhizogenes* mediated transformation of *C. canephora*.

3.2. MATERIALS AND METHODS:

3.2.1. Sensitivity tests for selection of transformed tissue:

Kanamycin (Sigma), hygromycin (Duchefa) and basta (Glufosinate; AgrEvo India Ltd., Gujrat, India)) were used for the study. For kanamycin sensitivity test, the embryos that had grown in embryo germination medium (Table 2.3, chapter 2) were wounded and

inoculated in the same medium containing different levels of kanamycin (25, 50, 100, 200, 300, 400 & 500 m gl^{-1}). For hygromycin (10, 20, 30, 40 and 50 m gl^{-1}) and basta (1, 5, 10, 20 and 30 m gl^{-1}), *in vitro* leaf explants were inoculated in callus development medium (Table 2.2, chapter 2).

3.2.2. Plasmid and construct used:

pCAMBIA1301 (Fig. 3.1; Fig 3.2) bearing intron GUS and a GFP reporter (pSK53 construct) were obtained from Dr. Lokeshwari of the SPIC science Foundation, Chennai, India. pCAMBIA1301 has the *gus*A gene present as a reporter which can be used to assess the efficiency of transformation. The MCS (multiple cloning site) is between *gus*A (proximal to the right T-DNA border) and hygromycin (proximal to the left T-DNA border) resistance genes. Thus, transformed plants expressing GUS and showing hygromycin resistance will also have cloned gene. A catalase intron present in the *gus*A coding sequence ensures that the gene is not expressed in bacteria (Ohta *et al.*, 1990), but only upon transfer to plants. Unique restriction sites in the MCS available for cloning are EcoR I, Sac I, Kpn I, Sma I, BamH I, Xba I, Sal I, Pst I and Hind III. For bacterial (*E. coli* or *Agrobacterium*) selection, kanamycin was used at 50 µg ml^{-1}. Hygromycin was used for plant selection.

3.2.2.1. pSK53 Construct:

The vector (Figure 3.2) has been constructed by introducing green flurescent protein (GFP) gene into pCAMBIA1301 plasmid at the site of *Pst* I. The vector had three genes (1) hygromycin phosphotransferase (HPT), (2) *β*-glucuronidase (GUS) and (3) green flurescence protein (GFP). All the genes are controlled by cauliflower mosaic virus (CaMV) 35S promoter and 35S terminator except GUS, which is having nopaline synthase (NoS) terminator.

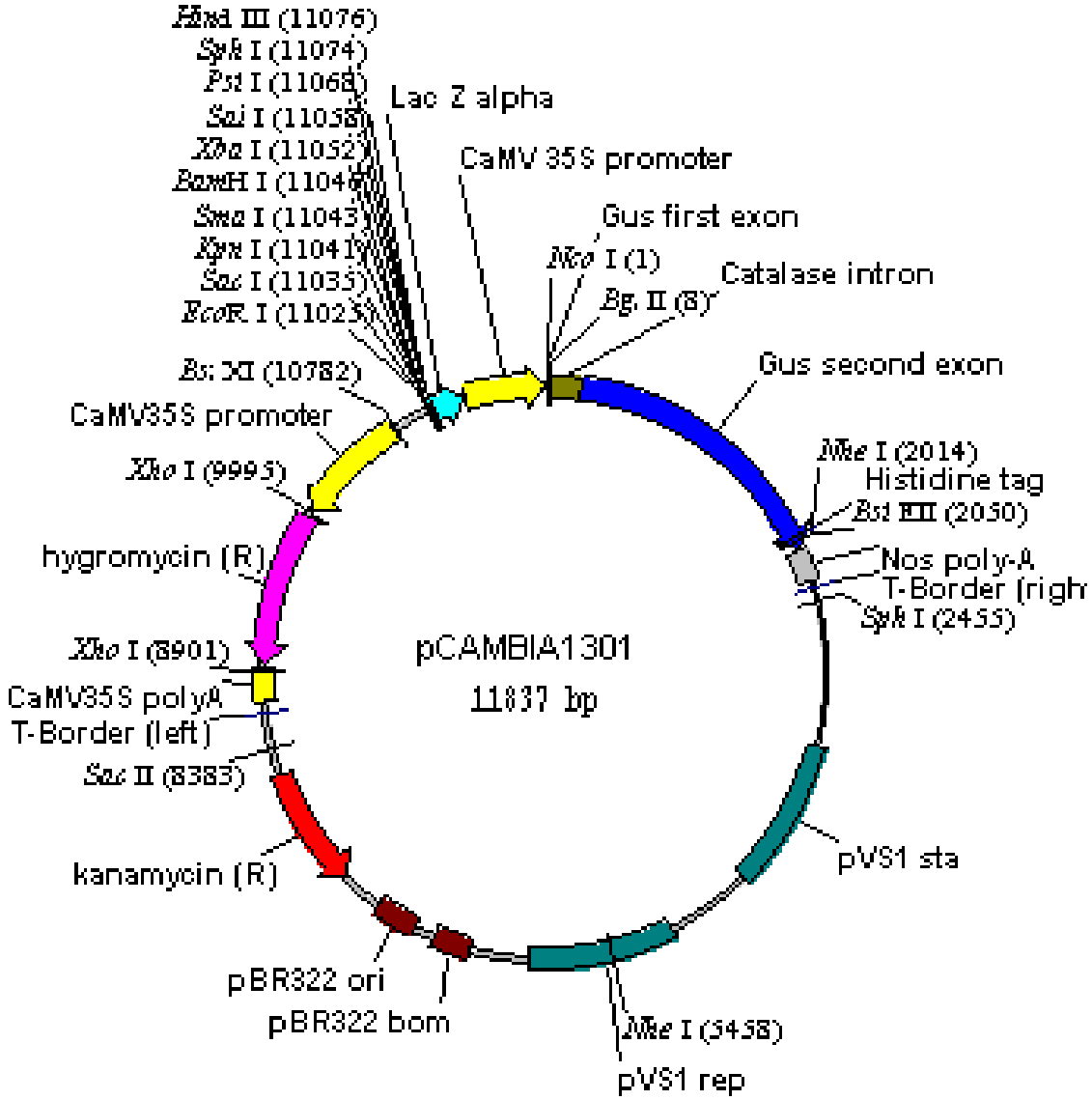


Figure 3.1 Restriction map of pCAMBIA1301 containing 'intron gus' as reporter

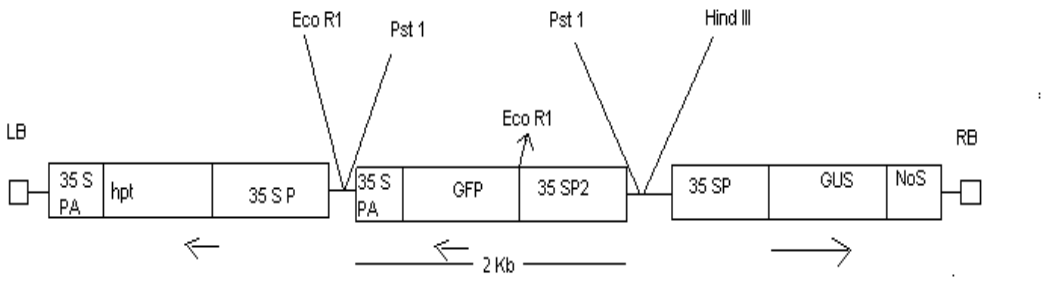


Figure 3.2 pSK53 construct showing hygromycin, GFP and GUS genes

3.2.2.2. *Agrobacterium* strains:

The vector had been mobilized into *Agrobacterium tumefaciens* EHA 101 and *A. rhizogenes* A4. Binary vectors were mobilized to *Agrobacterium* strains by freeze-thaw method of An *et al.* (1988). The bacterial transformants were selected based on kanamycin selection.

3.2.3. Plasmid isolation (Sambrook and Russel, 2001):

Materials:

LB (Luria Bertani) medium (g l^{-1}):

Tryptone	10.0
Yeast extract	5.0
NaCl	10.0

Adjusted pH to 7.2 by using 2.0 M NaOH and sterilized by autoclaving

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

The resulting solution was 3.0 M with respect to Potassium and 5.0 M with respect to acetate.

Protocol:

1. Inoculated single colonies from plates in 20 ml of LB broth in okaridge tubes.
2. Grown overnight at room temperature.
3. Harvested the cells by centrifugation at 5000 rpm for 10 min.
4. Added 200 µl of solution I to the pellet in okaridge tube and mixed well.
5. Transferred 100 µl to eppendorf tubes.
6. Added 20 µl of 20 mg ml^{-1} lysozyme solution (prepared freshly), mixed well by gentle vortexing and incubated at 37 ^{o}C for 15 min.
7. Added 200 µl of cell lysis solution (solution II), mixed completely by repeated gentle inversion of the tube. Incubated on ice for 5 min.
8. Added 150 µl of solution III and vortexed gently. Placed the tubes on ice for 5 min.
9. Centrifuged the tubes at 10,000 rpm for 15 min at 4 ^{o}C .
10. Transferred the supernatant to a fresh tube and added equal volume of phenol-chloroform (1:1) and vortexed thoroughly. Separated the two phases by centrifuging at 10,000 rpm at 4 ^{o}C for 10 min.

11. Transferred the upper aqueous phase to a fresh tube and added equal volume of chloroform. Centrifuged the tubes at 12,000 rpm at 4°C for 10 min to remove traces of phenol.
12. Transferred the upper aqueous phase to a fresh tube and added distilled ethanol to fill the eppendorf tube. Kept the tubes in freezer overnight for precipitation.
13. Centrifuged the tubes at 10,000 rpm at 4 °C for 30 min.
14. Pellets redissolved in 20 µl sterile water and stored at -80 °C.

3.2.4. Mobilization of binary vectors to *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*:

Protocol:

Direct *Agrobacterium* transformation: Freeze-thaw method (An *et al.*, 1988).

1. *Agrobacterium* was grown in 5 ml of LB medium overnight at 28°C.
2. Added 2 ml of the overnight culture to 50 ml LB medium in a 250 ml conical flask and shaken vigorously (9000 rpm) at 28°C until the culture grew to an OD of 0.5-1 at 600 nm.
3. Chilled the culture on ice. Centrifuged the cell suspension at 3000 rpm for 5 min at 4 °C.
4. Discarded the supernatant. Resuspended the cells in 1 ml of 20 mM $CaCl_2$ solution (ice-cold). Dispensed 0.1 ml aliquots into prechilled eppendorf tubes.
5. Added about 1 µg of plasmid DNA into the cells.
6. Froze the cells in liquid nitrogen.
7. Thawed the cells by incubating the test tube in 37 °C water bath for 5 min.
8. Added 1 ml of LB medium to the tube and incubated at 28 °C for 2-4 hours with gentle shaking. This period allows the bacteria to express the antibiotic resistance genes.

9. Centrifuged the tubes for 30 sec., discarded the supernatant and resuspended the cells in 100 µl of LB medium.
10. Spreaded the cells on an LB agar plate containing 50 mg l^{-1} kanamycin. Incubated the plate at 28 ^{o}C. Transformed colonies appeared in 2-3 days.

The transformation frequency of this method is low (ca. 103 transformation per mg DNA) compared to triparental mating. The technique is reliable and rapid. It eliminates much of the plasmid rearrangement that often occurs during triparental mating.

3.2.5. Agarose gel electrophoresis:

Materials:

1. Agarose (Electrophoretic grade)
2. 50X TAE buffer

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

Sterilized by autoclaving

3. DNA loading dye (6X)

Xylene cyanol	0.25%
Bromophenol blue	0.25%
Glycerol	30%

Stored at 4 ^{o}C

4. Standard DNA size marker
5. Gel running boat
6. Electrophoresis unit
7. Ethidium bromide stock solution

10 mg ml^{-1} in distilled water. Used at a working concentration of 0.5 µg ml^{-1}. Stored at 4^{o}C.

Protocol:

1. Sealed the boat with adhesive tape and placed the comb for wells.
2. Used agarose at 0.8% level and melted in 1X TAE.
3. Allowed agarose to cool to about 50^{o}C and poured into the sealed boat.
4. Allowed the gel to set (30 min). Removed the comb and adhesive tape and placed the gel in the electrophoresis tank.
5. Added 1X TAE buffer to the electrode chamber to cover the gel to a depth of about 1 mm.
6. Loaded the samples by mixing 10 µl aliquot with 2 µl of loading dye.
7. Run the samples at 50 volts till the leading dye reached ¾th of the gel.
8. Removed the gel from the tank and placed in ethidium bromide solution for 15 min.
9. Destained and examined the gel on a UV transilluminator.

3.2.6.1. Coffee varieties used:

Two different varieties of *C. canephora* viz., CxR and Old Robusta were used for the experiments.

3.2.6.2. Induction of embryos:

Embryos were obtained from hypocotyl explants cultured on modified callus development medium (Table 2.2; see Chapter 2). Later more embryos obtained by culturing the primary embryos in embryo germination medium (Table 2.3). The embryos were wounded and incubated in MS medium containing 2iP (4.9 µM) for one month just before using for transformation.

3.2.6.3. Preparation of bacterial strains:

A. rhizogenes and *A. tumefaciens* strains which had pSK53 plasmid were used for transformation. Single colonies were inoculated

in 10 ml of LB broth. One ml of the overnight grown bacterial cultures were inoculated in 50 ml of LB broth. The bacterial cultures were grown for one day in LB broth till the OD reached one at 600 nm. The cultures were centrifuged in 1.5 ml eppendorf tubes twice to harvest the fresh culture, resuspended in one ml of the MS liquid medium containing acetosyringone (100 µM), and kept for activation of the *vir* genes for half an hour on shaker.

3.2.6. Transformation of coffee:

The general protocol followed for transformation is given in the Fig. 3.3

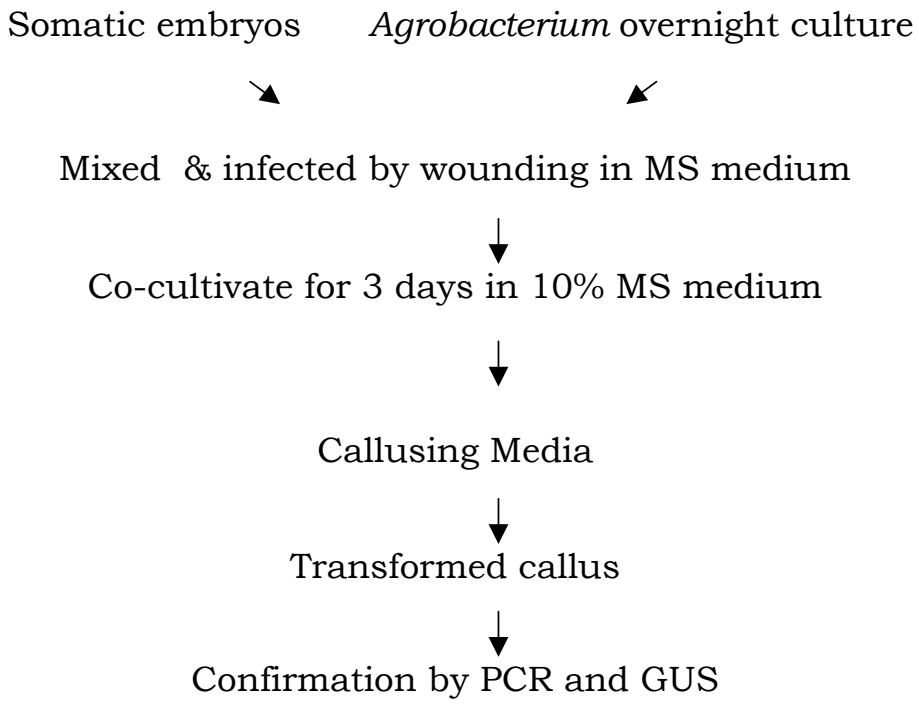


Fig 3.3. Schematic representation of *Agrobacterium* mediated transformation.

3.2.6.4. Transformation:

The embryos were wounded by manual cutting and transferred to the okaridge tubes containing MS liquid medium and

acetosyringone (100 µM). Subsequently 1 ml of bacterial culture was added and kept the whole thing for one hour incubation. Later the explants were blotted dry and inoculated in the half strength MS medium (solid) containing acetosyringone.

The embryos were co-cultivated with bacteria for 3 days and transferred to callus development medium (Table 2.2) containing half strength MS salts with growth regulators, 2-iP (2 9.8 µM), IAA (5.7 µM) and NAA (5.3 µM). Cefotaxim (Alkem Laboratories Ltd., Mumbai, India; 500 mg l^{-1}), augmentin (Medreich Sterilab Ltd., Bangalore, India; 500 mg l^{-1}) and hygromycin (3 mg l^{-1}) were also added to the medium to kill the bacteria and for the selection of transformed cells.

After one month the embryos were transferred to modified embryo induction medium having half strength MS salts containing the growth regulators 2,4-D (9 µM) and BAP (35.5 µM), hygromycin (10 mg l^{-1}) and antibiotics. This medium was used for further proliferation of the transformed callus. The embryos were kept in this medium for forty days.

The surviving calli were transferred to the same medium containing 2,4-D (9 µM) and BAP (35.5 µM) with hygromycin (20 and 50 mg l^{-1}) and antibiotics. Transformants were confirmed by GUS assay and PCR.

3.2.7. GUS assay (modified method of Hatanaka *et al.*, 1999):

Reagents:

N, N-Dimethyl formamide	1 or 2 drops
X-gluc* (Cyclohexylammonium salt, MW=520.8; Sigma)	1 mg
0.1 M Phosphate buffer (pH 7.0)	980 µl
5 mM Potassium ferricyanide	10 µl
5 mM Potassium ferrocyanide	10 µl
Triton X-100	1 µl

*5-bromo-3-chloro-3-indolyl-*β*-D-glucuronic acid

Added N, N - Dimethyl formamide to X-gluc and mixed until dissolved. Added 0.1 M Phosphate buffer, 5 mM Potassium ferricyanide, 5 mM Potassium ferrocyanide and Triton X-100. Prepared fresh before use.

Around 100 mg of callus, which had survived in selection pressure (hygromycin 10 mg l^{-1}) was taken in eppendorf tubes. 100 µl of GUS assay mixture was added to the tubes just enough to cover the callus. The tubes were incubated at 37^{o}C in water bath for 16 hours. Dark blue colour was developed in transformed callus.

3.2.8. Isolation of genomic DNA from coffee by Paillard (1996) method:

Reagents:

Composition of extraction buffer (50 ml):

This buffer contains Sorbital, Sodium bisulphate, Tris HCl and EDTA.

Preparation of Tris HCl and EDTA solutions:

Chemical	Concentration	Weight for 100 ml
Tris-HCl	0.5 M	6.057 g
EDTA	0.5 M	14.613 g

Weighed Tris-HCl and dissolved in distilled water (Milli Q). Adjusted the pH to 8.0 with 1N NaOH, autoclaved and stored. Weighed EDTA and dissolved in distilled water (Milli Q). Adjusted the pH to 8.0 with NaOH pellets, autoclaved and stored.

Preparation of extraction buffer:

Chemical	Conc. in this mix	Amount for 50 ml
D-sorbital	0.35 M	3.188 g
Sodium bisulphate	0.5%	0.25 gm
Tris-HCl (pH 8.0)	0.1 M	Added 5 ml from solution above (0.5 M)
EDTA (pH 8.0)	0.005 M	Added 0.5 ml from solution above (0.5 M)

Dissolved sorbital in 35 ml of distilled water (Milli Q autoclaved) then added sodium bisulphate, dissolved and added 5 ml of Tris HCl solution (pH 8.0) and 0.5 ml of EDTA solution (pH 8.0), autoclaved and stored.

Lysis buffer (10 ml):

Chemical	Conc. in this mix	Amount for 10 ml
CTAB	2%	0.2 gm in 1 ml distilled water
NaCl	2 M	4 ml from a 5 M NaCl solution
Tris-HCl (pH 8.0)	0.2 M	Added 4 ml Tris-HCl (0.5 M)
EDTA	0.05 M	Added 1 ml of EDTA (0.5 M)

Preparation: Dissolved CTAB in 1 ml distilled water and added the remaining solution as indicated above. Autoclaved and stored.

0.1X TE (20 ml):

Preparation: Stock solutions of Tris-HCl and EDTA are given above. Added 40 µl of 0.5 M Tris-HCl (pH 8.0) and 4.0 µl of 0.5M EDTA (pH 8.0) and made up to 20 ml with distilled water and autoclaved.

1. Took 100 mg of callus survived in selection pressure (hygromycin 10 mg l^{-1}) and ground to fine powder with liquid N_2 at room temperatue.
2. Suspended the frozen powder in 10 ml of extraction buffer (preheated to 65 ^{o}C).
3. Filtered the sample using muslin cloth.
4. Centrifuged at 12,000 rpm for 20 min at 4 ^{o}C.
5. Suspended the pellet in 20 µl of cold extraction buffer.
6. Added 30 µl of lysis buffer and 10 µl of sodium lauryl sarcosine (5%, Sigma).
7. Incubated the above mixture at 65 ^{o}C for 30 min.

8. Added 40 µl of chloroform:isoamylalcohol (24:1).
9. Inverted the tube to form an emulsion.
10. Centrifuged at 12,000 rpm for 15 min. at 4 °C.
11. Transferred the supernatant to a new tube.
12. Added 2 µl of 3 M sodium acetate and 40 µl of cold ethanol.
13. Centrifuged at 12,000 rpm for 15 min. at 4 °C.
14. The pellet was resuspended in 70% ethanol and kept at 4 °C overnight.
15. Centrifuged at 12,000 rpm for 15 min. at 4 °C.
16. Suspended the pellet in 5 µl of 0.1X TE.

3.2.9. Polymerase chain reaction (PCR):

Materials:

DNA from putative transformants and untransformed tissue

Downstream oligonucleotide primer

Upstream oligonucleotide primer

Taq DNA polymerase

10X reaction buffer

Nuclease-free water

dNTP mix (10 mM of each dNTP)

PCR reaction mixture:

Components	Volume (µl)	Final concentration
Nuclease-free water	39.4	--
10X reaction buffer	5.0	1X
dNTP mix (10 mM)	1.0	0.2 mM
Taq polymerase	0.6	1 unit
Downstream primer	1.0	1.0 µM
Upstream primer	1.0	1.0 µM
Template DNA	2	--
	50 µl (Total volume)	

1. Combined the first five reaction components in a thin-walled PCR tube. Initiated the reaction by adding the primers and the template. Mixed well.
2. Placed tubes in the thermocycler (Gene Amp® PCR System 9700) and proceeded with the thermal cycling programme chosen for the reaction.

PCR conditions:

1. Initial denaturation-94 ^{o}C for 4 min.
2. Denaturation-94 ^{o}C for 30 sec.
   Annealing-50 ^{o}C for 45 sec.
   Extension-72 ^{o}C for 1 min and 15 sec (29 cycles)
3. Final extension-72 ^{o}C for 10 min.

3.2.10. Scanning electron microscopy:

Co-cultivated embryos were subjected to scanning electron microscopy to see the bacteria.

The samples were fixed in 2% gluteraldehyde and 0.5% paraformaldehyde in phosphate buffer (pH 7.5) for 4-5 hours at 4°C. Later the samples were passed through alcohol series (10-90%, 30 minutes each) to bring to absolute alcohol. Finally, they were taken out of the alcohol and kept in the desiccator until the scanning was performed.

Embryos were mounted on stubs and gold coated (Polaron SEM coating system) and then viewed using a LEO 435 VP scanning electron microscope (LEO Electron Microscope Ltd., Cambridge).

3.3. RESULTS AND DISCUSSION:

3.3.1. Selection of transformed tissues:

3.3.1.1. Kanamycin sensitivity test:

Embryos grown in embryo germination medium were placed in the same medium with different concentrations of kanamycin (25 mg l^{-1}, 50, 100, 200, 300, 400 & 500) after wounding them. Callusing was observed in samples placed at 25 and 50 mg l^{-1} of kanamycin concentrations (Table 3.1). Slight callusing with leaf growth was found in cultures placed in 100 mg l^{-1}. Higher concentrations of kanamycin prevented successful callusing. Muniswamy and Sreenath (1996) reported that callus development from leaf tissue was reduced at 50 mg l^{-1} of kanamycin, while callus induction was almost completely suppressed at 100 mg l^{-1} and higher concentrations.

Table 3.1: Kanamycin sensitivity test with wounded embryos:

Kanamycin (mg l^{-1})	**Callusing (%)**		**Browning (%)**	
	34days	**48days**	**34days**	**48days**
Control	94.60	96.20	0	0
25	95.65	92.60	0	0
50	95.80	84.60	0	0
100	7.70	0	15.4	0
200	0	0	15.4	100
300	0	0	31.0	100
400	0	0	3.7	100
500	0	0	15.4	100

3.3.1.2. Hygromycin sensitivity test:

Table 3.2: Hygromycin sensitivity test with *in vitro* leaves:

Hygromycin (mg l^{-1})	**Callusing (%)**		**Browning (%)**	
	14days	**30days**	**14days**	**30days**
Control	-	54	16	25
10	-	-	46	71
20	-	-	92	96
30	-	-	100	100
40	-	-	100	100
50	-	-	100	100

In hygromycin medium no callusing was observed in any of the concentrations. Hygromycin at 10 mg l^{-1} itself prevented callusing. This concentration (10 mg l^{-1}) was chosen as the ideal level for the successful selection of the transformants because it prevents callusing and also kills the untransformed cells (Table 3.2). In our experiments, transferring the transformed callus to the 20 mg l^{-1} hygromycin medium prevented the callus growth, only Old Robusta callus survived in the medium, which shows that the selection pressure should be increased in the medium gradually giving enough time for the transformed cells to survive.

3.3.1.3. Basta (glufosinate) sensitivity test:

Table 3.3: Basta (glufosinate) sensitivity test with *in vitro* leaves:

Basta (mg l^{-1})	**Callusing (%)**		**Browning (%)**	
	14days	**30days**	**14days**	**30days**
Control	-	55	30	56
1	-	-	42	74
5	-	-	77	93
10	-	-	97	100
20	-	-	79	100
30	-	-	96	100

Even 1 mg l^{-1} of basta in the medium completely prevented the callusing (Table 3.3). 5 mg l^{-1} basta was selected for the confirmation transformation because at that concentration most of the tissues died which are not transformed.

3.3.1.4. Summary of the sensitivity tests:

Selection marker	**Minimum concentration that prevents growth of callus**
Kanamycin	200 mg l^{-1}
Hygromycin	10 mg l^{-1}
Basta	5 mg l^{-1}

It was seen that kanamycin had to be used at very high levels to obtain killing and very often regeneration of plants and multiplication of untransformed somatic embryos occurred even at high levels of kanamycin. Basta was very effective but turned the media brown and the detergent used to keep the herbicide in solution interfered with regeneration. Therefore hygromycin was used as the selection marker for further experiments.

3.3.2. Mobilization of binary vectors to *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*:

PCR for the bacterial selection marker kanamycin (neomycin phoshpho transferase gene: npt II) was used to check the presence of the introduced plasmid in the *Agrobacterium*. This is presented below (Fig. 3.4). This gene is outside the two borders of the T-DNA.

1 2 3 4

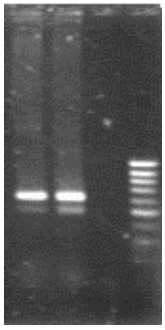


Figure 3.4: PCR amplification of NPTII gene in DNA from 1.*A. rhizogenes* transformed with pCAMBIA1301, an *A. tumefaciens* vector. 2. pCAMBIA1301 from *A. tumefaciens*. 3. Untransformed *A. rhizogenes*. 4. 100bp Marker

3.3.3. Period of co-cultivation:

The embryos were co-cultivated with bacteria for different number of days (Table 3.4). Much difference was not observed between 3 days and 4 days of incubation for successful transformation.

Table 3.4: Percentage of transformed embryos surviving in selection pressure after one month (hygromycin, 10 mg l^{-1}):

Control	Surviving embryos
3 days of co-cultivation	18.03
4 days of co cultivation	19.04

3.3.4. Scanning Electron Microscopy:

Long rod shaped *Agrobacteria* were clearly visible at 15 K X magnifications (Fig. 3.5).

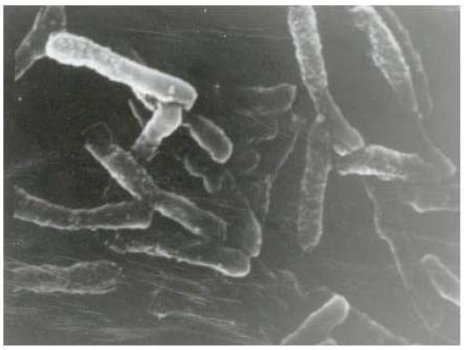


Figure 3.5: Scanning electron micrograph of *A. rhizogenes* at a magnification of 15 K X

3.3.5. Transformation and callus formation:

Experiments were conducted in duplicates involving approximately 200 embryos each. Slight callus formation was observed in some surviving explants as visible under microscope after one month in callus development medium containing half strength MS medium with growth regulators, 2-iP (2 9.8 µM), IAA (5.7 µM) and NAA (5.3 µM), Cefotaxim, augmentin (500 mg l^{-1} each) and hygromycin (3 mg l^{-1}). However, this was not visible with the naked eye. There were some surviving embryos in control with selection pressure. Around 91-95 % of the transformed explants retained their freshness in the medium (Table 3.5). Embryos also turned brown slightly in the control without selection pressure.

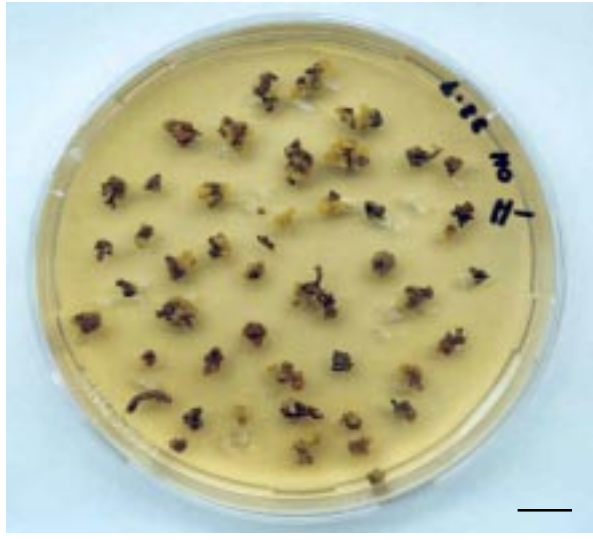
Later these embryos were transferred to embryo induction medium with higher selection pressure (hygromycin, 10 mg l^{-1}), having half strength MS medium containing the growth regulators 2,4-D (9 µM) and BAP (35.5 µM) and antibiotics (Table 3.5). After one-month callus was formed from these embryos. The control without selection pressure also showed less survival. This could be because of the antibiotics present in the medium. There were a few survivals in controls in the medium with hygromycin. But Old Robusta showed vigorous growth in controls as well as transformed embryos compared to CxR (Fig. 3.6, 3.7, 3.9). The calli were yellow in colour and granular in appearance.

A. tumefaciens transformed CxR embryos at higher frequency (12.03%) than *A. rhizogenes* (4.50%; Fig. 3.9). Difference in frequency of transformation was also observed between the varieties. Old Robusta showed high virulence for transformation with 49.29% compared to CxR (12.03%). In general, *A. tumefaciens* infected embryos showed higher frequency of callus formation than *A. rhizogenes*

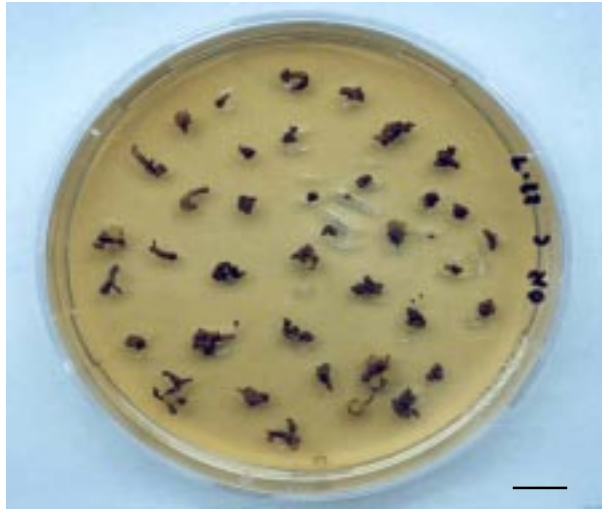
Table 3.5: Callus formation from the transformed embryos in 30 days intervals in media containing hygromycin (3, 10. 20 mg l^{-1}):

	Variety	**% of survival in hygromycin (3 mg l^{-1})**	**% of survival in hygromycin (10 mg l^{-1})**	**% of survival in hygromycin (20 mg l^{-1})**
Control (Without hygromycin)	CxR	90.90	32.14	100
Control (Without hygromycin)	Old Robusta	94.82	91.75	100
Control (With hygromycin)	CxR	17.74	0.62	0
Control (With hygromycin)	Old Robusta	9.4	12.50	0
A. rhizogenes	CxR	95.53	4.50	0
A. tumefaciens	CxR	94.46	12.03	0.04
A. tumefaciens	Old Robusta	91.89	49.29	34.04

(a)



(b)



(c)

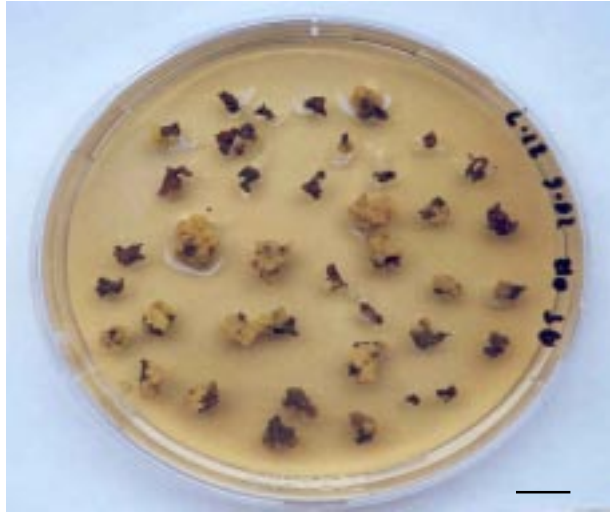
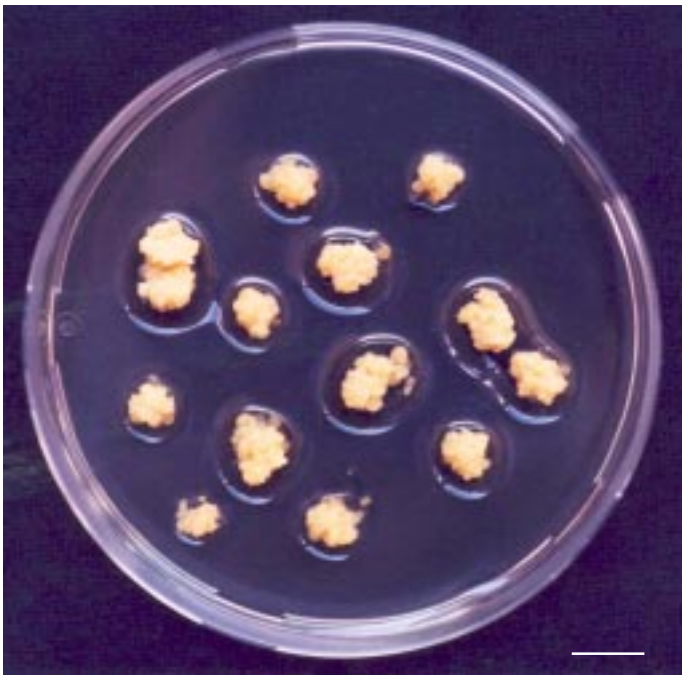


Figure 3.6: Old Robusta embryos transformed with *A. tumefaciens*. a. Surviving calli in the medium without hygromycin; b. Tissues in the medium containing hygromycin (10 mg l^{-1}); c. Transformed calli surviving in hygromycin (10 mg l^{-1}); Callusing can be seen (bar 6 mm)

(a)



(b)

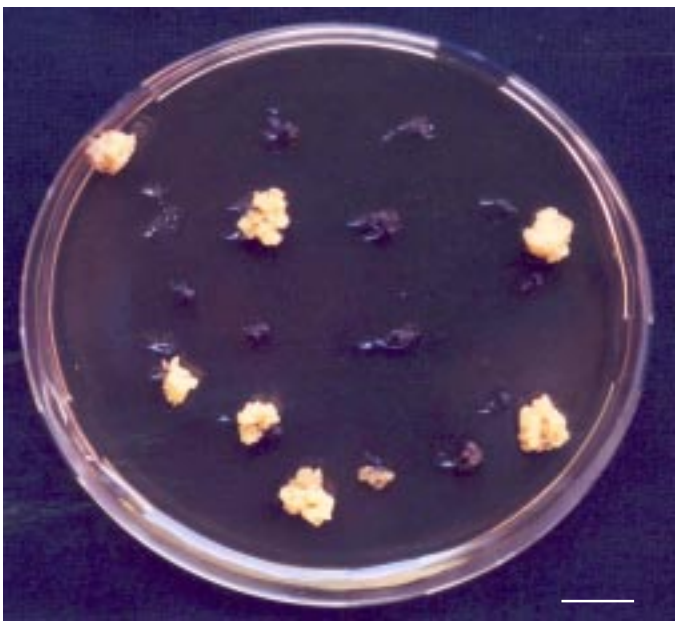


Figure 3.7: Old Robusta embryos transformed with *A. tumefaciens*.
a. Surviving calli in the medium without hygromycin;
b. Transformed calli surviving in hygromycin (20 mg l^{-1})
(bar 10 mm)

(a) (b)

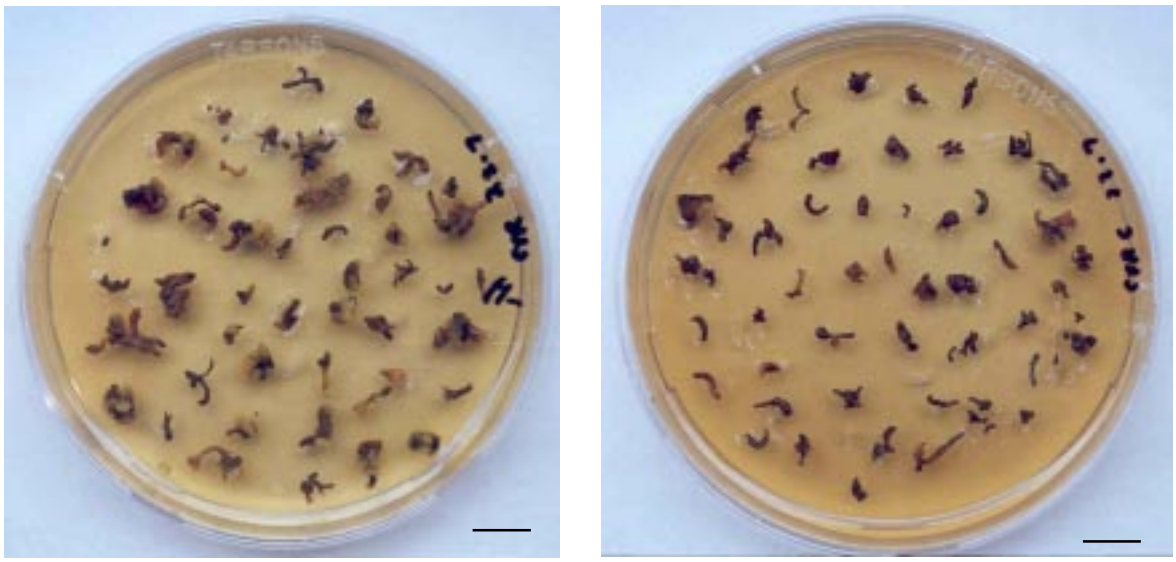
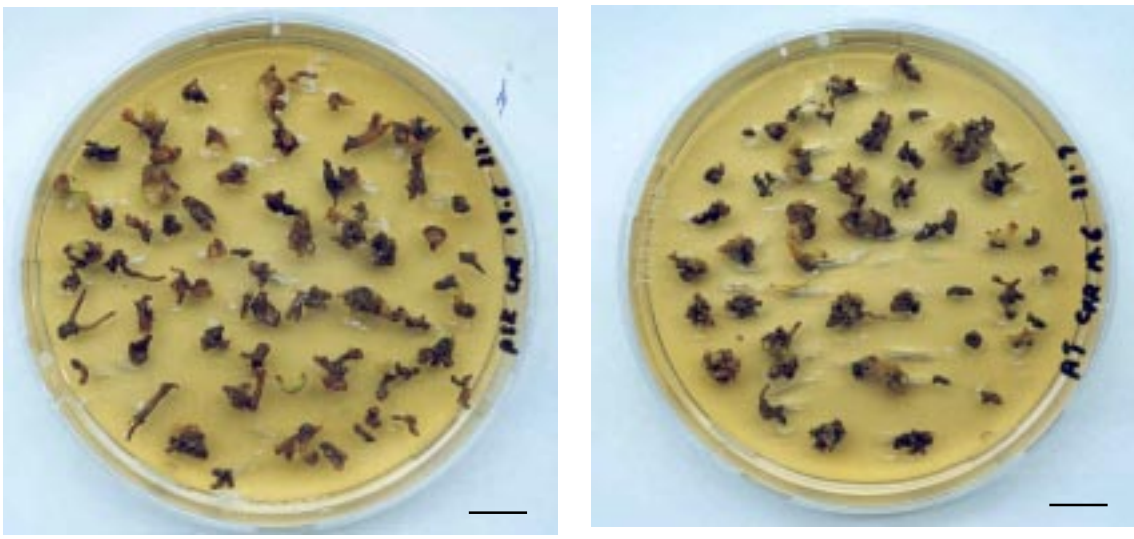


Figure 3.8: CxR embryos. a. Surviving calli in the medium without hygromycin
b. Tissues in the medium containing hygromycin (10 mg l^{-1})
(bar 11 mm)

(a) (b)



Firgure 3.9: CxR embryos transformed with *A. rhizogenes* (a) and *A. tumefaciens* (b) strains. Callusing could be seen (bar 11 mm)

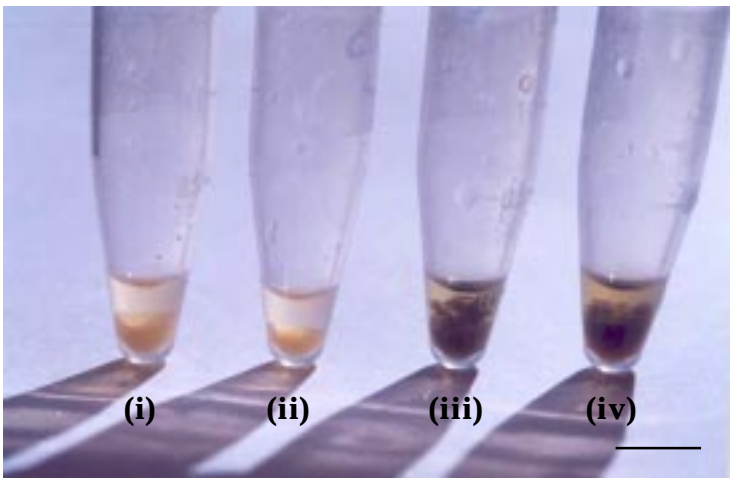
3.3.6. Confirmation of transformants:

The transformants were confirmed by GUS and PCR of the *gus* A gene. The X-gluc reaction was positive for callus obtained by transformation of CxR with *A. tumefaciens* (Fig. 3.9b). Other calli did not show the colour formation but were positive in the PCR (Fig. 3.11). The reason could be the stable integration of the gene but lack of proper expression or false positive from the bacterial DNA. However, very high levels of cefotaxim and augmentin (500 mg l^{-1}) were used to eliminate bacteria during initial stages of incubation itself, under the selection pressure (hygromycin, 3 mg l^{-1}). The transformed calli were observed after two months time under higher selection pressure (hygromycin, 10 mg l^{-1}). The calli were carefully removed for the DNA extraction avoiding contact with embryos.

3.4 CONCLUSION:

The sensitivity of the particular explant of the plant species to the corresponding selection marker has to be ascertained first before taking up genetic transformation studies. In the study we have used hygromycin as the selectable marker. It was seen that kanamycin had to be used at very high levels (200 mg l^{-1}) to obtain killing and very often regeneration of plants and multiplication of untransformed somatic embryos occurred even at high levels of kanamycin. Basta was very effective but turned the media brown and the detergent used to keep the herbicide in solution interfered with regeneration. The frequency of transformation with *A. rhizogenes* and *A. tumefaciens* was found to be varying and varietal difference in the frequency of transformation was observed.

(a)



(b)

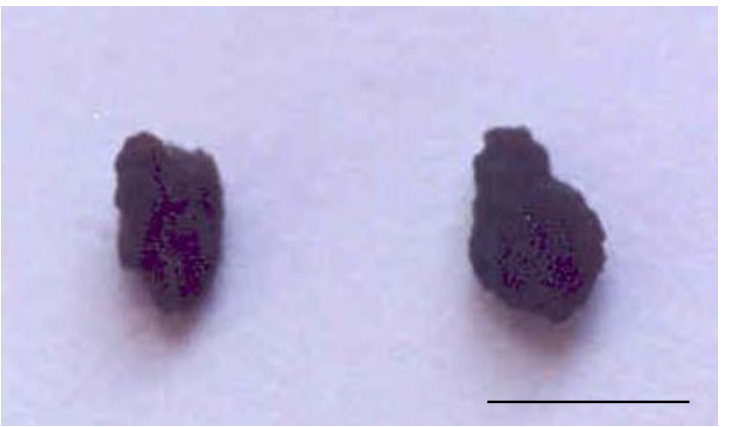


Figure 3.10: (a), (b) X-gluc staining of non-transformed and transformed embryogenic calli of CxR transformed with *A. tumefaciens*. (bar 7 mm)
The latter (b) stained deep blue (a-iii) (a-iv) and (b) while the former is negative (a-i) and (a-ii). (bar5 mm)

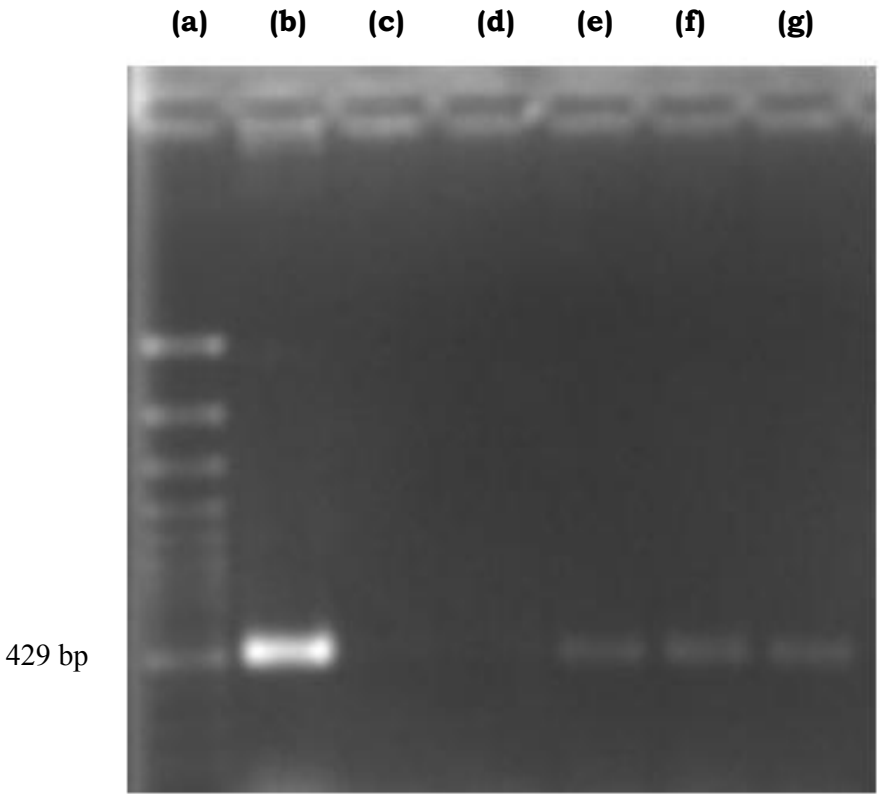


Figure 3.11 Detection of *gus* gene in transgenic coffee callus by PCR amplification of the fragment.

A. 100bp Marker;
B: pCAMBIA 1301 vector,
C, D: DNA extracted from untransformed *C. canephora* calli (CxR and Old Robusta respectively)
E: DNA extracted from CxR transformed calli, transformed with *A. rhizogenes*
F. DNA extracted from Old Robusta transformed calli, transformed with *A.tumefaciens*
G. DNA extracted from CxR transformed calli, transformed with *A. tumefaciens*

CHAPTER IV

Coffea Seed Germination & Caffeine

4.1. INTRODUCTION:

4.1.1. Seed germination:

Plant tissue culture studies for development of *in vitro* propagation of coffee to produce elite plantlets are very important. Several reports on successful development of *in vitro* propagation in *Coffea* are available (Yasuda *et al.,* 1985; Hatanaka *et al.,* 1991; Van Boxtel and Berthouly, 1996). The source of explants viz. cotyledons, hypocotyl etc. are very crucial for effective micropropagation and transformation studies. Effective seed germination is required to obtain healthy seedlings for *in vitro* studies. However, slow and erratic seed germination and long production time are drawbacks. Apart from this, coffee seedlings grow very slowly in the field, and this has been found to be responsible for over 60% of the cases of failure to establish, since the plants are feeble to withstand the first dry season following transplanting (Obatolu, 1999). Coffee being a perennial crop, field planting of vigorous and healthy seedlings is one of the pre-requisites to establish a successful and viable plantation (Glory Swarupa, 1996). Even if there are a few methods known for efficient germination of coffee seeds i*n vivo* (Ramaiah and Venkataraman, 1988; Opile, 1977), there has been no systematic study with efficient germination percentage *in vitro*.

4.1.2. Caffeine:

In chemical terms the purine alkaloid caffeine is a three-fold methylated xanthine (1,3,7-trimethylxanthine) (Waldhausser *et al.*, 1997a). Caffeine is the major alkaloid in coffee, and variations in caffeine content are found within the same tree with the highest levels in seeds, flowers and leaves (Raju and Gopal, 1979). Variability is also observed among varieties of the same species and among different species of coffee (Charrier, 1978; Mazzaferra and Carvalho, 1992).

Purine alkaloids in plants may have ecological significance, by influencing the interrelation between organisms or by improving adaptation to unfavorable environmental conditions (Suzuki *et al.*, 1992). The activities of the *N*-methyl transferases were maximum during the first stage of emergence of the leaflets on *C. arabica*. When the leaves enter the phase of rapid expansion both the relative enzyme activities and the purine alkaloid concentrations decrease concomitantly. The nutritive value of the tissue is proportionately diminished (Waldhausser *et al.*, 1997a).

Plant tissue and cell cultures are suitable systems for studying the metabolism of caffeine (Buckland and Townsley, 1975). Waller *et al.* (1983) reported that the caffeine is produced by callus and excreted into the medium and that brown callus (older) produced less caffeine than white callus. In recent years there has been an increased interest in the regeneration of coffee through somatic embryogenesis on the one hand and an increase in the consumption of caffeine free drinks. As a prelude to the development of transgenic coffee plants that are caffeine free it was of interest to record the levels of caffeine during somatic embryogenesis. Since there is a similarity between germination of the seed and that of the somatic embryos, caffeine levels were compared during the two processes.

The objectives of the present research were:

- To improve the speed and percentage of germination of coffee seeds by scarification treatment with sulphuric acid
- Effect of gibberelic acid in coffee seed germination and seedling growth
- Analysis of caffeine. The following tissues were studied for caffeine content:
    1. Different parts of the plant like leaves, seeds etc.
    2. Different varieties of *Coffea* seeds and hybrids

3. *In vitro* callus cultures
4. Coffee seedlings (effect of light on chlorogenic acid content was also analyzed)
5. Somatic embryos (different developmental stages)

Other caffeine related metabolites like, 7-methylxanthine, theobromine and theophylline were also analyzed in seedlings and somatic embryos.

4.2. MATERIALS AND METHODS:

4.2.1. Seed material:

Fresh ripened red colored coffee fruits i.e. *C. canephora* and *C. arabica* were collected from coffee growing areas in Mysore (Fig. 4.1). The seeds either were extracted freshly from the fruits (Fig. 4.2) or were stored along with the testa for one month under laboratory conditions for different experiments. Hard testa and silver skin (Fig. 1.12, chapter I) were removed manually before sterilization.

4.2.2. Sterilization of coffee seeds:

The seeds were washed for 3 minutes in 70% alcohol followed by water. They were then sterilized in 0.2 % (w/v) mercuric chloride ($HgCl_2$) solution for 10 min and washed five times in sterile distilled water. The dry seeds were soaked in sterile distilled water and kept on shaker (98 rpm) in medium light for one day. Water presoaking treatment of the seeds for 24 to 48 hours has proven to be an effective way to advance germination and to increase germination capacity, and is claimed to harden seedlings, which grow faster and more vigorously when planted in the open sun (Guiscafré, 1946).

4.2.3. Sulphuric acid treatment of coffee seeds:

After soaking, the seeds were treated with sterile sulphuric acid solution (0.23, 0.46, 0.93, 1.40, 1.87 and 2.81M). In each

concentration, 10 seeds were treated for 1.5 min. Later the seeds were immediately washed with sterile distilled water thrice. The blotted seeds were inoculated on to 1/4th MS basal medium (Murashige and Skoog, 1962) prepared in culture bottles (3 seeds/bottle) and incubated in dark at 25±2^{0}C.

4.2.4. Gibberelic acid (GA_3) pretreatment of coffee seeds:

Seeds extracted from the fresh fruits were with a slimy coating (Fig. 4.2). Just below the slimy surface, there is a very hard testa (Fig. 1.12). Acid treatment was given to these seeds with 9 M sulphuric acid for 1.5 min with vigorous shaking. Later the seeds were thoroughly washed thrice with tap water with vigorous shaking to remove slimy material completely. The seeds were blotted dry (Fig. 4.3) and testa along with silver skin was removed (Fig. 4.4). Germination is delayed by the hard parchment (testa) and this effect is most likely due to its relative impermeability to water (Bendaña, 1962), though Huxley (1964) suggested that it restricts the passage of oxygen to the internal tissues. It seems, however, that the parchment (testa) does not constitute a mechanical constraint upon the growth of the embryo (Bendaña, 1962; Huxley, 1964). Parchment removal is a standard procedure in germination tests (Huxley, 1965), and is also recommended to speed up germination as a practice to raise coffee seedlings (Suárez de Castro, 1960). They were sterilized and then treated with gibberelic acid (0.07, 0.14, 0.22 and 0.29 mM) by soaking the seeds in GA_3 solution for 36 hr in dark after sterilization. After the pretreatment, the seeds were dried by blotting on filter paper and inoculated on to 1/4th MS basal medium (Murashige and Skoog, 1962) prepared in culture bottles (3 seeds/bottle). Twelve seeds were used for each treatment including control that did not receive GA_3 treatment. The cultures were kept in dark at temperature 25± 2 ^{0}C.

Figure 4.1: Fresh ripened fruits of *C. arabica* (bar 10 mm)

Figure 4.2: Coffee seeds extracted freshly from the fruits with slimy surface (*C. arabica*.) (bar 10 mm)

Figure 4.3: Acid washed coffee seeds with testa. No slimy material remains on the surface (*C. arabica)* (bar 10 mm)

Figure 4.4: Coffee seeds in which hard testa and silver skin have been removed (*C. arabica*) (bar 10 mm)

4.2.5. Scanning electron microscopy of sulphuric acid treated coffee seeds:

Explants were fixed overnight in a buffered 2% gluteraldehyde and 0.5% paraformaldehyde (pH 7.5, 50 mM phosphate buffer) and dehydrated through an alcohol series (10% to absolute alcohol). They were dried and mounted on stubs and gold coated (Polaron SEM coating system) and were viewed using a LEO 435 VP scanning electron microscope (LEO Electron Microscope Ltd, Cambridge, England) at a magnification of 500-1000 X.

4.2.6. Plant material for caffeine estimation:

4.2.6.1. Leaf:

Leaf samples were obtained from one year old greenhouse grown plants (S-274). The developmental stages of the leaves were categorized as (i) first leaves, (ii) second leaves and (iii) third leaves from shoot apex. Young leaves or the first leaves were ca. 3.5 cm long and 1.5 cm in width. They were very glossy, shining with light green in colour. The second leaves were greener than the first and were ca. 14.8 cm long and 6.8 cm in width. The third leaves were dark green in colour and slightly longer than the second and were ca. 16 cm long and 6.7 cm in width.

4.2.6.2. Seeds:

Embryos were extracted from the fresh endosperm (S-274) with a scalpel and a needle. It has been reported that with the exception of the embryo site, the caffeine is homogeneously distributed within the endosperm (Baumann *et al.*, 1998). Ripened/unripened fruits were obtained from trees of Old Robusta, C x R, *Coffea bengalensis, C. salvatrix, C. eugenioides***,** Hybrido de Timor, Paradenia robusta, Kaggenhalla robusta, *C. canephora* (Uganda robusta), and from the hybrids of Racemosa x Robusta, Liberica x Eugenioides, Robusta x

Wightiania, Congensis x Robusta and Robusta x Travancorensis during the months of September-October from Central Coffee Research Institute, Balehonnur, Karnataka, India. Seeds were extracted from them by removing pulp and hard endocarp (testa) manually. Silver skin was removed using a scalpel and 100-150 mg of tissue was excised from the endosperm with a scalpel for the extraction of caffeine.

4.2.6.3. Seedlings:

The seeds (CxR) were germinated *in vitro* in ¼ MS solid medium after the scarification treatment with 2.81 M sulphuric acid and seedlings were obtained by 30-60 days. Folded cotyledons were ca. 1.1 cm broad and ca. 0.77 cm long and the hypocotyls were ca. 3.93 cm long with roots of around 5-6 cm. The expanded cotyledons were ca. 3.4 cm broad and ca. 2.5 cm long. They were having hypocotyls of length ca. 8.4 cm and the roots were 8-9 cm long.

4.2.7. Callus culture method:

The excised hypocotyl from the *in vivo* germinated seedlings were surface sterilized as mentioned in the Chapter II. The hypocotyl segments were cut into pieces ca. 0.75 cm long. In one experiment (Table 4.8) the calli of CxR, Old Robusta and S-274 were obtained from the callus development medium and modified callus development medium (Chapter II).

In another experiment, the hypocotyl explants (S-274) were transferred to 150 ml conical flasks containing 40 ml medium and incubated in light at 25±2°C. The medium contained Murashige and Skoog salts (1962) supplemented with B5 vitamins (Gamborg *et al.*, 1968), sucrose (3%) and agar 0.8%. Two combinations of growth regulators were tried for callus formation viz., 2.2 µM BAP containing 9 µM 2,4-D and 2.4 µM 2-iP containing 9 µM 2,4-D. Three types of

calli were formed in these media viz., white spongy, yellow friable and green hard. White callus appeared generally after two weeks. Yellow and green callus started forming after one and a half months. The callus along with the hypocotyl explant was subcultured once after 45 days. Vigorously growing fresh calli were used for caffeine estimation.

4.2.8. Somatic embryogenesis:

Embryogenic callus of CxR obtained from hypocotyls in modified callus development medium as mentioned in the Chapter II was inoculated in embryo germination medium (Table 2.3; Van Boxtel and Berthouly, 1996). Somatic embryos, which later developed into plantlets, were obtained when the embryogenic callus was incubated for about two and a half months. Embryos of different stages of development as well as callus were collected at the same time and estimated for caffeine. Fresh tissue (20-30 mg) was used for the extraction.

4.2.9. Sample preparation for HPLC for caffeine estimation:

For seed analysis, the endocarp (see Fig. 1.12, Chapter I) was peeled off manually and the silver skin was removed with a scalpel. The endosperm was excised with a scalpel. Samples (100-150 mg) were ground to fine slurry with a mortar and pestle and using neutralized sand in 80% ethanol (9ml total extract including the washing step). The slurry was centrifuged and the supernatant was flash-evaporated to dryness. The residue was redissolved in 1 ml of 80% ethanol, transferred to eppendorf tubes and kept in -80°C until the analysis was done. An aliquot of 10 µl was generally used for HPLC analysis. Leaves and other *in vitro* samples like callus, embryos of different stages and plantlets were also estimated for caffeine in the same manner.

4.2.10. Chemicals and solvents:

Distilled alcohol was used for extraction and distilled, deionized water was used to prepare aqueous solutions. The solvents (HPLC grade) were degassed under vacuum prior to use for HPLC. All standards were purchased from Sigma Chemical Co. (St. Louis, MO). Standards were prepared at concentrations of 1 mg ml^{-1} (caffeine and theophylline), 0.3 mg ml^{-1} (theobromine) and 0.1 mg ml^{-1} (7-methyl xanthine) in 80% ethanol.

4.2.11. HPLC analysis of caffeine:

Isocratic method of analysis was followed when only caffeine was estimated whereas gradient method was used to estimate other metabolites like 7-methylxanthine, theobromine and theophylline, in the caffeine pathway along with caffeine.

4.2.11.1. Isocratic method:

Caffeine analysis was carried out by high-performance liquid chromatography (HPLC; Shimadzu LC-10) by isocratic method on a *μ*-Bondapak C_{18} column, (3.9 x 30 cm, and 10 μm, Pharmacia) with a solvent system of acetonitrile:water (8: 92, v/v) or a 5 μ SGE ODS column (250 x 4.6 mm i.d.) (SGE Laboratory Accessories PVT Ltd., Mumbai, India), with 1% tetrahydrofuran (THF) in 30% MeOH, at a flow rate of 1 ml min^{-1} (Blauch and Tarka Jr., 1983). In the former column/solvent combination, caffeine peak appeared at around 8 min., while the later gave the peak at 7^{th} min. The injection system used a 20 μl Rheodyne loop. Caffeine eluting from the column was monitored with an on-line UV monitor operating at 245nm with a sensitivity of 0.08 AUFS, and the signals were integrated in a Shimadzu C-R6A Chromatopac integrator. The data processor was set with a chart speed of 2.5 mm min^{-1}. Values were expressed as dry

mass basis. Each sample was extracted and analyzed thrice with similar results.

4.2.11.2. Gradient method:

Solvents were delivered at flow rate of 1 ml min^{-1}. Samples were injected using Rheodyne injector of loop capacity of 20µl. Reversed phase HPLC utilized a 5 µ SGE ODS column (250 x 4.6 mm i.d.) (SGE Laboratory Accessories PVT Ltd., Mumbai, India). The alkaloids were eluted from the column with a 30 min. gradient of 40% methanol in 50 mM NaOAc (sodium acetate), pH 5.0 (Ashihara *et al.*, 1995). Column eluate was directed to a detector Model Shimadzu SPD-6AV UV-VIS spectrophotometric detector and data processing was done with a data processor Model Shimadzu CR-4A operating at 270 nm. The another system used was LC-10AT *VP* liquid chromatograph having SPD-10AT *VP* detector, SCL 10AT *VP* system controller and data processing was done with a PC based data acquisition and processing system having class-VP software. Peak identification was achieved by comparing retention time (R_t) of authetic standards. The R_t values (min) were as follows: 7-methylxanthine (15), theobromine (19), theophylline (22) and caffeine (26) (Fig. 4.11).

4.2.12. Chlorogenic acid staining:

CxR seeds were germinated (See Chapter 3 for method) both in dark and light conditions. The dark grown seedlings were completely bleached (Fig. 14).

The method of Reeve (1968) was followed for the staining. If catechol or chlorogenic acid were first treated with HNO_2 (nitrous acid), in the absence of copper, and then with dilute alkali a deep cherry-red colour was produced.

Separate aqueous solutions of 10% sodium nitrite, 2% urea, 1% glacial acetic acid and 2% sodium hydroxide were prepared.

The leaf, hypocotyl and root sections were made using a blade. Mixed aliquots of the first three reagents in a glass petriplate containing sections and allowed the initial yellow colour to develop (2-4 minutes). Added an aliquot of 2% NaOH to it. An intense red colour was developed.

4.3. RESULTS AND DISCUSSION:

4.3.1. Sulphuric acid treatment of coffee seeds:

The treatment was given both to fresh and stored seeds. There was no marked difference in germination (Fig. 4.5) between *C. canephora* and *C. arabica* seeds.

Scarification treatment given to the *C. canephora* seeds with different concentration of H_2SO_4 was highly effective in enhancing germination percentage compared to controls (Table 4.1; Table 4.2; Fig. 4.6). Scarification helps in faster germination and better seedling growth. Almost 100% of seed germination and good seedling length was noticed in samples treated with 2.81 M H_2SO_4 by four weeks itself. There was a gradual increase in the length of the seedlings (hypocotyls) starting from 0.97±0.26 cms (controls) to 4.58±1.28 cms (2.81 M H_2SO_4) in 8 weeks. Browning was more severe in controls. By eight weeks time almost 70% of the seeds showed browning even if they germinated. There was noted reduction in browning even in the 0.23 M sulphuric acid treated samples and no seeds turned brown in any of higher concentrations of acid treatment. The main reason for freshness of seeds and higher germination percentage rate seems to be due to the reduction in oxidized phenolics present in the seeds due to the scarification treatment. Rooting was also more efficient in higher acid concentrations (1.40 M; 1.87 M and 2.81 M H_2SO_4). Cotyledons fully opened in 67% of the seedlings treated with 2.81 M H_2SO_4 , by eight weeks time.

Figure 4.5: Different stages of germination of coffee seeds. In the folded cotyledonary leaf stage, the seeds were germinated but the cotyledons were not expanded, and remained inside the peripheral layers of the seed and in the expanded state, the two cotyledons were fully opened (bar 12 mm)

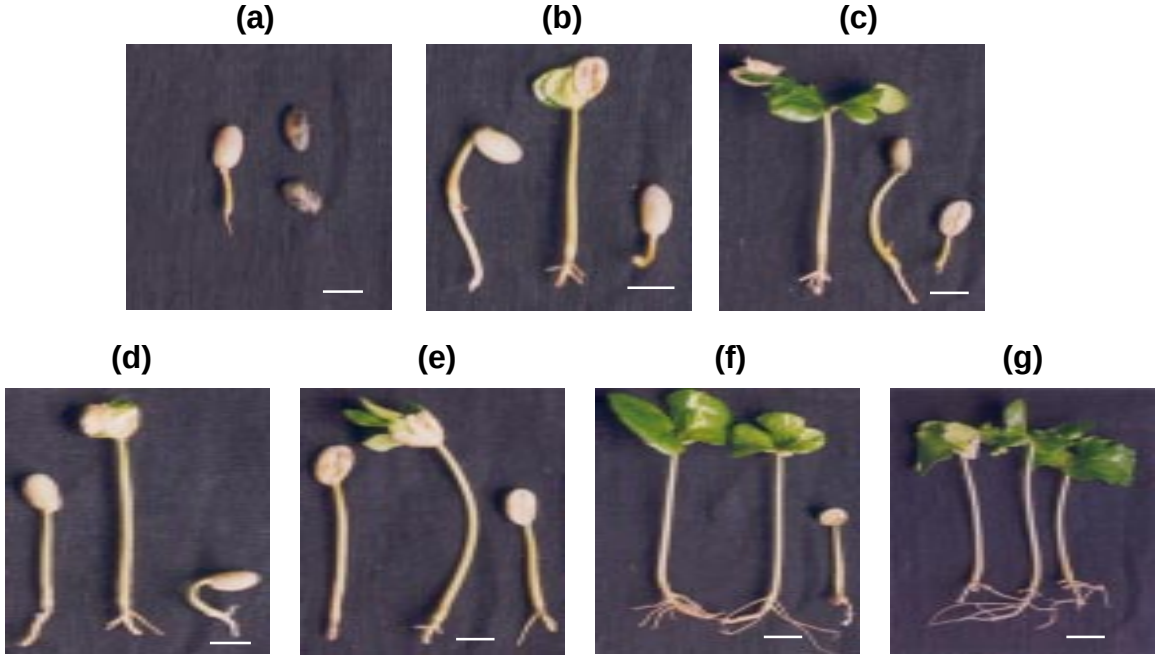


Figure 4.6: Coffee seedlings (*C. canephora*) showing the effect of sulphuric acid treatment in germination after 8 weeks growth in 1/4th MS basal medium (bar 12 mm)
a. Control seeds with no acid treatment;
Seeds treated with 0.23 M(b); 0.46 M (c); 0.93 M (d); 1.40 M (e); 1.87 M (f); 2.81 M (g) H_2SO_4

Table 4.1. Effect of sulphuric acid treatment on fresh *C. canephora* seed germination *in vitro*

H_2SO_4 (M)	Incubation period (weeks)	Seed germination (%)	Average seedling length (cms)	Browning (%)	Rooting efficiency
Control	2	35	-	-	-
	4	41.17	0.56±0.13	5.8	-
	8	82.35	0.97±0.26(h*)	70.58	-
0.23	2	50	-	-	
	4	62.5	0.87±0.59	-	-
	8	81.25	1.6±0.90(h)	12.5	+
0.46	2	57.14	-	-	-
	4	100	0.9±0.67	0	++
	8	100	1.95±0.94(h)	0	++
0.93	2	62.5	-	-	-
	4	87.5	1.26±0.56	0	++
	8	100	2.06±1.06(h)	-	++
1.40	2	85.71	-	-	-
	4	100	0.92±0.76	0	++
	8	100	2.84±2.00(h)	0	+++
1.87	2	46.66	-	-	-
	4	93.33	1.80±0.70	0	+++
	8	100	3.98±1.77(h)	0	+++
2.81	2	86.66	-	-	-
	4	100	1.97±0.63	0	+++
	8	100	4.58±1.28(h)	0	+++

* Length of hypocotyl; - not measured; +, > 1 cm; ++, 1-3 cm; +++, <3 cm with branches

Table 4.2. Effect of sulphuric acid treatment on stored *C. arabica* seed germination *in vitro*

H_2SO_4 (M)	Incubation period (weeks)	% of seed germination	Average seedling length (cms)	% of seeds showing browning	Rooting efficiency
Control	3	40.0	0.5±0.0	20.0	-
	4	40.0	1.0±0.0	20.0	+
0.23	3	30.0	0.23±0.04	50.0	-
	4	60.0	0.47±0.06	20.0	+
0.46	3	40.0	0.47±0.11	20.0	-
	4	60.0	0.98±0.18	20.0	+
0.93	3	60.0	2.20±0.21	10.0	-
	4	70.0	2.23±0.99	20.0	++
1.40	3	70.0	2.21±0.24	10.0	-
	4	70.0	2.42±1.08	10.0	++
1.87	3	70.0	2.28±0.93	-	-
	4	70.0	3.35±1.30	10.0	+++
2.81	3	100.0	2.15±1.0	-	-
	4	100.0	3.2±0.23	-	+++

- not measured; +, > 1 cm; ++, 1-3 cm; +++, <3 cm with branches

The scarification treatment given to the stored (one-month-old) *C. arabica* seeds was highly efficient in enhancing germination percentage compared to controls and for better seedling growth. Even at lower concentration of H_2SO_4 (0.93 M) used for scarification, 2.23 cm length seedlings were produced by one month. Upto 1.40 M and in controls 10-20% of seeds partially germinated. Similarly, 10-20% of partially germinated or nongerminated seeds turned dark brown and eventually dead. This may be due to the leaching out of the oxidized phenolic compounds present in the seeds, which might have happened because of the stress induced by the sterilization treatment.

Since stored seeds are more prone to contamination than freshly collected ones, harsh sterilization measures are required to get sterile cultures. It has to be assumed that 2.81 M sulphuric acid may help to make the cell wall more permeable to water, leading to better absorption and thus faster germination. Fully-grown germinated seedling with expanded cotyledons and roots were obtained after 7-8 more weeks of incubation in the same medium.

Almost similar trend was observed in both the varieties in terms of percentage of germination and seedling length. The percentage of germination in the acid treated seeds was high. Storage of seeds affects the percentage of germination irrespective of acid treatment. The seedling length was less in canephora compared to arabica variety in the first four weeks. However, they showed vigorous growth afterwards and obtained seedlings with 4.58 cm long hypocotyls by 8 weeks time in the 2.81 M acid treated ones. Browning percentage was more in the stored seeds. Even at 1.87 M acid slight browning was observed, while in fresh seeds browning observed only in 0.23 M. There was significant reduction in browning due to acid treatment.

When seeds were germinated in different culture vessels viz., 150 ml conical flasks and 200 ml jars, there was significant difference in the growth of seedlings. The growth was less in conical flasks compared to those grown in jars. In two months, 43% of the seedlings grown in jars reached the length in the range of 4.1-5 cm (4.59 ± 0.29) while it was only 10% in the case of seedlings grown in flasks. There were no seedlings with more than 5 cm height in the flasks even after 8 weeks. One reason for the difference in growth may be the relatively high humidity maintained in the bottles.

4.3.2. Scanning electron microscopy of sulphuric acid treated seeds:

The control and sulphuric acid treated seeds were examined under scanning electron microscope. The external surface of the epidermal cells showed that these cells were relatively elongated (Fig. 4.7) but there were no difference in the structure between the treated and non-treated ones except in the alignment of the long cells. In the control, they were found to be arranged more regular while in the acid treated ones, the cells found to be spread (Fig. 4.8 a & b). So it could be assumed that the acid treatment does not cause any major structural changes on the seed surface. Dentan (1985) reported that the seed surface was covered with folded waxes when examined under the scanning electron microscope (Dentan, 1985). We could not observe any waxy covering (Fig. 4.8a). On the reverse sides of the seed cuttings (internal view), the parenchymatous cells seemed to be expanded in the acid treated samples, while in the controls the cells were found to be constricted (Fig. 4.9a & b).

4.3.3. Gibberelic acid treatment of coffee seeds:

When fresh seeds were subjected to gibberelic acid (GA_3) treatment, at all concentrations of GA_3, seed germination was slightly prevented in the initial four weeks and at higher concentrations (0.14-0.29 mM) it was found to be inhibitory to seedling growth itself with less seedling length compared to the controls. Nevertheless, 100% seed germination was observed in all GA_3 treatment samples by four weeks time except in 0.22 mM, which showed full germination percentage by 11 weeks. When GA_3 at 0.07 mM was used for treatment the seedling length was slightly more (3.5±1.1) after 2 weeks incubation (Table 4.3), compared to controls (3.06±0.83).

Figure 4.7: SEM (Scanning electron microscopy) of epidermal cells. External view of the bean, showing the elongated epidermal cells at different magnifications, 50 x (a), 500 x (b) and 1 k x (c)

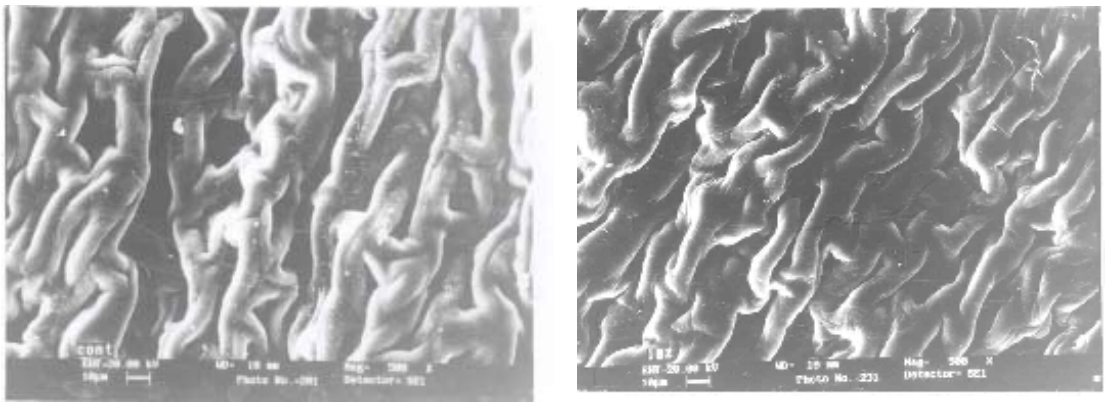


Fig. 4.8: SEM of external view of the seed surface
a. control b. 2.81 M H_2SO_4 treated seeds. The acid treatment does not cause any major structural changes on the seed surface.

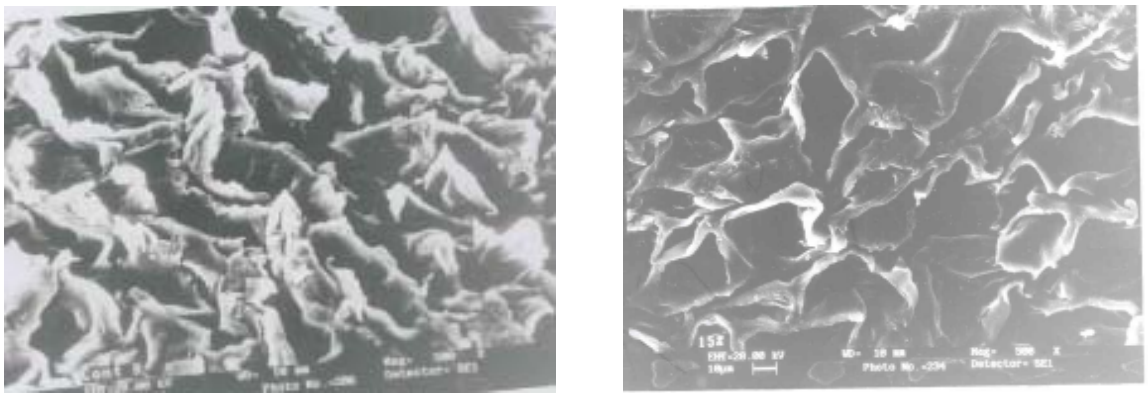


Figure 4.9: SEM of the internal view of the coffee seeds

a. control b. 2.81 M H_2SO_4 treated seeds

Table 4.3: Effect of Gibberelic acid (GA_3) treatment on *Coffea arabica* seed germination* *in vitro*:

GA_3 (mM)	Incubation periods (weeks)	Average seedling length (cms)	% of partially germinated seeds	% of seedlings with fully opened cotyledon	Rooting efficiency
Control	2	3.06±0.83	-	-	-
	4	7.11±1.78	-	8	++
	11	3.67± 0.49 (hyp. only)	-	83	+++
0.07	2	3.50±1.12	8.3	-	-
	4	7.12±1.35	-	42	++
	11	3.60±0.47	-	92	+++
0.14	2	2.60±0.32	8.3	8	++
	4	3.77±2.38	-	8	++
	11	3.09±0.89	-	75	+++
0.22	2	1.6±0.48	28.6	-	-
	4	4.63±1.39	8.3	-	+
	11	2.67±0.94	-	58	+++
0.29	2	2.30±1.26	16.7	-	-
	4	4.32±1.73	-	25	-
	11	2.71±1.04	-	58	+++

*Seed germination was 100% in all treatments including controls by 11 weeks; +, > 1 cm; ++, 1-3 cm; +++, <3 cm with branches

However, by next 4 weeks of incubation the seedling length of both controls and 0.07 mM samples were almost identical. After 11 weeks incubation, complete seedlings (92%) with fully opened cotyledonary leaves and rooting were established in 0.07 mM GA_3 treatment, which was higher than controls (83%). There was a gradual reduction in the total seedling length as the GA_3 concentration increases in four weeks time. Ramaiah and Venkataraman (1988) reported that pre-sowing soaking of seeds in GA_3 (0.29 mM) prior to

sowing hastened the germination by about 10 days in field trials. In our study 100% germination was observed in the control in two weeks time. All the treated samples showed 100% germination later, by 4-11 weeks. We found that high doses of GA_3 suppressed the seedling growth clearly indicated by the reduction in length and also less percentage of seedlings with opened cotyledons. Most treatments to speed up germination in coffee were innocuous or injurious (Barros *et al.*, 1999). Gibberellic acid (GA_3) inhibits germination (Maestri and Vieira, 1961; Gopal and Ramaiah, 1971; Válio, 1976; Takaki *et al.*, 1979; Takaki and Dietrich, 1980). As GA_3 enhances the hydrolysis of polysaccharides, it was suggested that the resulting mannose inhibits germination through inhibition of the growth of the embryo (Takaki and Dietrich, 1980). Since the growth of isolated embryos is not inhibited by GA_3 (Válio, 1976), an indirect effect of this hormone in germination inhibition may occur (Takaki and Dietrich, 1980).

4.3.4. Level of caffeine in leaves:

The amount of caffeine present in first leaf, second leaf and third leaf from the shoot apex of mature plant; and in parts of seeds, like endosperm and embryo has been estimated using an HPLC method (Table 4.4). HPLC chromatogram of caffeine obtained when an extract from first leaves analyzed is shown in Fig. 4.10.

Table 4.4: Caffeine estimation in the mature plant parts and seeds (S-274):

Sample	Amount of caffeine
1st leaf	1.81 ± 0.21
2nd leaf	0.67 ± 0.11
3rd leaf	0.63 ± 0.05
Embryo	3.58 ± 1.41
Endosperm	1.481 ± 0.14

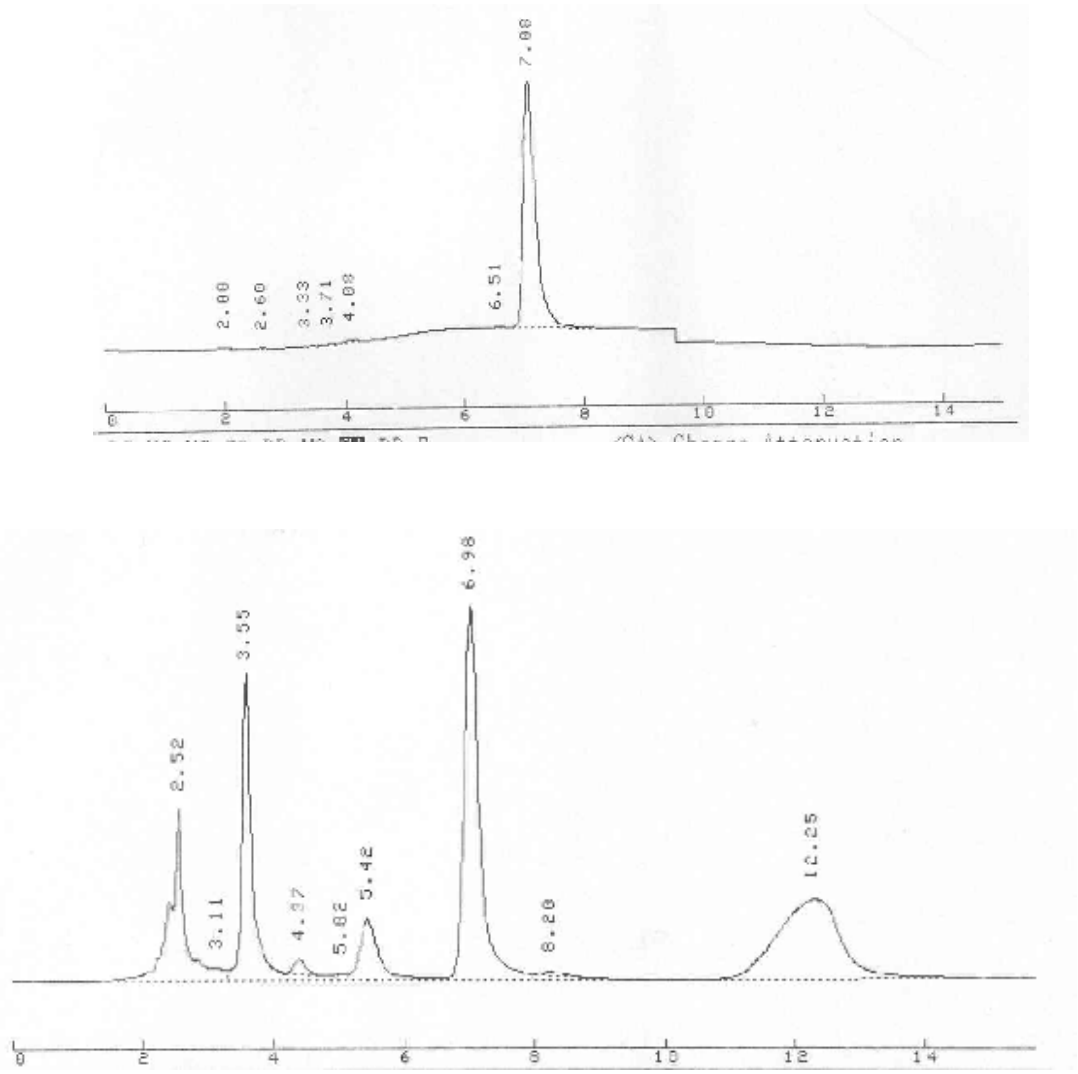


Figure 4.10: Reversed-phase HPLC analysis of caffeine in 80% EtOH extract from the first leaf of *C. canephora* (S-274). Column: 5µ SGE ODS column (250 x 4.6 mm i.d.). Mobile phase: 1% THF in 30% methanol. Flow rate: 1ml/min. Detector: Model Shimadzu SPD-6AV UV-VIS spectrophotometric detector. Absorbance: 270 nm. Samples: A. standard, caffeine. B. 5µl extract.

Tender tissues had higher caffeine content and there was gradual reduction in caffeine as the tissue matures. The youngest leaves contained the highest concentration of caffeine (1.81 ± 0.21%). The second and third pairs of leaves showed around one-third reduction in the caffeine level (0.67±0.11% & 0.63±0.05% respectively). Similar observation was reported by Ashihara *et al.* (1995). Buds and young leaves contained highest concentration of caffeine with about half as much being detected in mature and aged leaves. In a study conducted by Koshiishi *et al.* (2001) where methylations of putative caffeine precursors by tea leaf segments was demonstrated *in situ* using methyl-^{14}C-labelled methionine and SAM (*S*-adenosyl methionine), more than 50% of radioactivity was taken up by the segments of young tea leaves and was incorporated into caffeine. The incorporation was greatly reduced in mature and aged leaves. The activities of 7-*N*-methyl transferase and caffeine synthase was high in young tea leaves where active caffeine biosynthesis takes place than in mature and aged leaves. The activities of the most of the enzymes such as *S*-adenosyl homocysteine (SAH) hydrolase, 7-*N*-Methyl transferase and caffeine synthase were higher in young leaves, where active caffeine biosynthesis takes place, than in mature and aged leaves (Koshiishi *et al.*, 2001). Adenine and guanine derived from the nucleotide pool are the primary precursors of caffeine in coffee (Suzuki and Waller, 1984b). Thus, its level might be related to general metabolic activity and tissues such as young leaves, flowers, and immature fruits should be expected to contain more caffeine than mature or old tissues (Raju and Gopal, 1979; Frischknecht *et al.*, 1986;Clifford and Kazi, 1987).

4.3.5. Level of caffeine in seed parts:

Caffeine level was estimated in different parts of the seed. The embryo contained higher level of caffeine (3.58±1.41%; Table 4.4).

Endosperm was found to have considerable amount of caffeine (1.48%). In average, embryo represents only ca. 0.5% of the seed and the remaining 99.5 % of the embryo is occupied by endosperm. It is the endosperm that contributes maximally to the caffeine of the seed.

4.3.6. Caffeine in different varieties of *Coffea*:

The amount of caffeine present in endosperm of different varieties of *Coffea* as well as its hybrids was determined by reverse-phase HPLC. A quantitative estimate of the amounts of caffeine in endosperm from immature and mature fruits of various species of *Coffea* and different hybrids is presented in Table 4.5.

Table 4.5: Caffeine level in endosperm of different varieties of *Coffea*

Name of the variety	Immature	Mature
Coffea.bengalensis	0.06±0.01	-
C. salvatrix	0.07±0.04	-
C. eugenioides	0.46±0.04	0.69±0.11
Hybrido de Timore	1.52±0.17	1.37±0.18
C. racemosa	-	0.98±0.06
Paradenia robusta	-	1.60±0.10
Kaggenhalla robusta	4.08±0.18	2.27±0.15
C. canephora (Uganda robusta)	5.43±0.92	3.29±0.13
Old Robusta	6.57±1.73	3.48±0.42
C. racemosa x *C. canephora* (Line-1)	-	0.82±0.07
C. racemosa x *C. canephora* (Line-2)	-	1.25±0.07
C. canephora x C. wightiana	3.78±0.31	2.95±0.18
Congensis x Robusta (CxR)	3.64±0.88	3.33±0.17
C. canephora x C. travancorensis	2.53±0.13	3.56±0.17

The content of caffeine varies among species between 0.06 - 3.56%. Higher levels of caffeine are found in diploid species. Estimates of endogenous contents of caffeine are in agreement with data reported in the literature (Raju and Gopal, 1979; Mazzafera *et al.*, 1991). Immature fruits of *C. bengalensis* (0.06±0.01) and *C.salvatrix* (0.07±0.04%) contain the lowest amount of caffeine while those of Old Robusta (3.48±0.42%) and *C. canephora* collection from Uganda (3.29±0.13%) contain the highest. The caffeine content of immature fruits is generally higher than that in mature fruits as also reported by Roberts and Waller (1979). The caffeine content of immature and mature fruits in *C. eugenioides*, Hibrido de Timor, *C. canephora* x *C. wightiana* and Congensis x Robusta was similar. *Congensis* is a species with compact bunch size and bean quality better than *C. canephora* but is a poor yielder (Srinivasan, 1996). S-274 robusta (*C. canephora*) is a high yielder with bold bean character. The interspecific hybrid Congensis x Robusta (CxR) is popular because of compact bunch size, bold bean character and superior cup quality (Srinivasan, 1996). Caffeine was not detected in extracts from mature fruits of *C. bengalensis* (Mazzafere *et al.*, 1991). *C. bengalensis* is not suitable for production of naturally decaffeinated coffee because of the form of the plant coupled with low seed production and poor quality of the resultant beverage (Mazzafera *et al.*, 1991).

Mature seeds from different selections of the hybrid *C. racemosa* x *C. canephora* contain different levels of caffeine ranging from 0.82±0.07% and 1.25±0.07% (mature seeds). The difference might be due to difference in caffeine biosynthesis rate (Mazzafera *et al.*, 1994b). *C. racemosa* seeds contain 0.98±0.06% caffeine while *C. canephora* seeds are rich in caffeine (3%). *C. racemosa* x *C. canephora* F1 hybrids are highly sterile, produce very little pollen and subsequent poor seed set (Ram *et al.*, 1981). The seeds gave rise to

arabica like plants. Some of which are tetraploids and self-compatible (Reddy *et al.*, 1991). These tetrapoloid lines contain as much caffeine as do the arabica lines (0.8%-1.3%). *C. wightiana* and *C. travencorensis* were said to be caffeine (Sreenath *et al.*, 1992). However, they were observed to produce very low levels of caffeine in the present study. Parthenogenetic development occurs in *C. canephora* x *C. travancorensis* and *C. canephora* x *C.wightiana* and the resulting seeds resemble *C. canephora* (Sreenath *et al.*, 1992). Interspecific hybridization therefore appears one way of lowering caffeine content in seeds but not in all cases.

Caffeine content of coffee fruits rapidly increases in seeds of age 2.5-4 months (immature seeds). When L- [$^{14}CH_3$]-methionine (which is the precursor of SAM) was fed to detached coffee fruits, the results showed that the methyl group of L-methionine is actively incorporated into the caffeine of coffee fruits during the ripening process (2-3 months old). However, in older fruits (7-8 months old), addition of theobromine enhanced caffeine formation, which suggests a shortage of dimethylated xanthine substrates at this stage of development, in young fruits theobromine is readily available (Roberts and Waller, 1979).

4.3.7. Inheritance of caffeine in interspecific hybrids:

Experiments were conducted to find out how caffeine is inherited in interspecific hybrids and to assess the reduction of caffeine in the endosperm after breeding (Table 4.6). Caffeine was estimated in seed samples of the allotetraploid derived from *C. liberica* X *C. eugenioides* i.e. Ligenioides, Hibrido de Timor (HDT), their F1 (S.4595), four F2 families (S.4696, S.4697, S.4698 and S.4699) and two back cross (BC) families S.4700 (BC to HDT) and S.4702 (BC to Ligenioides).

HDT is a natural hybrid of robusta and arabica, spotted in Timor Island. It was introduced to India during 1961. After screening for resistance at Coffee Rust Research Centre, Portugal, it was found to possess highest vertical resistance to rust among arabica types. It is a moderate yielder (Srinivasan, 1996). Ligenioides (1.30%) is a tetraploid and contains caffeine at an intermediate level between that of the parents i.e., *Coffea liberica* (2.01±0.06%) and *C.eugenioides* (0.69±0.11%), which are diploids. *C. liberica* is having higher caffeine content and shows higher disease resistance and resistance to pests like stem borer. Eugenioides have large field tolerance to diseases, draught tolerance, tolerance to nematodes and leaf miner. Ligenioides also has resistance to diseases like rust. Eugenioides is the female progenitor of arabica. Liberica is very closely related to robusta and the caffeine level (2.01±0.06%) comes almost equal level to that of robusta.

Table 4.6: Estimation of caffeine in the inbred lines:

Variety	Specification	Caffeine level	Comments
Coffea liberica	Grand parent	2.01±0.06	Diploid
C.eugenioides	Grand parent	0.69±0.11	Diploid
Ligenioides	Parent	1.30±0.27	Tetraploid
Hibrido de Timore(HDT)	Parent	1.37±0.18	Tetraploid
4595	F1	1.55±0.23	Tetraploid
4700	F1 X HDT	1.65±0.18	Tetraploid
4702	F1 X *C.ligenioides*	1.54±0.20	Tetraploid
4696	F2	1.61±0.20	Tetraploid
4697	F2	1.56±0.32	Tetraploid
4698	F2	1.50±0.24	Tetraploid
4699	F2	1.57±0.21	Tetraploid

Ligenioides and HDT possess about 1.3% caffeine in their seeds. However, their F1, F2 and BC families manifested slightly higher caffeine ranging from 1.50%-1.65%. Even this caffeine content value is not much higher than that of *C. arabica* (0.6%-1.5%). Thus, it appears that caffeine content in tetraploids is stable in this range and may not vary significantly. Possibly caffeine levels can be further decreased by using an arabica selection reputedly carrying low levels of caffeine (<1.0%) as one of the parents in crossing followed by appropriate selection of the progenies (Ram *et al.*, in preparation).

4.3.8. Caffeine production at different developmental stages of germination (CxR):

Caffeine content and other related compounds content in different parts of germinating seeds was estimated by reversed-phase HPLC (Table 4.7). Two stages of development were selected, i.e. folded and expanded. In the folded stage, the seeds were germinated but the cotyledons were not expanded and remained inside the peripheral layers of the seed while in the expanded state, the two cotyledons were fully opened (Fig. 4.5). Reversed-phase HPLC analysis of caffeine and other related compounds in 80% EtOH extract of the folded cotyledons of *C. canephora* (CxR) is shown in Fig. 4.11.

Embryos were extracted from dry seeds. The endosperm (2.62±0.12%) appears to have the same amount of caffeine as the embryo (2.05±0.11%). The level of caffeine in the cotyledons was the same in both developmental stages (5.29% and 5.38% in folded and expanded cotyledons respectively) *visa vis* the hypocotyls from the two different stages wherein there was marked reduction in the caffeine content in the expanded stage. Earlier studies have shown that the total amount of caffeine in expanded cotyledons remains unchanged as long as they stay green. In addition, the concentration of caffeine is more or less constant during the entire cotyledon lifetime that may last several months (Frischknecht *et al.*, 1986).

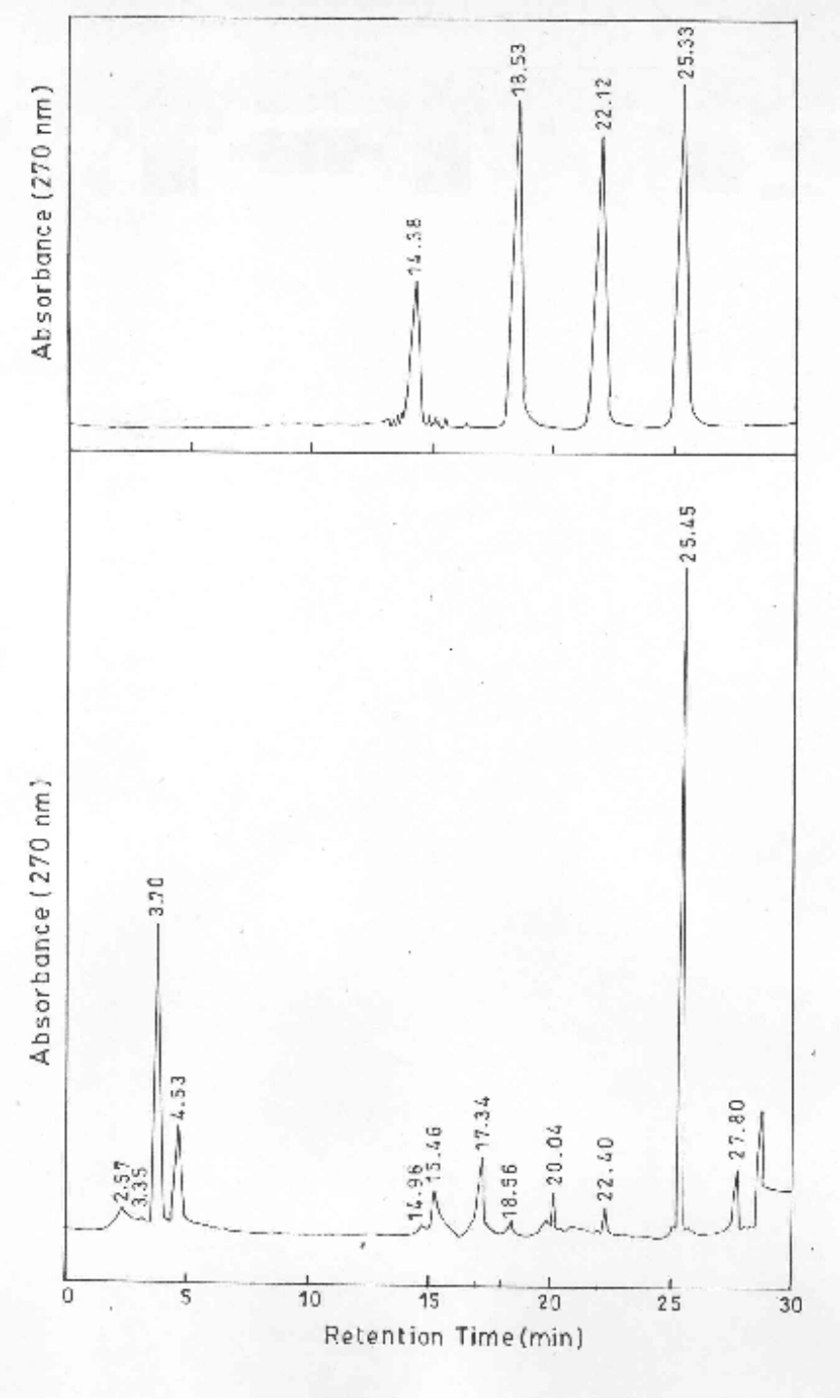


Figure 4.11: Reversed-phase HPLC analysis of caffeine and other related compounds in 80% EtOH extract of the folded cotyledons of *C. canephora* (CxR). Column: 5µ SGE ODS column (250 x 4.6 mm i.d.). Mobile phase: 30 min. 0-50% gradient of methanol in 50mM NaOAc, pH 5.0. Flow rate: 1ml/min. Detector: Model Shimadzu SPD-6AV UV-VIS spectrophotometric detector. Absorbance: 270 nm. Samples: A. standards (1) 7-methylxanthine, (2) theobromine, (3) theophylline and (4) caffeine. B. 10µl extract.

Table 4.7: Levels of caffeine and other metabolites at different stages of germination (CxR):

Stage	7-Methyl xanthine	Theobromine	Caffeine	Theophylline
Embryo	-	0.012±0.01	2.05±0.11	0.009±0.007
Endosperm	0.006±0.0	0.014±0.0	2.62±0.12	0.023±0.01
Folded cotyledon	0.051±0.024	0.055±0.002	5.29±0.19	0.124±0.046
Unfolded cotyledon	0.03±0.016	0.018±0.003	5.38±1.07	0.072±0.024
Folded, hypocotyls	0.076±0.074	0.099±0.044	4.08±0.77	0.007±0.005
Unfolded, hypocotyls	-	0.008±0.007	3.64±0.69	0.009±0.0
Folded, root	-	0.006±0.0005	0.1±0.05	0.008±0.004
Unfolded, root	-	0.005±0.001	0.06±0.02	0.005±0.001

Cotyledons were having the highest amount of caffeine. Baumann *et al.* (1998) reported that in general, the cotyledons had higher caffeine content (percentage dry weight) than the related endosperm, and they concluded that the relative caffeine increase was due to the loss of dry weight (dissimilation) during the endosperm-cotyledon transition, and that the endosperm caffeine simply permeates into the developing cotyledons. Therefore, the cotyledons were regarded as a "caffeine blot" of the seed. Hypocotyls also maintained relatively higher levels of caffeine. Hypocotyls from the folded stage contained the highest amount of 7-methylxanthine and theobromine while the levels of caffeine was not as high. All actively growing tissues showed the presence significant level of theophylline also, which is due to the active biosynthesis as well as degradation of caffeine in them (Ashihara *et al.*, 1995).

Our data indicates (Table 4.7) low, but measurable levels of caffeine in the root. Roots were not completely devoid of caffeine but

showed some amount of caffeine and other intermediates, which could be because of the assimilation of caffeine from the endosperm (Baumann *et al.*, 1998) as well as due to low level of caffeine biosynthesis in them.

So far, no study has been conducted on the distribution of caffeine in different parts of the germinating seedlings, which will be helpful for the experiments on regulation of caffeine biosynthesis either through breeding or genetic transformation. Baumann *et al.* (1998) conducted an experiment to compare the genetic variability of the caffeine content of *C. arabica* cv. laurina seeds (endosperm) by a non-destructive method that would allow caffeine determination of a single bean and thereafter, the germination of that same bean. In order to conserve the germination power of coffee beans, only a tiny portion of endosperm was excised for caffeine determination. With the exception of embryo site, the caffeine is homogeneously distributed within the endosperm. The study revealed a definite correlation between the caffeine contents of bean and cotyledons emerged from it. Simply analyzing the cotyledons for this character will facilitate breeding for low-caffeine coffee.

4.3.9. Caffeine production in callus:

The level and distribution of caffeine in the callus grown in two different media (i.e. callus development medium and modified callus development medium) was analyzed. The results are given in the table (Table 4.8). In our experiments this was a required preliminary prior to attempting the transformation of coffee and the analysis of caffeine in transgenic plants. Callus in the modified callus development medium produced more caffeine in CxR and S-274 varieties while Old Robusta showed a reverse trend. Different strains of *Coffea* differ markedly in their biotransformation capacity of theobromine to caffeine (Baumann *et al.*, 1983). The callus formed in modified callus

development medium was more compact in texture and greener in colour than the callus produced in callus development medium. Caffeine synthesis appears, as shown below (Table 4.8), more in greener tissue. It has been reported in tea plants that the presence of the activity of two of the three *N*-methyltransferases involved in the final two steps of caffeine biosynthesis is located in chloroplast (Kato *et al.*, 1998; Koshiishi *et al.*, 2001). The growth regulators also might play a role in caffeine regulation.

There are contradicting views regarding the formation of caffeine in *in vitro* cultures. Waller *et al.* (1983) reported that caffeine formation paralleled callus tissue growth. Caffeine is produced by callus and excreted into the medium and brown callus (older) produced less caffeine than white callus. They reported that the extract from vigorously growing cells in which active caffeine biosynthesis was occurring, exhibited *N*-methyl-N^9-nucleoside hydrolase and *N*-methyl transferase enzyme activities. Frischknecht and Baumann (1985) hypothesized that low stress culture conditions optimized for rapid cell growth are responsible for lack of accumulation of substantial amounts of secondary metabolites spontaneously.

Table 4.8: Caffeine levels in callus tissue formed in callus development (C) and modified callus development (mC) media:

Variety	Medium	Caffeine
C x R	C	0.30
C x R	mC	0.49
Old Robusta	C	1.76
Old Robusta	mC	0.91
S-274	C	0.59
S-274	mC	0.65

C- $^1/_2$MS+B5 Vitamins, 2-iP 9.8 µM, IBA 4.9 µM & 2, 4-D 2.2 µM; mC- $^1/_2$MS+B5 Vitamins, 2-iP 4.9 µM

In coffee this hypothesis does not seem to work regarding caffeine accumulation because callus showing no growth or production of considerable polyphenolic compounds, evidenced by a light to dark brown colour, produced much less caffeine that did cultures of white vigorously growing callus (Waller *et al.*, 1983). There are many factors involved in the regulation of caffeine biosynthesis and one of them could be organization of the cells.

In another experiment, hypocotyl explants were inoculated in two media differing only in the types of cytokinins i. e., BAP and 2-iP. Three types of calli were produced in these media, viz., white-spongy, yellow-friable and green-hard callus (Fig. 4.12). The caffeine levels in these calli are shown in Table 4.9. The white callus appeared to grow faster than the yellow callus. The green callii grew slowly. Caffeine content was much higher in the green calli than in the others. Compact or organized and slow growing cultures synthesize higher amounts of secondary products than do friable and rapidly growing cultures. Organization of cells is essential for normal cell metabolism to proceed. Such organization appears to be a major difference between high- and low- producing cultures (Haldimann and Brodelius, 1987). Cell suspension cultures of *C. arabica* with large aggregates show a higher production of methylxanthine alkaloids than cultures with finely suspended cells (Haldimann and Brodelius, 1987). The interesting point however is that in all our experiments caffeine levels were directly correlated with the greenness of tissue. The reason why green tissue contains more caffeine needs to be explored and may be related to greater synthesis, lesser catabolism or greater retention. There is definite indication as suggested above that there is greater synthesis in green tissue (Koshiishi *et al.*, 2001).

Figure 4.12: Three different types of calli viz., white spongy, yellow friable and green hard obtained from hypocotyl explants cultured in MS medium supplemented with 9 µM 2,4-D and 2.2 µM BAP for two months (bar 6 mm)

Table 4.9: Caffeine estimation in different types of calli* (S-274):

Medium	Colour and texture of tissue	Caffeine level (% Dry wt.)
-	Original tissue	0.24±0.10
BAP 2.2 µM	White & spongy	0.57±0.13
2-iP 2.5 µM	White & spongy	0.68±0.16
BAP 2.2 µM	Yellow & friable	0.77±0.08
2-iP 2.5 µM	Yellow & friable	0.80±0.17
BAP 2.2 µM	Green & hard	4.21±0.65
2-iP 2.5 µM	Green & hard	3.34±1.08

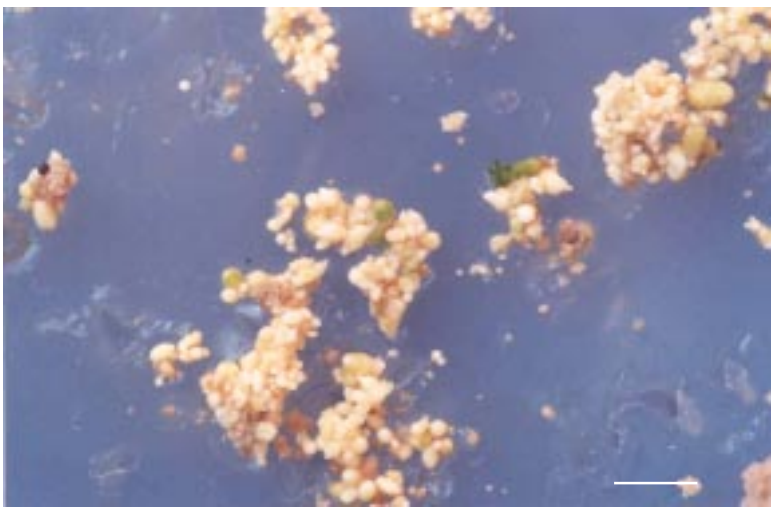
* The basic medium consisted of ½ MS salts, vitamins and 2,4-D (9 µM)

Alkaloid concentration (% DW) achieved by *in vitro* cultivation of plant tissues is generally distinctly lower than in the original plant. Among few exceptions are the purine alkaloids theobromine and caffeine which are produced by callus and suspension cultures of *C. arabica* and *C. canephora* in concentrations almost as high as in the plant (Baumann *et al.*, 1983) or more (Waller *et al.*, 1983). One of the explanations for this ready formation is the connection of these secondary plant substances to adenine-containing compounds of primary metabolism (Baumann *et al.*, 1978).

4.3.10. Caffeine at different stages of somatic embryogenesis (CxR):

Caffeine level was estimated during the somatic embryogenesis in two varieties of *C. canephora* i.e., CxR and Old Robusta. (Table 4.10; Fig.4.13). There was an increase in the caffeine level when the globular embryos were formed from callus. Caffeine level had almost doubled when calli (0.21±0.04% & 0.24±0.07% in CxR and Old Robusta respectively) turned to globular embryos (0.54±0.04% & 0.42±0.02%).

(a)



(b)

Figure 4.13: Different developmental stages of embryogenesis in coffee, globular (a) (bar 5 mm), torpedo and plantlet (b) (bar 5 mm) stages of development can be seen.

There was a slight decrease in the caffeine when the globular embryos reached torpedo stage. The torpedo embryos germinated into plantlets with green leaves. These leaves contained highest levels of caffeine. The differentiation of plant cells in culture is correlated with the accumulation of secondary products (Haldimann and Brodelius, 1987).

Table 4.10: Caffeine level at different stages of somatic embryogenesis:

Stage	CxR	Old Robusta
Callus	0.21±0.04	0.24±0.07
Globular embryo	0.54±0.04	0.42±0.02
Torpedo embryo	0.35±0.01	0.34±0.09
Plantlet	0.68±0.08	0.96±0.24

Table 4.11: Levels of caffeine and other metabolites at different stages of somatic embryogenesis obtained from yellow friable callus in embryo germination medium (CxR):

Stage	7-Methyl xanthine	Theobromine	Caffeine	Theophylline
Callus	0.003±0.002	0.004±0.002	0.21±0.04	-
Globular embryo	-	0.007±0.001	0.54±0.04	0.006
Torpedo embryo	-	0.008±0.0	0.35±0.01	-
Plantlet	-	0.020±0.001	0.68±0.08	0.002

It is interesting to note that both the varieties shows similar trend in the pattern of caffeine accumulation. Old robsuta leaves appeared to contain slightly more caffeine than that from CxR.

In the detailed analysis of the caffeine and its metabolites during the somatic embryogenesis the following observation have been recorded (Table 4.11). Callus showed the presence of 7-methylxanthine. When the caffeine level is high, it is converted to

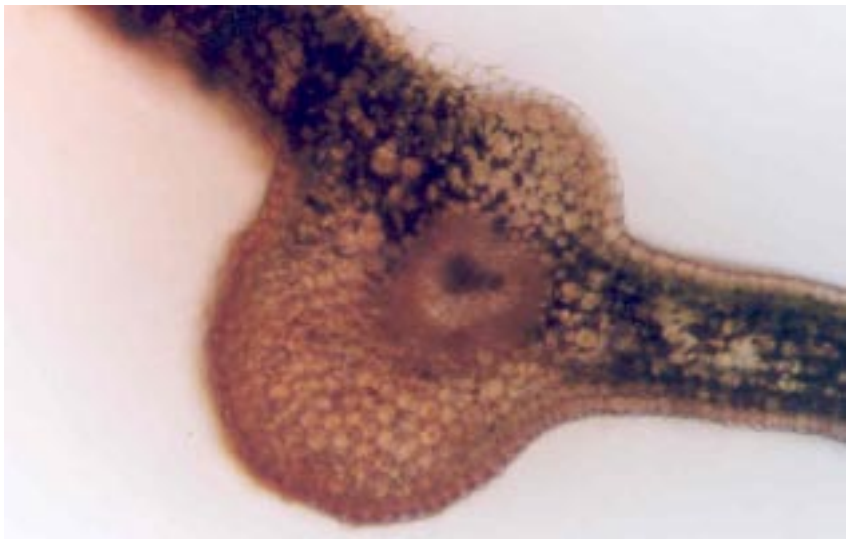
theophylline as in globular embryos and plantlets. At torpedo stage, the caffeine level was less compared to globular but the theobromine is more. Plantlets formed highest amounts of theobromine and caffeine. Caffeine is found to be accumulating during embryogenesis. These results again clearly conjoin caffeine synthesis with greenness of tissue.

4.3.11. Chlorogenic acid and caffeine:

Coffee seedlings of the variety CxR were germinated both in dark and light conditions (Fig. 14). Earlier reports have shown that chlorogenic acid and caffeine levels are higher in light grown than in dark grown tissues (Schulthess and Baumann, 1995). It has also been reported that cholrogenic acid and caffeine are bound together in the cell (Waldhausser and Baumann, 1996). We wished to use a histochemical method to visualize cholrogenic acid in developing seedlings. The reagent produces a red colour on reaction with cholrogenic acid. Intense red colour was observed in the lower epidermal region of green leaves (Fig. 4.15a) while of lower intensity in other parts while in white leaves (Fig.4.15b) the overall intensity of staining was less. In the hypocotyl red colouration was observed in the epidermal layer in both green and white ones (Fig. 4.16a & b) with more intense staining in the green. The colouration was found in the vascular region also in the green ones. But such colouration was very meager in white ones. Slight colouration was found in the pith region also. The leaves showed colour mainly in the lower epidermis and for the hypocotyls it was concentrated mainly in the epidermal and vascular regions. Root hairs were observed in the epidermis of the white roots, which was totally absent in light-grown roots. Roots showed a little or no colouration. Red colour was found to be spread in the parenchyma tissue below the epidermis in light grown roots, which was found to be more concentrated in certain cells (Fig. 4.17a). No such colour formation was observed in the white roots (Fig. 4.17b). Nowhere else the colour formation was observed in light-grown root.

Figure 4.14: *C. canephora* (CxR) seedlings germinated *in vitro* in both light and dark conditions (bar 30 mm)

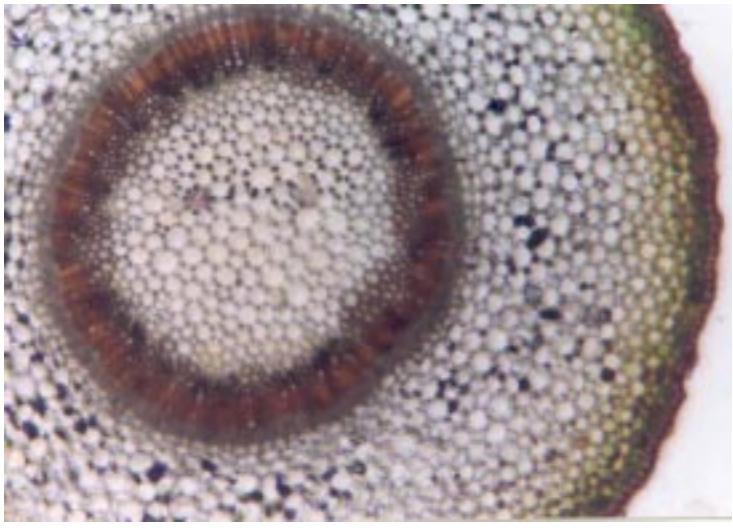
(a)



(b)

Fig. 4.15 Light microscopy. Chlorogenic acid. Cotyledonary leaf sections of *C. canephora* (CxR) seedlings obtained by germinating the seeds in both light (a) and dark (b) conditions. Intense red color was observed in the lower epidermal region of green leaves (a) while of lower intensity in other parts. In white leaves (b) the intensity of staining was less. (400 x)

(a)



(b)

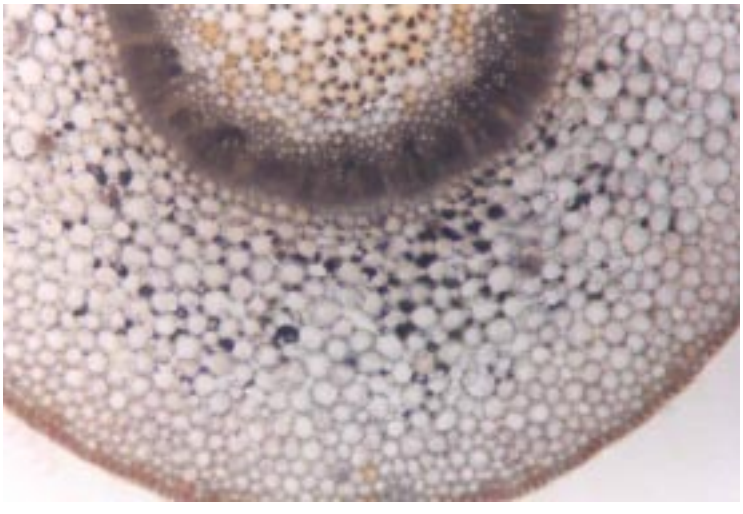
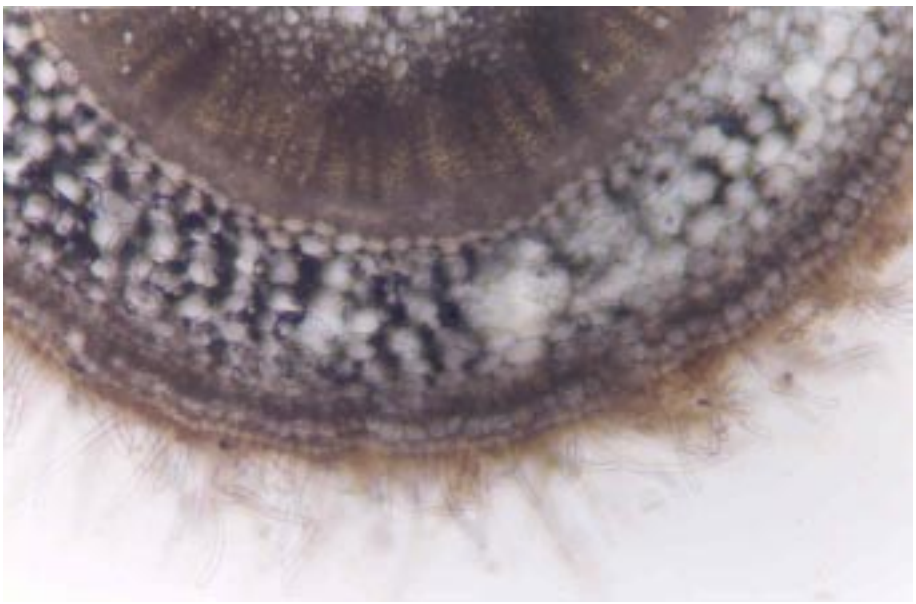


Fig. 4.16 Light microscopy. Chlorogenic acid. Hypocotyl sections of *C. canephora* (CxR) seedlings obtained by germinating the seeds in both light (a) and dark (b) conditions. Red coloration was mainly observed in the epidermal layer in both green (a) and white ones (b) with more intense staining in the green (400 x)

(a)



(b)

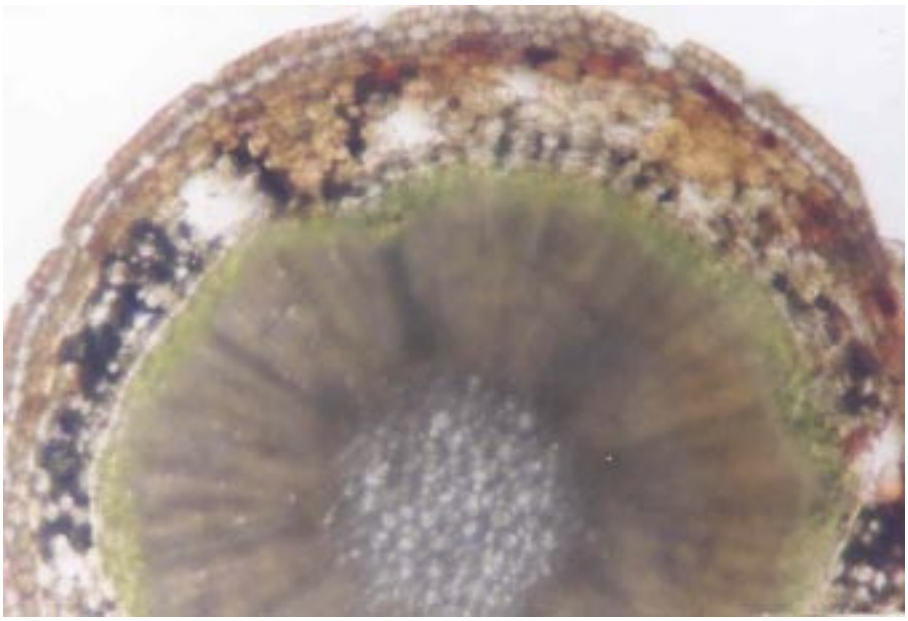


Fig. 4.17 Light microscopy. Chlorogenic acid. Root sections of *C. canephora* (CxR) seedlings obtained by germinating the seeds in both light (a) and dark (b) conditions. Root hairs were observed in the epidermis of the white roots (b), which was totally absent in light-grown roots (a). Red color was found to be spread in the parenchyma tissue below the epidermis in light grown roots, which was found to be more concentrated in certain cells (a). No such color formation was observed in the white roots (b) (400 x)

Slight difference was observed in the caffeine levels in both light-grown and dark-grown leaf tissues. There was an increase in the caffeine content between the light-grown and dark-grown tissues, which were more prominent in hypocotyl. Light-grown leaves contained 6.19±0.23% caffeine and the dark-grown leaf showed 5.84±0.17%. Light-grown hypocotyls had 2.85±0.25% and dark-grown hypocotyls tissue produced 1.92±0.23% caffeine. Schulthess and Baumann (1995) reported that high caffeine concentration reached under photoperiod in suspension cultures of *C. arabica* were always accompanied and governed by high chlorogenic acid concentrations.

4.4 CONCLUSION:

Coffee seedlings are valuable source of explants for various *in vitro* studies. Sulphuric pretreatment and use of jars are recommended to increase the rate of germination of coffee seeds as well as growth of seedlings in *in vitro* conditions. There are several reports on increasing viability of seeds by adopting different storing techniques. Sulphuric acid treatment along with such techniques can be very helpful in field trials to increase the rate of germination and growth of seedlings.

From the study of caffeine analysis the following conclusions were drawn. The older leaves contain less caffeine and in the coffee seeds even if the germ contained (3.58±1.41%) almost double the level of caffeine in endosperm (1.481±0.14%), of the two parts of the dried coffee seed, the endosperm occupies 99.5% by weight while the germ occupies the remaining 0.5%. It is the endosperm therefore that contributes maximally to the caffeine of the seed. The content of caffeine in endosperm varies among species between 0.06 - 3.56%. Higher levels of caffeine are found in diploid species. Immature seeds contained higher caffeine than mature ones. In interspecific hybrids it

appeared that caffeine content in tetraploids is stable in 1.5-1.6% range and may not vary significantly. Possibly caffeine levels can be further decreased by using an arabica selection reputedly carrying low levels of caffeine (<1.0%) as one of the parents in crossing followed by appropriate selection of the progenies. Cotyledons were having the highest amount of caffeine in the germinating seedlings. Caffeine level in callus found to be related to the greenness and the organization of the tissue. Caffeine and related compounds found to be accumulated during the somatic embryogenesis. Light-grown seedlings showed higher chlorogenic acid content in different parts of the seedling and caffeine level was also slightly higher in light grown seedlings. In overall observation caffeine was found to be accumulated in green tissues.

CHAPTER V

Summary and Conclusion

5. 1. SUMMARY:

Coffee is an extremely important agricultural crop with more than 6.5 million tonnes of green beans produced every year on about 11 million hectares. In terms of economic importance on the international market, it is second only to oil and contributes more than 9000M U.S.$ (Leroy *et al.*, 2000).

Coffee is a perennial crop belonging to *Rubiacea* and among more than 80 species of coffee, the most economically important are *Coffea 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. canephora* is becoming popular particularly in the lower elevation areas where *C. arabica* doesn't thrive well. It is hardier, more resistant to leaf diseases and stem borer and better adapted to varying climates and soils. It is a prolific bearer, produced large number of light red berries, begins to yield earlier and average yield is more than *C. arabica*. (The Wealth of India, 1950).

India is one of the major coffee growing countries with around 3 lakh hectares of land under coffee cultivation, producing 2.3 lakh tonnes of green coffee (Sreenath, 2000). India is a producer of both arabica (104,400 tonnes) and robusta (196,800 tonnes) variety of coffee in proportion of 35:65. India produces the best robustas in the world in terms of quality (Reddy *et al.*, 2001).

There is an urgent need to increase the productivity in coffee bean through the development of disease and pest resistant varieties. Moreover, there is need to improve the quality of coffee. 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 most notable behavioral effects of caffeine occur after low to moderate doses (50-300mg) and are increased alertness, energy and ability to

concentrate. Higher doses of caffeine induce negative effects such as anxiety, restlessness, insomnia and tachychardia. Currently, use of decaffeination processes results in degradation of quality in terms of aroma and taste. In this context demand for decaffeinated coffee is increasing and internationally there is market for such product with high coffee aroma.

Conventional breeding has limitations for coffee improvement because of the genetic barriers of chromosome number (diploid *vs*. tetraploid), auto- incompatible alleles (diploid species) and long breeding cycles (Sreenath, 2000). Furthermore, coffee has lengthy period of fruit development and 2-4 years bean to bean generation time, which make such traditional approaches costly and time consuming.

The caffeine content of the beans varies in different species, eg: *C. arabica* contains 1.0-1.2% and *C. canephora* has 1.5-2.5%. There is a possibility of developing transgenic coffee with reduced caffeine content or caffeine-free by metabolically engineering the pathway of caffeine production. This needs development of transformation protocol for coffee using suitable vector. Hence the proposal focuses on developing rapid propagation system to propagate elite coffee variety and getting transformants using *Agrobacterium* strains. In addition to these major objectives, we conducted studies on the caffeine analysis in different parts of the coffee plant, seedlings, hybrids with major concentration on seeds and *in vitro* cultures. Summary of the results and conclusion made are as follows:

- It was found that embryogenic callus could be derived from the hypocotyl region of germinating coffee seeds. High frequency somatic embryogenic callus was obtained from hypocotyl explants by two different methods of culture in three different selections of *Coffea canephora* P. ex Fr. i.e., CxR, Old Robusta and S-274.

- In method I, embryos were formed from hypocotyls by following a two-step method i.e., callus induction and embryo induction by incubating the explants in callus development and embryo induction media containing different growth regulators, later embryos were regenerated.
- The hypocotyl explants inoculated in callus development medium containing half strength MS (Murashige and Skoog, 1962) salts, B5 vitamins, casein hydrolysate 100 mg l^{-1}, malt extract 400 mg l^{-1} with 2.26 µM 2,4-D, 4.9 µM IBA and 9.8 µM 2-iP as growth regulators (Table 2.5; Fig. 2.1).
- After 55 days the explants with primary calli were transferred to MS medium having 4.5 µM 2,4-D and 17.6 µM BAP and other supplements (embryo induction medium) for embryo induction (Table 2.5; Fig. 2.2).
- The yellow friable callus formed in 60-57 days was transferred to regeneration medium containing five different growth regulator levels viz., (a) 4.4 µM BAP, (b) 8.8 µM BAP, (c) 13.2 µM BAP, (d) 2.2 µM BAP and 2.4 µM 2-iP, (e) 6.6 µM BAP and 7.2 µM 2-iP and subcultured once in 45 days (Table 2.6; Fig. 2.3).
- CxR variety started producing embryos in all the media in five months with culture frequency of 55.5%, 30%, 22.2%, 18.18% and 11.1% respectively while Old robusta showed response in 8.8 µM BAP (11.1%), 2.2 µM BAP and 2.4 µM 2-iP (12.5); and 6.6 µM BAP and 7.2 µM 2-iP (88.8%). 13.2 µM BAP (22.2%) medium was the only one, which could produce proembryogenic callus mass in 274 cultures but that did not turn to embryos.
- Media were selected for further culture from these results with frequency of response of 30% and above. So, 4.4 µM BAP and 8.8 µM BAP media were selected for CxR (Table 2.7; 2.8) while 6.6 µM BAP and 7.2 µM 2-iP; and 13.2 µM BAP media were used for Old robusta and 274 respectively (Table 2.7; 2.8). Later on,

8.8 μM BAP medium was found very effective for CxR variety (Fig. 2.4), which produced 81.1±49.7 embryos per 100 mg tissue in seven and a half months while 6.6 μM BAP and 7.2 μM 2-iP medium has been used for Old Robusta (84±37.6 embryos per 100 mg tissue) for further production of embryos (Table 2.7; 2.8).

- In single step procedure (method II) modified callus development medium was used which consisted of MS salts, B5 vitamins, casein hydrolysate 100 mg l^{-1}, malt extract 400 mg l^{-1} and 2-iP (5 μM).
- Embryogenic callus developed in about 4 months and embryos started appearing by another one month (Table 2.9; Fig. 2.5; 2.6; 2.10). Somatic embryos could be obtained faster by 5 months with frequencies of 16% (CxR), 21.42% (Old Robusta) and 17.14% (S-274), in single step procedure. Upon subculturing in the same medium torpedo shaped embryos were formed (Fig. 2.12; Fig. 2.13), which germinated in embryo germination (EG) medium containing 1.1 μM BAP and 2.5 μM IAA as growth regulators, with 67-81% frequency (Table 2.12; Fig. 2.14).
- Primary somatic embryos gave rise to secondary embryos in embryo germination medium upon prolonged incubation and also produced callus (Table 2.13; Fig. 2.15; 2.16; 2.17). These calli upon transfer to the same medium subsequently regenerated to produce embryos at high frequency (90±40 embryos per 100 mg of callus in CxR variety). Secondary embryogenesis is important for using them for genetic transformation via *Agrobacterium*.
- The developed embryos were grown in half strength MS medium and when the plantlets (50-77%) reached 3-4 pairs of leaves, they were hardened (Fig. 2.19; 2.21).
- Moreover, 30% of hypocotyl explants of all the varieties

responded to formation of shoots (Table 2.9; Fig. 2.5; 2.6; 2.7), which gives additional advantage of using hypocotyl explants for organogenesis.

- In both methods, almost same time has been taken for embryogenic callus induction (4 months; Table 2.6; 2.9). But in method I, when the embryogenic callus was transferred to regeneration media, it took minimum five-six months for the somatic embryos to be formed (Table 2.7). However, as in method II, when the embryogenic callus was incubated in the medium further, embryos could be induced in another one month (Table 2.9). Further incubation produced more number of embryos. Secondary somatic embryos also could be induced from the primary ones. Thus, modified callus development medium (method II, single-step procedure) gave faster embryogenesis response.
- A highly reproducible method for regeneration of *C. canephora* plants via direct somatic embryogenesis method from *in vitro* leaf and stem segments of regenerated plants was developed. Embryogenesis was influenced by the presence of triacontanol (TRIA) in the medium (Table 2.16; 2.17; 2.18 and 2.19). TRIA incorporated at 4.55 and 11.38 μM in half strength MS basal medium containing 1.1 μM 6-benzylaminopurine (BA) and 2.5 μM indole-3-acetic acid (IAA) induced direct somatic embryogenesis. A maximum of 58.59 ±10.3 (Fig. 2.22) somatic embryos per culture were induced from *in vitro* leaf explants of *C. canephora* (S-274). TRIA also induced embryo formation from *in vitro* stem segment callus tissues along with multiplication of primary embryos into secondary embryos.
- The rapid direct and repetitive somatic embryogenesis response of *C. canephora* genotypes was tested on Murashige and Skoog medium (MS) containing thidiazuron (2.27–11.35 μM). Segments

taken from cotyledons, leaf, first leaf and stalk of regenerated plantlets, produced clusters of somatic embryos directly from cut portions of explants on TDZ (9.08 µM) containing medium within a period of two months (Table 2.20; 2.21; 2.22; 2.23; Fig. 2.23; 2.24). These direct somatic embryogenesis from leaf and hypocotyl explants in *Coffea* sp. is a strong evidence of their cell totipotency. The rapid somatic embryo induction protocol would be useful for the mass propagation, direct regeneration and genetic transformation of selected elite lines.

- Zygotic embryos were inoculated in three media, viz., MS basal medium, MS salts supplemented with Morel and Wetmore vitamins (Morel and Wetmore, 1951) and Heller's medium (Heller, 1953) with other supplements. In all the media, response was good with 80-90% survival. 2-2.5 cm long plantlets could be obtained in 60-75 days with proper rooting and shoot growth.
- Roots were removed and shoots were propagated in MS medium containing BAP (4.4 µM) as the growth regulator (Fig. 2.25).
- Callus proliferation was observed from the shoot ends when shoots were cultured in MS medium containing 2-iP (4.9 µM), which produced embryos on further incubation in the same medium (Fig. 2.26). The embryos were developed into fully-grown plantlets.
- Contamination is the major problem while using explants from the natural environmental conditions and zygotic embryo derived plantlets are excellent source of aseptic plant material for the embryogenesis in coffee.
- Studies on establishment of nodal cultures were done using field grown explants but successful *in vitro* cultures could not be obtained due to severe contamination problem (Table 2.24)

- Silver nitrate enhanced growth of the coffee shoots. The greatest shoot length and more number of leaves were observed in 10 µM silver nitrate, but 5 and 10 µM induced more leaf area (Table 2.25; Fig. 2.27).
- *Agrobacterium* mediated transformation of coffee was carried out. Two different varieties of *C. canephora* viz., CxR and Old Robusta were used for the experiments. Embryos were obtained from hypocotyl explants. *A. rhizogenes* (having pSK53 construct; Fig.3.1; 3.2) and *A. tumefaciens* strain (having pSK53 construct; Fig.3.1; 3.2) were used for infection.
- Optimum levels of selection pressure required for the selection of transformed tissues were standardized: hygromycin (10 mg l^{-1}), kanamycin (200 mg l^{-1}) and basta (glufosinate; 5 mg l^{-1}) (Table 3.1; 3.2; 3.3). It was seen that kanamycin had to be used at very high levels to obtain killing and very often regeneration of plants and multiplication of untransformed somatic embryos occurred even at high levels of kanamycin. Basta was very effective but turned the media brown and the detergent used to keep the herbicide in solution interfered with regeneration. Therefore hygromycin was used as the selection marker in the study.
- Binary vectors were mobilized to *A. tumefaciens* and *A. rhizogenes* (Fig. 3.4)
- The embryos were wounded and incubated with the bacteria (Fig. 3.5). After co-cultivation for 3 days they were transferred to modified callus development medium containing half strength MS medium with growth regulators, 2-iP (9.8 µM), IAA (5.7 µM) and NAA (5.3 µM) and other growth supplements like casein hydrolysate and malt extract. Cefotaxim, augmentin (each 500 mg l^{-1}) and hygromycin (3 mg l^{-1}) were also added to the medium to kill the bacteria and for the selection of transformed cells.

Slight callus formation was observed in some surviving explants as visible under microscope after one month in hygromycin (3 mg l^{-1}). Around 91-95 % of the transformed explants retained their freshness in the medium (Table 3.5).

- After one month, the embryos were transferred to half strength MS medium containing the growth regulators 2,4-D (9 µM) and BAP (35.5 µM), hygromycin (10 mg l^{-1}) and antibiotics. The embryos were kept in this medium for forty days. After one-month, callus proliferation could be seen on the embryos. Old Robusta (49.29%) showed vigorous growth in transformed embryos compared to CxR (12.03%) (Fig. 3.6; 3.7; 3.9). The calli were yellow in colour and granular in appearance. *A. tumefaciens* infected embryos (12.03% in CxR) showed higher frequency of callus formation than *A. rhizogenes* (4.50%) (Table 3.5). The calli, which were surviving in hygromycin (10 mg l^{-1}), were used for GUS analysis and PCR (Fig. 3.10; 3.11). GUS expression was found in the CxR callus transformed with *A. tumefaciens*. PCR amplification of *gus* gene was observed in the CxR and Old Robusta calli transformed with *A. rhizogenes* and *A. tumefaciens* respectively.
- After this the surviving calli were transferred to the same medium containing 2,4-D (4.4 µM) and BAP (35.5 µM) with higher selection pressure (hygromycin, 20 mg l^{-1}) and antibiotics. Old Robusta calli survived well in higher selection pressure (34.04%) (Table 3.5; Fig. 3.7).
- An attempt was made to find effective method to improve the germination of coffee seeds. The objective of the present research was to improve the speed and percent germination in fresh and stored seeds by scarification treatment with different concentrations (0.23, 0.46, 93, 1.40, 1.87 and 2.81 M) of H_2SO_4

(Table 4.1; 4.2; Fig. 4.6) and to see the effect of GA_3 on germination of seeds and growth of seedlings (Table 4.3).

- Speed of germination was influenced significantly by treatment with H_2SO_4 both in fresh (Table 4.1) and stored (Table 4.2) seeds, but not with GA_3. The highest total germination of 100% in 4 weeks was achieved when stored seeds were exposed to 2.81 M H_2SO_4 for 1.5 minutes (Table 4.2). In fresh seeds first pre-treated with 9 M sulphuric acid and further treated with GA_3 100% germination was observed (Table 4.3). Maximum seedling length and growth was recorded in seeds treated with 0.07 mM GA_3 (3.2±0.23). This protocol will be useful in initiation of tissue culture of coffee with high rate of *in vitro* seedling formation and growth.
- No basic structural difference was observed in scanning electron microscopy of the acid treated samples except a slight expansion of epidermal cells in the acid treated ones (Fig. 4.8; 4.9).
- Caffeine analysis was conducted in the following tissues:
- **Mature plant parts and seeds (*C. canephora*, S-274):** The levels of caffeine in first leaf, second leaf and third leaf from apex were estimated (Table 4.4). The first leaves, which were the most tender ones was having the highest amount (1.81±0.21%) followed by second (0.67±0.11%) and third (0.63±0.05%) pair. In the seed, embryo was having higher caffeine with 3.58±1.408% and endosperm contains 1.48±0.13%.
- **Different varieties of *Coffea*:** Endosperm tissues were analysed for caffeine from ripened/unripened fruits obtained from different varieties of *Coffea* (Table 4.5). *C. salvatrix* and *C. bengalensis* were having the least amount (0.06%) while the highest was in Old Robusta (3.48%). The other varieties tested were *C. canephora* x *C. travancorensis* (3.55%). *C. canephora* x

*C. ui*ghtiana (2.94%), Hybrido de Timore (1.36%). *C. eugenioides* (0.68%), *C. racemosa* x *C. canephora* (0.71-1.24%), CxR (3.33%), Liberica x Eugenioides(1.39%), Kaggenhalla Robusta (2.27%) and Paradenia robusta (1.60%).

- **Interspecific hybrids and their progenies:** This study was conducted to assess the reduction of caffeine in the endosperm after breeding (Table 4.6). Seed samples of the allotetraploid derived from *C. liberica* (2.01%) X *C. eugenioides*(0.69%) (Ligenioides-1.3%), Hybrido de Timore (HDT-1.3%), their F1 (S.4595-1.5%), four F2 families (S.4696-1.61%, S.4697-1.56%, S.4698-1.50%, S.4699-1.56%) and two back cross (BC) families S.4700 (1.65% - BC to HDT) and S.4702 (1.53% - BC to Ligenioides) were analyzed.
- Ligenioides (1.30%) showed a caffeine level of middle range between the parents. Ligenioides and HDT possess about 1.3% caffeine in their seeds. However their F1, F2 and BC families manifested slightly higher caffeine ranging from 1.50%-1.65%. Even this caffeine content value is not much higher than that of *C. arabica* (0.6%-1.5%). Thus it appears that caffeine content in tetraploids is stable in this range and may not vary significantly until a choice of arabica carrying very low caffeine (<1.0%) is used as one of the parents in crossing followed by appropriate selection to reduce this value in progenies.
- **Different developmental stages of germination (CxR):** Seeds of CxR were germinated *in vitro* and caffeine and other three metabolites viz., 7-methylxanthine, theobromine and theophylline, in the pathway were analyzed at different stages of growth in different parts of the seedling namely cotyledonary leaf, hypocotyl and root (Table 4.7). Tissues from different parts were taken when the seedlings were in unopened cotyledonary stage and fully opened stage. Leaf tissues (5.29±0.18%)

expressed more caffeine than hypocotyls (4.08±0.77%) and leaves at unopened and fully opened stage showed almost same level viz., 5.29±0.18% and 5.38±1.06% respectively. Roots were also having some amount of caffeine (0.1±0.04%). 7-methyl xanthine, theobromine and theophylline were also found to be present in leaves and hypocotyls (unopened stage), which showed that caffeine was being produced and degraded in these tissues. The zygotic embryo from which germination had started is having 2.62±0.11% caffeine.

- **Callus:** Three different kinds of calli namely, white spongy, yellow friable and green hard were obtained when hypocotyl explants of 274 were cultured in modified callus development medium with two different cytokines viz., BA and 2-iP for about three and a half months (Table 4.9). Green tissue, which was hard, had the highest amount of caffeine (4.20±0.65%) followed by yellow (0.76±0.08%) and white (0.56±0.13%), which shows that the caffeine level depends on the texture and organization of the tissue. White and yellow callus produced slightly higher caffeine in 2-iP medium and green callus showed reverse trend.
- **Different stages of embryogenesis (CxR):** The tissue was taken from different stages viz., callus, globular embryo, torpedo embryo and plantlet (Table 4.10; 4.11). The plantlets were having the highest amount of caffeine (0.7±0.07) followed by globular embryos (0.54±0.03), torpedo embryos (0.35±0.01) and callus (0.21±0.03). Theobromine was present in all (0.001-0.008%; Table 4.11) while in callus there was some amount of 7-methyl xanthine (0.003%). Caffeine was found to be accumulated when the callus tissue differentiated to somatic embyos.
- In Old robusta same trend of caffeine level was observed with plantlets having the highest amount (0.96±0.24%) followed by

globular embryos (0.42±0.02%), torpedo embryos (0.34±0.08%) and callus (0.24±0.04%).

- Histochemical method was used to visualize cholrogenic acid in developing seedlings (Fig. 4.15; 4.16; 4.17). Coffee seedlings of the variety CxR were germinated both in dark and light conditions (Fig. 4.14). The reagent produces a red color on reaction with cholrogenic acid. A correlation between caffeine and chlorogenic acid content was observed in coffee seedlings germinated in dark and light conditions. Light induced both chlorogenic acid and caffeine content slightly in cotyledons and hypocotyls. Leaves from Light-grown seedlings contained 6.19±0.23% caffeine and leaves from dark-grown seedlings showed 5.84±0.17%. Light-grown hypocotyls had 2.85±0.25% and dark-grown hypocotyls tissue produced 1.92±0.23% caffeine.

5.2. CONCLUSION:

- Somatic embryogenesis has been carried out successfully – regenerated plantlets were transferred to green house
- Enhancement in the efficiency of embryogenesis was obtained by using triacontanol and thidiazuron
- *Agrobacterium* mediated transformation has been obtained in coffee embryos – transformation has been confirmed by GUS assay and PCR
- Protocol for high efficient germination of coffee seeds has been developed
- Caffeine level in leaves depends on the age – tender tissues show higher caffeine level
- Caffeine content in the endosperm is variable among species viz., 0.06–3.56%

- In the coffee seedlings, cotyledonary leaves contain the highest level of caffeine
- Caffeine level depends on the organization of tissue in *in vitro* cultures and caffeine accumulates during embryogenesis
- Caffeine and chlorogenic acid were found to be induced slightly by light

The results are useful for studies related to coffee crop improvement through genetic transformation using somatic embryos and for mass multiplication. The study also gives an insight to the distribution of caffeine in different *in vivo* and *in vitro* tissues and its relation to chlorogenic acid, which is highly helpful for the transformation studies of coffee for regulation of caffeine biosynthesis.

BIBLIOGRAPHY

Ammirato P. V. and Styer D. J. (1985) Strategies for large-scale production of somatic embryos in suspension culture. *In*: M. Saitlin, P. Day and A. Hollaender (eds.) Biotechnology in Plant Science: Relevance to Agriculture in the 1980's. Academic Press, New York pp. 161-178.

Ammirato P.V. (1983) Embryogenesis. *In*: D. A. Evans, W. R. Sharp, P. V. Ammirato and Y. Yamada (eds.) Handbook of Plant Cell Cultures, Techniques for propagation and Breeding. Macmillan Publishing Co., New York and London pp. 82-133.

An G., Ebert P.R., Mitra A. and Ha S.B. (1988) Binary vectors. *In*: S. B. Gelvin, R. A. Schilperoort and D. P. S. Verma (eds.), Plant Molecular Biology Manual. Kluwer Academic Publishers, Dordrecht, The Netherlands pp. 1-19.

Anderson L. and Gibbs M. (1962) The biosynthesis of caffeine in the coffee plant. *J. Biol.Chem*. 237: 1941-1944.

Ashihara H. and Crozier A. (1999) Biosynthesis and metabolism of caffeine and related purine alkaloids in plants. *In*: J. A. Callo (ed.) Advances in Botanical Research, Vol. 30. Academic Press, London pp. 117-205.

Ashihara H. and Kubota H. (1986) Patterns of adenine metabolism and caffeine biosynthesis in different parts of tea seedlings. *Physiol. Plant.* 68: 275-281.

Ashihara H., Monteiro A. M., Gillies F. M. and Crozier A. (1995) Biosynthetic pathways of caffeine in *Coffea arabica* leaves. *In*: Proceed. 16th International Scientific Colloquium on Coffee, ASIC Kyoto pp. 589-598.

Barros R. S., Maestri M. and Rena A. B. (1999) Physiology of growth and production of the coffee tree – a review. *J. Coffee Res*. 27(1): 1-54.

Barton C. R., Adams T. L. and Zorowitz M. A. (1991) Stable transformation of foreign DNA into *Coffea arabica* plants. *In*: Proceed. 14th International Scientific Colloquium on Coffee, ASIC San Fransisco pp. 460-464.

Baumann T. W. and Röhrig L. (1989) Formation and intracellular accumulation of caffeine and chlorogenic acid in suspension cultures of *Coffea arabica*. *Phytochem.* 28: 2667-2669.

Baumann T. W., Dupont-Looser E. and Wanner H. (1978) 7-methylxanthosine – an intermediate in caffeine biosynthesis. *Phytochem.* 17: 2075-2076.

Baumann T. W., Koets R. and Morath P. (1983) *N*- methyltransferase activities in suspension cultures of *Coffea arabica* L. *Plant Cell Rep*. 2: 33-35.

Baumann T. W., Schulthess B. H. and Hänni K. (1995) Guaraná (*Paullinia cupana*) rewards seed dispersers without intoxicating them by caffeine. *Phytochem.* 39: 1063-1070.

Baumann T. W., Sondahl M. R., Waldhausser M. and Kretschmar J. A. (1998) Non-destructive analysis of natural variability in bean caffeine content of Laurina coffee. *Phytochem.* 49: 1569-1573.

Bendaña F. E. (1962) The physiology of coffee seeds. II. Factors retarding germination: parchment. *Turrialba*, 4: 76-79.

Berthouly M. and Etienne H. (1999) Somatic embryogenesis of coffee. *In*: S. M. Jain, P. K. Gupta and R. J. Newton (eds.) Somatic Embryogenesis in Woody Plants, Volume 5, Kluwer Academic Publishers, Dordrecht pp. 259-288.

Berthouly M. and Michaux-Ferriere (1996) High frequency somatic embryogenesis in *Coffea canephora* - Induction conditions and histological evolution. *Plant Cell Tiss. Org. Cult.* 44: 169-176.

Berthouly M., Dufour M., Alvard D., Carasco C., Alemano L. and Teisson C. (1995) Coffee micropropagation in liquid medium using temporary immersion system. *In*: Proceed. 16th International Scientific Colloquium on Coffee, ASIC Vevey, Switzerland pp 514-517.

Bertrand-Desbunais A., Noirot M. and Charrier A. (1991) Minimal growth *in vitro* conservation of coffee (*Coffea* spp.). 1. Influence of reduced concentrations of 6-benzyl adenine. *Plant Cell Tiss. Org. Cult.* 27: 333-339.

Bertrand-Desbunais A., Noirot M. and Charrier A. (1992) Slow growth *in vitro* conservation of coffee (*Coffea* spp.). 2. Influences of reduced concentrations of sucrose and low temperature. *Plant Cell Tiss. Org. Cult.* 31: 105-110.

Bevan M. (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acid Res.* 12: 8711-8721.

Bheemaiah M. M. (1992) Coffee and its management in South India- a brief account. *Indian Coffee* 56(12): 9-18.

Biratu T., Omondi C. and Hindorf H. (1996) Caffeine content in relation to resistance of *Coffea arabica* L. to coffee berry disease (*Colletotrichum coffeanum* Noack). *J. Plant Dis. Protec.* 103: 15-19.

Blauch J. L. and Tarka Jr. S. M. (1983) HPLC determination of caffeine and theobromine in coffee, tea and instant hot cocoa mixes. *J. Food Sci.* 48: 745-750.

Blecher R. and Lingens F. (1977) The metabolism of caffeine by a *Pseudomonas putida* strain. Hoppe-Seyler's Zeitschrift Fur Physiology und Chemistry 358: 807-817.

Bonga J. M. (1982) Vegetative propagation in relation to juvenility, maturity and rejuvenation. *In*: J. M. Bonga, D. L. Durzan and W. Junk (eds.) Tissue Culture in Forestry. Martinus Nijhoff. pp. 387-412.

Brun L. O. and Constant R. F. (1997) Insecticide resistance in the coffee berry borer: state of current knowledge. *In*: Proceed. 17th International Scientific Colloquium on Coffee, ASIC Nairobi Abstract, p. 96.

Buchanan R. L., Tice G. and Marino D. (1981) Caffeine inhibition of Ochratoxin A production. *J. Food Sci.* 47: 319-321.

Buckland E. and Townsley P. M. (1975) Coffee cell suspension cultures, caffeine and chlorogenic acid content. *J. Inst. Can. Sci. Tech.* 8: 164-165.

Capelle S. C., Mok D. W. S., Kirchner S. C. and Mok M. C. (1983) Effects of thidiazuron on cytokinin autonomy and the metabolism of N^6-(Y^2- isopentyl) [8-14c] adenosine in callus tissues of *Phaseolus lunatus* L. *Plant Physiol.* 73: 796-802.

Carelli M. L. C., Lopes C.R. and Monaco L. C. (1974) Chlorogenic acid content in species of *Coffea* and selections of *C. arabica*. *Turrialba* 24: 398-401.

Carneiro M. F. (1999) Advances in coffee biotechnology – review. Ag Biotechnet, Vol.1, ABN 006 pp. 1-14. Available from Internet: (http://www.agbiotecnet.com/reviews/Jan99/HTML/Carneiro.htm).

Carvahlo A., Ferwerda F. P., Frahm-Leliveld J. A., Medina D. M., Mendes A.J. T. and Monaco L. C. (1969) Coffee - *Coffea arabica* L. and *Coffea canephora* Pierre ex Froehner. *In*: F. P. Ferwerda and F. Wit (eds.) Outlines of Perennial Crop Breeding in the Tropics. H. Veenman and Zonen N. V. (Publishers), Wageningen pp. 189-241.

Carvalho A., Tango J. S. and Monaco L. C. (1965) Genetic control of the caffeine content of coffee. *Nature* 205: 314.

***Chevalier A.** (1947)'Les caféiers du globe III. Systématique des caféiers et faux caféiers. Maladies et insects nuisibles', *Encyclopedie biologique 28*, Fascicule III, Paul Le Chevalier, Paris p. 356.

***Charrier A.** (1978) La structure génétique des caféiers spontanés es la region Malgache (*Mascarocoffea*). Leurs relations avec les caféiers d'origine africaine (*Eucoffea*). Mémoires ORSTOM (Paris) no.87 p.141.

***Charrier A. and Berthaud J.** (1975) Variation de la teneur en caffeine dans le genre *Coffea*. *Café Cacao Thé* 19: 251-264.

Charrier A. and Berthaud J. (1985) Botanical classification of coffee. *In*: M. N. Clifford and K. C. Willson (eds.) Coffee - Botany, Biochemistry and Production of Beans and Beverage. The avi publishing company, Inc. Westport, Connecticut pp. 13-47.

Chilton M. D., Drummond M. H., Merlo D. J., Sciaky D., Montoya A. L., Gordon M. P. and Nester E. W. (1977) Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. *Cell* 11: 263-271.

Cho U. H. and Kasha K. (1989) Ethylene production and embryogenesis from anther cultures of barley (*Hordeum vulgare*). *Plant Cell Rep*. 8: 415-417.

Cho U. H. and Kasha K. (1992) Relationships of senescence to androgenesis in barley (*Hordeum vulgare* L. cv. *Klages*). *J. Plant Physiol.* 139: 299-302.

Clifford M. N. (1985) Chemical and physical aspects of green coffee and coffee products. *In*: M.N Clifford and K. C. Willson (eds.) Coffee- Botany, Biochemistry and Production of Beans and Beverage. The avi publishing company, Inc. Westport, Connecticut pp. 305-374.

Clifford M. N. and Kazi T. (1987) The influence of coffee bean maturity on the content of chlorogenic acids, caffeine and trigonelline. *Food Chem.* 26: 59-69.

Clifford M. N., Gibson C. L., Rakotomalala J. –J., Cros E. and Charrier A. (1991) Caffeine from green beans of *Mascarocoffea. Phytochem.* 30: 4039-4040.

Clifford M. N., Williams T. and Bridson D. (1989) Chlorogenic acids and caffeine as possible taxonomic criteria in *Coffea* and *Psilanthus*. *Phytochem.* 28: 829-838.

Compton M. E. and Gray D. J. (1992) Somatic embryogenesis on immature cotyledons of water melon. *In Vitro Cell Dev. Biol.* 28: 98.

Crozier A., Monteiro A. M., Moritz T., Gillies F. M. and Ashihara H. (1995) Purine alkaloid catabolism pathways in *Coffea arabica* leaves. *In*: Proceed. 16th International Scientific Colloquium on Coffee, ASIC Kyoto 606-615.

D'Silva I. and D'Souza L. (1993) Controlling contamination and browning of *in vitro* cultures of cashew. *J. Plantn. Crops* 21(1): 22-29.

***De Garcia E. and Menéndez A.** (1987) Embryogénesis somática a partir de explantes foliares del cafeto 'Catimor'. *Café Cacao Thé* 31: 15-22.

De Pena M. (1995) Development of a stable transformation procedure for the protoplasts of *Coffea arabica* cv. Columbia. Doctoral thesis. University of Purdue p. 75.

Dentan E. (1985) The microscopic structure of the coffee bean. *In*: M. N. Clifford and K. C. Willson (eds.) Coffee- Botany, Biochemistry and Production of Beans and Beverage. The avi publishing company, Inc. Westport, Connecticut pp. 284-304.

***Dublin P.** (1981) Embryogenése somatic directe sur fragments de feuilles de caféier Arabusta. *Café Cacao Thé* 25: 237-241.

Ducos J. P., Zamarripa A., Eskes A. B. and Petiard V. (1993) Production of somatic embryos of coffee in bioreactor. *In*: Proceed. 15th International Scientific Colloquium on Coffee, ASIC, Montpellier, France pp. 89-96.

Eriksen A. B., Haugstad M. K. and Nilsen S. (1982) Yield of tomato and maize in response to foliar and root applications of triacontanol. *Plant Growth Regul.* 1:11-14.

Etienne-Barry D., Bertrand B., Vasquez N. and Etienne H. (1999) Direct sowing of *Coffea arabica* somatic embryos mass-produced in a bioreactor and regeneration of plants. *Plant Cell Rep.* 19: 111–117.

Fagerlind F. (1939) Perisperm oder Endosperm bei Coffea? *Svensk Bot. Trdskrift* 33: 303-309.

***Fazuoli L. C.** (1976) Avaliacao de progenies de café Mundo Nove (*Coffea arabica* L.) University of Sao Paulo - ESALQ. M. Sc. Thesis.

Feng Q., Yang M. Z., Zheng X. Q., Zhen X. S., Pan N. S. and Chen Z. L. (1992) *Agrobacterium*-mediated transformation of coffee (*Coffea arabica* L.). *Chin. J. Biotechnol.* 8: 255-260.

Fernandez-Da Silva R. and Menéndez-Yuffa A. (2003) Transient gene expression in secondary somatic embryos from coffee tissues electroporated with the genes *gus* and *bar*. *Electronic J. Biotechnology* ISSN: 0717-3458,Vol 6: No.1, April, 15.

Fisk H. J. and Dandekar A. M. (1993). The introduction and expression of transgenes in plants. *Scientia Horticulturae* 55: 5-36.

Freire A. V., Lightfoot D. A. and Preece L. E. (1994) Genetic transformation of coffee (*Coffea arabica* L.) by *Agrobacterium* spp. *Hort. Science* 29: 454.

Frischknecht P. M. and Baumann T. W. (1985) Stress induced formation of purine alkaloids in plant tissue cultures of *Coffea arabica*. *Phytochem.* 24: 2255-2257.

Frischknecht P. M., Eller B. M., Wanner H. and Baumann T. W. (1982) Purine alkaloid formation and CO_2 gas exchange in dependence of development and of environmental factors in leaves of *Coffea arabica* L. *Planta* 156: 295-301.

Frischknecht P. M., Ulmer-Dufek J. and Baumann T. W. (1986) Purine alkaloid formation in buds and developing leaflets of *Coffea arabica*: Expression of an optimal defence strategy? *Phytochem.* 25: 613-616.

Fuentes S. R. L., Calheiros M. B. P., Manetti-Filho J. and Vieira L. G. E. (2000) The effects of silver nitrate and different carbohydrate sources on somatic embryogenesis in *Coffea canephora*. *Plant Cell Tiss. Org. Cult.* 60: 5-13.

Fujimori N. and Ashihara H. (1994) Biosynthesis of theobromine and caffeine in developing leaves of *Coffea arabica*. *Phytochem.* 36: 1359-1361.

Furuya T., Koge K. and Orihara Y. (1990) Long-term culture and caffeine production of immobilized coffee (*Coffea arabica*) L. cells in polyurethane foam. *Plant Cell Rep.* 9: 125-128.

Gamborg O. L., Miller R. A. and Ojima K. (1968) Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* 50: 151-158.

Ganesh S. D. and Sreenath H. L. (1996) Silver nitrate enhanced shoot development in cultured apical shoot buds of *Coffea arabica* cv. *Cauvery* (S. 4347). *J. Plantn. Crops* 24: 577-580.

Ganesh S. D. and Sreenath H. L. (1999) Embryo culture in coffee: technique and applications. *Indian Coffee* 4: 7-9.

Gill R. and Saxena P. K. (1992) Direct somatic embryogenesis and regeneration of plants from seedling explants of peanut (*Arachis hypogea*) promotive role of thidiazuron. *Can. J. Bot.* 70: 1186-1192.

Gillies F. M., Jenkins G. I., Ashihara H. and Crozier A. (1995) *In vitro* biosynthesis of caffeine: the stability of *N*-methyltransferase activity in cell-free preparations from liquid endosperm of *Coffea arabica*. *In*: Proceed. 16th International Scientific Colloquium on Coffee, ASIC, Kyoto pp. 599-605.

Glory Swarupa S. (1996) Study on the effect of biofertilizer on the growth of CxR seedlings. *J. Coffee. Res.* 26(2): 62-66.

Gopal N. H. and Ramaiah P. K. (1971) Studies on hastening of germination of *Arabica* coffee seed and further growth of the seedlings. I. Hastening of germination. *Indian Coffee* 35: 459-464.

Gray D. J., Mccolley D. W. and Compton M. E. (1992) Effects of cytokinins, genotype and other factors on somatic embryogenesis from cotyledons of *Cucumis melo* . *In Vitro Cell. Dev. Biol.* 28:101.

Gundlach H., Muller M. J., Kutchan T. M. and Zenk M. H. (1992) Jasmonic acid is a signal transducer in elicitor induced plant cell cultures. *Proc. Nat. Acad. Sci.* 89: 2389-2393.

Hakil M., Voisinet F., Viniegra-Gonzalez G. and Augur C. (1999) Caffeine degradation in solid state fermentation by *Aspergillus tamarii*: effects of additional nitrogen sources. *Process Biochem.* 35: 103-109.

Haldimann D. and Brodelius P. (1987) Redirecting cellular metabolism by immobilization of cultured plant cells: A model study with *Coffea arabica*. *Phytochem.* 62: 1431-1434.

Hamidi A. and Wanner H. (1964) The distribution pattern of chlorogenic acid and caffeine in *Coffea arabica*. *Planta* 61: 90-96.

***Hannig E.** (1904) Zur Physiologie pflanzlicher Embryonen. I. Ueber die Cultur von Cruciferen-Embryonen ausserhalb des Embryosacks. Bot. Ztg. 62:45-80.

Hanson K. R. and Zucker M. (1963) The biosynthesis of chlorogenic acid and related conjugates of the hydroxycinnamic acids. *J. Biochem*. 238: 1105.

Harborne J. B. (1982) Introduction to Ecological Biochemistry. (IInd Ed.) Academic Press, London pp. 1-31.

Hashim O. B. and Lundergan C. A. (1985) Effect of triacontanol on yield and fruit composition of spring–harvested 'Tangi' and 'Dover' strawberries. *Hort. Science* 20: 73-74.

Hatanaka T., Arakawa O., Yasuda T., Uchida N. and Yamaguchi T. (1991) Effect of plant growth regulators on somatic embryogenesis in leaf cultures of *Coffea canephora*. *Plant Cell Rep.* 10: 179-182.

Hatanaka T., Azuma T., Uchida N. and Yasuda T. (1995) Effect of plant hormones on somatic embryogenesis of *Coffea canephora*. *In*: Proceed. 16th International Scientific Conference on Coffee Science, Kyoto pp. 790-797.

Hatanaka T., Choi Y. E., Kusano T. and Sano H. (1999) Transgenic plants of coffee *Coffea canephora* from embryogenic callus via *Agrobacterium tumefaciens* - mediated transformation. *Plant Cell Rep*. 19: 106-110.

Heller R. (1953) Researches on the mineral nutrition of plant tissues. *Ann. Sci. Nat. Bot. Biol. Veg.*, 11th ser. 14: 1-223.

Hermann F. R. P. and Hass G. J. (1975) Clonal propagation of *Coffea arabica* L. from callus culture. *Hort. Science* 10: 588-589.

***Herndlhofer E.** (1933) A distribuição das proteínas, da cafeína do mono-amino-ácidos no cafeeiro e as variações da porcentagem destas substâncias no percurso de um anno. *Bol. Agric. Sao Paulo* 34: 163-251.

Hoagland R. E. (1980) Effects of triacontanol on seed germination and early growth. *Bot.Gaz*. 141: 53-55.

Horman I. and Viani R. (1971) The caffeine-chlorogenate complex of coffee-an NMR study. *In*: Proceed. 5th International Scientific Colloquium on Coffee, ASIC, Paris pp. 102-111.

Horsch R.B., Fry J. E. and Hoffman N. (1985) A simple and general method for transferring genes into plants. *Science* 227: 1229-1231.

Houk W. G. (1938) Endosperm and perisperm of coffee, with notes on the morphology of the ovule and seed development. *Am. J. Bot.* 25: 56-61.

Huetteman M. J. and Preece J. E. (1993) Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant Cell Tiss. Org. Cult*. 33: 105-119.

Hutchinson M. J., Murch S. J. and Saxena P. K. (1996) Morphoregulatory role of TDZ: evidence of the involvement of endogenous auxin in TDZ-induced somatic embryogenesis of Geranium (*Pelargonium X hortorum* Bailey). *J Plant Physiol*. 149: 573-579.

Huxley P. A. (1964) Some factors which can regulate germination and influence ability of coffee seeds. *In*: Seed Test Assoc. Proc. 29: 33-60.

Huxley P. A. (1965) Coffee germination test recommendations and defective types. *In*: Seed Test Assoc. Proc. 30: 705-715.

Iantcheva A., Vlahova M., Bakalova E., Kondorosi E., Elliott M. and Atanassov A. (1999) Regeneration of diploid annual medics via direct somatic embryogenesis promoted by thidiazuron and benzylaminopurine. *Plant Cell Rep.* 18: 904-910.

Indian Coffee (1994) Facts about Indian coffee. 58(2): 28-30.

Indian Coffee (2000) Caffeine: physiological effects of caffeine and decaffeination. LXIV: 13-17.

International Coffee Organization (2003) Available from Internet (http://www.ico.org).

Jayaraman J. (1996) Chlorophylls in leaf development. *In*: Laboratory manual in Biochemistry. New Delhi, New Age International pp. 171-172.

John M. (1993) India, a source of unique speciality coffees. *Indian Coffee* 57(4): 17.

Jones J., Wert V. F. and Ries S. K. (1979) Specificity of 1-triacontanol as a growth stimulator and inhibition of its effect by other long-chain compounds. *Planta* 144: 277-292.

Kalberer P. (1965) Breakdown of caffeine in leaves of *Coffea arabica* L. *Nature* 205: 597-598.

Kato A., Crozier A. and Ashihara H. (1998) Subcellular localization of the *N*-3-methyl-L-transferase involved in caffeine biosynthesis in tea. *Phytochem.* 48: 777-779.

Kato M., Mizuno K., Fujimura T., Iwama M., Irie I., Crozier A. and Ashihara H. (1999) Purification and characterization of caffeine synthase from tea leaves. *Plant Physiol.* 120: 579-586.

Kawalleck P., Plesch G., Hahlbrock K. and Somssich I.E. (1992) Induction by fungal elicitor of *S*-adenosyl-L-methionine synthetase and *S*-adenosyl-L-homocysteine hydrolase mRNAs in cultured cells and leaves of *Petroselinum crispum*. *Proc. Nat. Acad. Sci.* 89: 4713-4717.

King F. J. (1980) Chemical engineering in coffee technology. *In*: Proceed. 9th International Scientific Colloquium on Coffee, ASIC, Paris pp. 237-50.

Komeda A. Y. and Yamada H. (1993) Microbial production of theobromine from caffeine. *Biosci. Biotech. Biochem.* 57: 1286-1289.

Koshiishi C., Kato A., Yama S., Crozier A. and Ashihara H. (2001) A new caffeine biosynthetic pathway in tea leaves: utilization of adenosine released from the *S*-adenosyl-L-methionine cycle. *FEBS Letters* 499: 50-54.

Krens F. A., Molendijk L., Wullens G. J. and Schilperoort R. A. (1982) *In vitro* transformation of plant protoplasts with Ti-plasmid DNA. *Nature* 296: 72-74.

Krikorian A. D. and Smith D. L. (1991) Somatic embryogenesis in carrot (*Daucas carota*). *In*: K. Lindsey (ed.) Plant Tissue Culture Manual, supplement 3. Kluwer, Dordrecht pp. 1-32.

Krug C. A. (1937) Cytological observations in *Coffea* III. *J. Gen.*, 34: 399-414.

***Krug C. A.** (1963) Cultura e abuabacao do cafeeiro. Sao Paulo: Instituto Brasileiro de Potassa pp. 7-32.

Langenheim J. H. and Thimann K. V. (1982a) Organizing diversity: how plants are classified. *In:* Botany-Plant Biology and its Relation to Human Affairs. John Wiley and Sons, New York pp. 219-239.

Langenheim J. H. and Thimann K. V. (1982b) How the flowering plant is constructed. *In:* Botany-Plant Biology and its Relation to Human Affairs. John Wiley and Sons, New York pp. 50-79.

Laughlin R. G., Munyon R. L., Ries S. K. and Wert V.F. (1983) Growth enhancement of plants by femtomole doses of colloidally dispersed triacontanol. *Science* 219: 1219-1220.

Lazzeri P. A., Hildebrand D.F. and Collins G.B. (1987) Soybean somatic embryogenesis. I. Effects of hormones and culture manipulations. *Plant Cell Tiss. Org. Cult.* 10:197-208.

***Leliveld J. A.** (1938) Vruchtzetting bij koffie (Fruit setting in coffee), *Arch. Koffiecult. Ned. Ind.* 12: 127-161.

***Leroy J. F.** (1962) 'Coffeae novae madagascariensis et mauritianae', *Journal d'Agriculture Tropicale et de Botanique Appliqu*ée 9(11, 12): 525-530.

***Leroy J. F.** (1980) 'Evolution et taxogenèse chez les caféiers. Hypothese sur leur origine', *Comptes Rendus de l'Academie des Sciences,* Paris 291D: 593-596.

***Leroy T. and Charrier A.** (1989) Mise en place d'un programme de sélection récurrente réciproque chez *Coffea canephora* Pierre. 13ème Colloque Scientifique Internationale sur le Café. Paipa, ASIC, Paris p. 457.

Leroy T., Henry A. M., Royer M., Altosar I., Frutos R., Duris D. and Philippe R. (2000) Genetically modified coffee plants expressing the *Bacillus thuringiensis cry*1Ac gene for resistance to leaf miner. *Plant Cell Rep.* 19: 382-389.

***Leroy T., Royer M., Paillard M., Berthouly M., Spiral J., Tessereau S., Legavre T. and Altosaar I.** (1997) Introduction de genès d'interêt agronomique dans l'espèce *Coffea canephora* Pierre par transformation avec *Agrobacterium sp. In*: Proceed. 17th International Scientific Colloquium on Coffee, ASIC Paris, France pp. 439-446.

Lindsey K. and Yeoman M. M. (1983) The relationship between growth rate, differentiation and alkaloid accumulation in cell cultures. *J. Exp. Bot.* 34:1055-1065.

***Linnaeus C.** (1737a) Corollarium generum plantarum exhibens genera plantarum sexaginta, addenda prioribus characteribus expositis in generibus plantarum. Accedit methodus sexualis, Leiden.

***Linnaeus C.** (1737b). Critica botanica, in qua nomina plantarum generica, specifica & variantia examini subjiciuntur, selectiora confirmantur, indigna rejiciuntur; simulque doctrina circa denominationem plantarum traditur. Seu Fundamentorum

botanicorum pars IV. Accedit J. Browallii De necessitate historiae naturalis discursus, Leiden.

Lippmann B. and Lippmann G. (1984) Induction of somatic embryos in cotyledonary tissue of soybean, *Glycine max* L. Merr. *Plant Cell Rep.* 3: 25-218.

***Lobreau-Callen D. and Leroy J. F.** (1980) 'Quelques données palynologiques sur le genre *Coffea* et autres du cercle des caféiers', *In*: Proceed. 9th International Scientific Colloquium on Coffee, ASIC Paris pp.479-506.

Loh C. S. and Rao A. N. (1989) Clonal propagation of guava (*Psidium guajava* L.) from seedling and grafted plants and adventitious shoot formation *in vitro*. *Scientia Hort.* 39: 31-39.

Looser E., Baumann T. W. and Wanner H. (1974) The biosynthesis of caffeine in the coffee plant. *Phytochem.* 13: 2515-2518.

Lorz H., Baker B. and Schell J. (1985) Gene transfer to cereal cells mediated by protoplast transformation. *Mol. Gen. Genet.* 199: 178-182.

Lu C. Y. (1993) The use of thidiazuron in tissue culture. *In Vitro Cell. Dev. Biol.* 29: 92-96.

Ma F. W., Wang J. C. and Rong W. (1990) Effects of plant growth regulator on *in vitro* propagation of apple cultivation. *J. Fruit Sci.* 7: 201-206.

Madhava Naidu M. and Sreenath H. L. (1999) *In vitro* culture of zygotic embryos for germplasm preservation. *Plant Cell Tiss. Org. Cult.* 55: 227-230.

Madhava Naidu M., Veluthambi K., Srinivasan C. S. and Naidu R. (1998) *Agrobacterium* mediated transformation in *Coffea canephora*. *Dev. Plantn Crops Res.* 46-50.

Madhava Naidu M., Samuel Ganesh D., Jayashree G. and Sreenath H. L. (1999) Effect of genotype on somatic embryogenesis and plant regeneration in arabica coffee. *In*: P. B. Kavi Kishor (Ed.) Plant Tissue Culture and Biotechnology-*Emerging Trends,* Universities Press (India) Limited pp. 90-95.

***Maestri M. and Vieira C.** (1961) Nota sobre a reduçao da porcentagem de germinaçao de sementes de café (*Coffea arabica* var bourbon), por efeito de ácido giberélico. *Rev. Ceres* 65: 237-249.

Mamat A. S. B., Fontenot J. F. and Newsom D. W. (1983) The effects of triacontanol on the growth and development of Tabasco pepper. *Hort. Science* 18: 247-249.

Marshal C. F. (1985) World Coffee Trade. *In*: M. N. Clifford and K. C. Willson (eds.) Coffee-Botany, Biochemistry and Production of Beans and Beverage. The avi publishing company, Inc. Westport, Connecticut pp. 251-283.

Martin H. (1982) Selective extraction of caffeine from green coffee beans and the application of similar processes on other natural products. *In*: Proceed. 10th International Scientific Colloquium on Coffee, ASIC Paris p. 21.

Martin R., Lilley T. H., Falshaw P., Haslam E., Begley M. J. and Magnolato D. (1987) The caffeine-potassium chlorogenate molecular complex. *Phytochem.* 26: 273-279.

Mazzafeara P. and Carvalho A. (1992) A breeding for low seed caffeine content of coffee (*Coffea* L.) by interspecific hybridization. *Euphytica* 59: 55-60.

***Mazzafera P.** (1990) Estudo sobre o papel da cafeína em plântulas de café (*Coffea arabica* L.) *Rev. Bras. Bot.* 13: 97-102.

***Mazzafera P.** (1991) Análises químicas em folhas de cafeeiros atacados por *Atta* spp. *Rev. Agric.* 66: 33-45

Mazzafera P. and Goncalves K. V. (1999) Nitrogen compounds in the xylem sap of coffee. *Phytochem.* 50: 383-386.

***Mazzafera P. and Magalhães A. C. N.** (1991) Cafeína em folhas e sementes de *Coffea* e *Paracoffea*. *Rev.Bras.Bot.* 14: 157-160.

Mazzafera P., Crozier A. and Magalhães A. C. N. (1991) Caffeine metabolism in *Coffea arabica* and other species of coffee. *Phytochem.* 30: 3913-3916.

Mazzafera P., Crozier A. and Sandberg G. (1994b) Studies on the metabolic control of caffeine turnover in developing endosperms and leaves of *Coffea arabica* and *Coffea dewevrei*. *J. Agric. Food Chem.* 42: 1423-1427.

Mazzafera P., Wingsle G., Olsson O. and Sandberg G. (1994a) *S*-adenosyl-L-methionine: theobromine 1-*N*-methyltransferase, an enzyme catalyzing the synthesis of caffeine in coffee. *Phytochem.* 37: 1577-1584.

McKey D. (1979) The distribution of secondary compounds within plants. *In*: G. A. Rosenthal and D. H. Janzen (eds.) Herbivores: Their Interaction with Secondary Plant Metabolites. Academic Press, San Diego pp. 56-133.

Medeiros M. A. P. X. L., Guedes M. E. M. and Sousa M. L. B. (1990) Has caffeine a role in the resistance of coffee to orange rust? *In*: Proceed. 13th International Scientific Colloquium on Coffee, ASIC Paipa, Colômbia pp. 733-744.

Medina H. P., Carvalho A., Sondahl M. R., Fazuoli L. C. and Costa W. C. (1984) Coffee breeding and related evolutionary aspects. *Pl. Breed. Rev.* 2: 157-193.

Mendes A. J. T. (1941) Cytological observations in *Coffea*. VI – Embryo and endosperm development in *Coffea arabica* L. *Amer. J. Bot.* 28: 784-789.

Menéndez-Yuffá A. and de Garcia E. G. (1997) Morphogenic events during indirect somatic embryogenesis in coffee 'Catimor'. *Protoplasma* 199: 208-214.

Menéndez-Yuffá A., de Garcia E. G. and Nieto M. S. (1994) Comparative study of protein electrophoretic patterns during embryogenesis in *Coffea arabica* cv Catimor. *Plant Cell Rep.* 13: 197-202.

Mizuno K., Kato M., Irino F., Yoneyma N., Fujimura T. and Ashihara H. (2003a) The first committed step reaction of caffeine biosynthesis: 7-methylxanthosine synthase is closely homologous to caffeine synthases in coffee (*Coffea arabica* L.). *FEBS Lett.* 547(1-3): 56-60.

Mizuno K., Okuda A., Kato M., Yoneyama N., Tanaka H., Ashihara H. and Fujimora T. (2003b) Isolation of a new dual-functional caffeine synthase gene encoding an enzyme for the conversion of 7-methylxanthine to caffeine from coffee (*Coffea arabica* L.). *FEBS Lett.* 534(1-3): 75-81.

Moens P. (1965) Development et embryogenese chez *Coffea canephora* Pierre. *La Cellule* 63: 129-147.

Moisyadi S., Neupane K. R. and Stiles J. I. (1999) Cloning and characterization of xanthosine-*N*-7-methyltransferase, the first enzyme of the caffeine biosynthetic pathway. *In*: Proceed. 18th International Scientific Colloquium on Coffee, ASIC, Paris pp. 327-331.

Mok M. C., Mok D. W. S., Armstrong D. J., Shudo K., Isogai Y. and Okampto T. (1982) Cytokinin activity of N-phenyl-N'-1,2,3,-thidiazol-5-yl urea (thidiazuron). *Phyotochem.* 21: 1509-1511.

Monaco L. C. and Carvalho A. (1963) Coffee breeding XXIII: New data on the variability of isogenic lines in coffee (Port: Engl. Sum.) *Bragantia.* 22: 117-123.

***Monaco L. C. and Carvalho A.** (1964) Genetica de Coffea. Hibrido entre espécies (Genetics of *Coffea*. Interspecific hybrids) Ciéncia e Cultura. S. Paulo 16(2): 144.

Monaco L. C., Söndahl M. R., Carvalho A., Crocomo O. J. and Sharp W. R. (1997) Application of tissue culture in the improvement of coffee. *In*: Reinert J. and Bajaj Y. P. S. (eds.) Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture. Narosa Publishing House pp. 109-129.

Morel G. and Wetmore R.H. (1951) Tissue culture of monocotyledons. *Amer. J. Bot* . 38:138-140.

Muniswamy B. and Sreenath H. L. (1995a) High frequency somatic embryogenesis form cultured leaf explants of *Coffea canephora* on a single medium. *J. Coffee Res*. 25(2): 98-101.

Muniswamy B. and Sreenath H. L. (1995b) Standardization of encapsulation technique for producing synthetic seeds in coffee. *J. coffee. Res*. 25(1): 24-29.

Muniswamy B. and Sreenath H. L. (1996) Effect of kanamycin on callus induction and somatic embryogenesis in cultured leaf tissues on *Coffea canephora* Pierre (Robusta). *J. Coffee Res.* 26(1): 44-51.

Muniswamy B. and Sreenath H. L. (1999a) Embryo culture in coffee: technique and application. *Indian Coffee*, April pp. 7-9.

Muniswamy B. and Sreenath H.L. (1999b) Standardization of encapsulation technique for producing synthetic seeds in coffee. *J. Coffee Res*. 25(1): 24-29.

Muniswamy B., Naidu M. M. and Sreenath H. L. (1993) Somatic embryogenesis and plant regeneration from cultured immature zygotic embryos of *Coffea canephora* Pierre. *J. Plantn. Crops* 21 (Suppl.): 346-350.

Murashige T. and Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-479.

Nakamura T., Taniguchi and Maeda E. (1992) Studies on somatic embryogenesis of coffee by scanning electron microscope. *Japan J. Crop Sci.* 61: 476-486.

Nathanson J. A. (1984) Caffeine and related methylxanthines: possible naturally occurring pesticides. *Science* 226: 184-187.

Navellier P. (1961) Compositions moyenne du café vert. *Les caféiers et les cafés dans le monde. In:* R. Coste (ed.), Larose (1955-61) 2: 163-220.

Negishi O., Ozawa T. and Imagawa H. (1985a) Conversion of xanthosine into caffeine in Tea plants. *Agric. Biol. Chem.* 49: 251.

Negishi O., Ozawa T. and Imagawa H. (1985b) Methylation of xanthosine by tea-leaf extracts and caffeine biosynthesis. *Agric. Biol. Chem.* 49: 887.

Neuenschwander B. and Baumann T. W. (1992) A novel type of somatic embryogenesis in *Coffea arabica. Plant Cell Rep.* 10: 608-612.

Nishibata T., Azuma T., Uchida N., Yasuda T. and Yamaguchi T. (1995) Amino acids on somatic embryogeneis in *Coffea arabica*. *In*: Proceed. 16th International Scientific Colloquium on Coffee, ASIC Kyoto pp. 839-844.

Noriega C. and Söndahl M. R. (1993) Arabica coffee micropropagation through somatic embryogenesis via bioreactors. *In*: Proceed. 15th International Scientific Colloquium on Coffee, ASIC, Montpellier, France pp. 73-81.

Nyange N. E., Williamson B. and McNicol R. J. (1997) Plant regeneration from suspension culture protoplasts established from hypocotyls-derived callus of two *Coffea arabica* genotypes. *In*: Proceed. 17th International Scientific Colloquium on Coffee, ASIC Nairobi pp. 779-782.

Nyange N. E., Williamson B., McNicol R. J. and Hackett C. A. (1995) *In vitro* screening of coffee genotypes for resistance to coffee berry disease (*Colletrichum kahawae*). *Ann. Appl. Biol.* 127: 251-261.

Nyanje N. E., Mc Nicol R. J., Williamson B. and Lyon G. D. (1993) High frequency induction and regeneration of multiple shoots from hypocotyl segments of *Coffea arabica*. *In*: Proceed. 15th International Scientific Colloquium on Coffee, ASIC Montpellier pp.123-132.

Obatolu C. R. (1999) Use of humic acid in promoting growth of young coffee robusta seedlings in Nigeria. *In*: Proceed. 18th International Scientific Colloquium on Coffee, ASIC Helsinki pp. 449-451.

Ocampo C. A. and Manzanera L. M. (1991) Advances in genetic manipulation of the coffee plant. *In*: Proceed. 14th International Scientific Colloquium on Coffee, ASIC San Francisco, USA pp. 378-382.

Ogawa M., Herai Y., Koizumi N., Kusano T. and Sano H. (2001) 7-methylxanthine methyltransferase of coffee plants. *J. Biol. Chem.* 276: 8213-8218.

Ogita S., Uefuji K., Choi Y. E., Hatanaka T., Ogawa M., Yamaguchi Y., Koizumi N. and Sano H. (2002) Genetic modification of coffee plants. *J. Plant Biotech.* 4(3): 91-94.

Ogutuga D. B. A. and Northcote D.H. (1970) Caffeine formation in tea callus tissue. *J. Exp. Botany* 21(67): 258-273.

Ohta S., Mita S., Hattori T. and Nakamura K. (1990) Construction and expression in tobacco of a *β*-glucuronidase (GUS) reporter gene containing an intron within the coding sequence. *Plant Cell Physiol.* 31:805-814.

Opile W. R. (1977) Effect of gibberelic acid on yield of arabica coffee in Kenya. *Kenya Coffee* 42: 395-403.

***Pagacz E. A.** (1960) Contribution a l'étude du mode de semis en caféiculture. INEAC (Belgique), Bul. Inf. 16: 1-6.

Paillard M., Lashermes P. and Petiard V. (1996) Construction of molecular linkage map in coffee. *Theor. Appl. Genet.* 93: 41-47.

Pan-American Coffee Bureau (1973) Annual Coffee Statistics, Newtype Inc., Wellington, New Jersey 37.

Pena M. de (1984) Somatic embryo induction and plant regeneration from *Coffea canephora* and *Coffea arabica*. Symposium Ferrugen Café, CIFC, Oeiras, Portugal pp. 494-512.

Pierson E. S., Van Lammeren A. A. M., Schell J. H. N. and Staritsky G. (1983) *In vitro* development of embryoids from punched leaf discs of *Coffea canephora*. *Protoplasma* 115: 208-216.

Prabhuji S. K., Srivastava G. C., Rizvi S. J. H. and Mathur S.N. (1983) 1,3,7-Trimethyl xanthine (caffeine), a new natural fish fungicide. *Experientia* 39: 177-179.

Quiroz-Figueroa F., Méndez-Zeel, Larqué-Saavedra A. and Loyola-Vargas V. M. (2001) Picomolar concentrations of salicylates induce cellular growth and enhance somatic embryogenesis in *Coffea arabica* tissue culture. *Plant Cell Rep.* 20: 679-684.

Qureshi J. A. and Saxena P. K. (1992) Adventitious shoot induction and somatic embryogenesis with intact seedlings of several hybrid seed geranium (*Pelargonium X hortorum* Biley) Varieties. *Plant Cell Rep.* 11: 443-448.

Raghuramulu Y., Purushotham K., Sreenivasan M. S. and Ramaiah P. K. (1987) *In vitro* regeneration of coffee plantlets in India (Brief note). *J. Coffee Res.* 17(2): 57-64.

Raghuramulu Y., Sreenivasan M. S. and Ramaiah P. K. (1989) Regeneration of coffee plantlets through tissue culture techniques in India. *J. Coffee Res.* 19(1): 30-38.

Rajasekaran P. and Raman, K. (1993) Propagation of tea by *in vitro* culture of nodal explants. *J. Plantn Crops* 21 (1): 8-14.

Raju K. I. and Gopal N. H. (1979) Distribution of caffeine in arabica and robusta coffee plants. *J. Coffee Res.* 9: 83-90.

Ram A. S., Indu E. P., Chandrashekar A., Ganesh D. and Ravishankar G. A. Breeding low caffeine coffees (in preparation).

Ram A. S., Ramachandran M. and Sreenivasan M. S. (1981) Pollen fertility of the interspecific hybrids of coffee. *In*: PLACROSYM IV Vishveshwara S. (Ed.). Indian Society for Plantation Crops, Kasaragod, India pp. 406-412.

Ramaiah P. K. and Venkataraman D. (1988) Studies on the effect of gibberelic acid on arabica coffee in India. *J. Coffee Res.* 18(1): 47-51.

Reddy A. G. S., Sreenivasan M. S. and Ahmad J. (1991) Isolation barriers of diploid species (*Coffea racemosa* Lour and *Coffea canephora* Pierre) as reflected in their hybrids. *In*: PLACROSYM VIII Premkumar T., Sarma Y. R., Ravindran P. N. and Madhusoodanan K. J. (Eds.) *J. Plantn. Crops* 18 (Suppl.): 24-30.

Reddy B. D. R., Shivaprasad P. and Naidu R. (2001) Cost of production of robusta coffee in major coffee growing regions of India. *J. Coffee Res.* 29 (1&2): 41-49.

Reddy B.O., Giridhar P. and Ravishankar G.A. (2002) The effect of Triacontanol on Micropropagation of *Capsicum frutescens* and *Decalepis hamiltonii* W & A. *Plant Cell Tiss. Org. Cult.* 71: 253-258.

Reeve R. M. (1968) Histochemical differentiation between tyrosine and chlorogenic acid in plant tissues. I. Nitrous acid reactions and metal chelation of nitrosotyrosine. *J. Histochem. Cytochem.* 16: 191-198.

Rhoades D. F. (1979) Evolution of plant chemical defence strategy against herbivores. Chapter 1. *In*: G. A Rosenthal and D. H. Janzen (eds.) Herbivores-Their Interaction with Secondary Plant Metabolites. Academic Press, London p. 3.

Ries S, K. and Wert V. F. (1977) Growth response of rice seedlings to triacontanol in light and dark. *Planta* 135: 77-82.

Ries S. K. and Houtz R. L. (1983) Triacontanol as a plant growth regulator. *Hort. Science* 18: 654-662.

Ries S. K., Wert V. F., Sweely C. C. and Leavitt R. A. (1977) Triacontanol: a new naturally occurring plant growth regulator. *Science* 195: 1337-1341.

Ries S., Wert V., Biernbaum J. A., Gibson T. and Bardley W. J. (1983) Factors alternating response of plants to triacontanol. *Hort. Science*. 108: 917-922.

Ries S. K., Wert V. F. and Biernbaum J. A. (1984) Interference of triacontanol activity by chemical constituents present in experimental and field sprayers. *J. Amer. Soc. Hort Sci.* 109: 145-150.

Rizvi S. J. H., Mukerji D. and Mathur S. N. (1980) A new report on a possible source of natural herbicide. *Indian J. Exp. Biol.* 18: 777-778.

Rizvi S. J. H., Rizvi V. and Mukerjee D. (1987) 1,3,7-Trimethylxanthine, an allelochemical from seeds of *Coffea arabica* : some aspects of its mode of action as a natural herbicide. *Plant Soil* 98: 81-91

Robbrecht E. and Puff C. (1986) A survey of the *Gardenieae* and related tribes (*Rubiaceae*). *Bot. Jahrb. Syst.* 108: 63-137.

Roberts M. F. and Waller G. R. (1979) *N*- methyltransferases and 7-methyl- N^9-nucleoside hydrolase activity in *Coffea arabica* and the biosynthesis of caffeine. *Phytochem.* 18: 451-455.

Rosillo A. G., Acuna J. R., Gaitan A. L. and De Pena M. (2003) Optimized DNA delivery into *Coffea arabica* suspension culture cells by particle bombardment. *Plant Cell Tiss. Org. Cult.* 74: 45-49.

Sambrook J. and Russell D. W. (2001) Preparation ofplasmidDNA by alkaline lysis with SDS: Minipreparation. *In*: Molecular Cloning – a Laboratory Manual. 3rd Edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York pp. 1.32-1.34.

Samuel Ganesh D. and Sreenath H. L. (1996) Silver nitrate enhanced shoot development in cultured apical buds of *Coffea arabica* cv. Cauvery (S. 4347). *J. Plantn. Crops* 2(Supplement): 577-580.

Samuel Ganesh D. and Sreenath H. L. (1999) Direct and Indirect plant regeneration in coffee. *J. Plantn. Crops* 27(3): 216-220.

Sauer M., Kappeli O. and Fiechter A. (1982) Comparison of the cytochrome P-450 containing monooxygenases originating from two different yeasts. *Developments in Biochem*. 23: 452-457.

Schulthess B. H. and Baumann T. W. (1995) Stimulation of caffeine biosynthesis in suspension-cultured coffee cells and the *in situ* existence of 7-methylxanthosine. *Phytochem.* 38: 1381-1386.

Schulthess B. H., Morath P. and Baumann T. W. (1996) Caffeine biosynthesis starts with the metabolically channeled formation of 7-methyl-XMP – a new hypothesis. *Phytochem.* 41: 169-175.

Schwimmer S., Kurtzman R. H. and Heftmann E. (1971) Caffeine metabolism by *Penicillium roqueforti*. *Arch. Biochem. Biophy*. 147: 109-113.

Seneviratne P. and Wijesekara G. A. S. (1996) The problem of phenolic exudates in *in vitro* cultures of mature *Hevea brasiliensis*. *J. Plantn. Crops* 24: 54-62.

Sideso O. F. P., Marvier A. C., Katerelos N. A. and Goodenough P. W. (2001). The characteristics and stabilization of caffeine demethylase enzyme complex. *Int. J. Food Sci. Tech.* 36: 693.

Sivetz M. and Foote H. E. (1963) Coffee processing technology. Westport, Conn: Avi Publ. Vol. I.

Smith R. F. (1985) A history of coffee. *In*: M. N. Clifford and K.C. Willson (eds.) Coffee - Botany, Biochemistry and Production of Beans and Beverage. The avi publishing company, Inc. Westport, Connecticut pp. 1-12.

Söndahl M. R. and Lauritis J. A. (1992) Coffee. *In*: R. E. Hammerschlog and R. E. Litz (eds.) Biotechnology of Perennial Fruit Crops. pp. 401-420.

Söndahl M. R. and Loh W. H. T. (1988) Coffee Biotechnology *In*: Coffee Agronomy, Vol.4, R. J. Clarke and R. Macrae (eds.) Elsevier Applied Science, London pp. 236-262.

Söndahl M. R. and Sharp W. (1977) High frequency induction of somatic embryos in cultured leaf explants of *Coffea arabica* L. *Z. Pflanzen*. 81: 395-408.

Söndahl M. R. and Sharp W. R. (1979) Research in *Coffea* spp. and applications of tissue culture methods. *In:* W. R. Sharp, P. O. Larsen, E. F. Paddock and V. Raghavan (eds.) Plant Cell and Tissue Culture Principles and applications. Ohio State University Press, Columbus pp. 527-584.

Söndahl M. R., Nakamura T. and Sharp W. R. (1985) Propagation of coffee. *In*: R. R. Henke, K. W. Hughes, M. J. Constantin and A. Hollaender (eds.) Tissue culture in Forestry and Agriculture. Plenum, New York pp. 215-232.

Söndahl M. R., Spahlinger A. and Sharp W. R. (1979) A histological study of high frequency and low frequency induction of somatic embryos in cultured leaf explants of *Coffea arabica* L. *Z.Pflanzenphysiol.* 94: 101-108.

Spoladore D. S., Boaventura M. A. M. and Saes L. A. (1987) Teor de cafeina em sementes de matrizes do guaranazeiro. *Bragantia* 46: 425-429.

Sreenath H. L. and Naidu R. (1997) Quarter century of research in coffee biotechnology-A review. *In:* G. A. Ravishankar and L. V. Venkataraman (eds.) Recent

Advances in Biotechnological Applications of Plant Tissue and Cell Culture. Oxford & IBH Publishing Co. Pvt. Ltd., New Delhi and Calcutta pp. 14-27.

Sreenath H. L. (2000) Biotechnology in the improvement of coffee. *In*: K. L. Chadha, P. N. Ravindran and Leela Sahijram (eds.) Biotechnology in Horticultural and Plantation Crops. Malhotra Publishing House, New Delhi India pp. 310-322.

Sreenath H. L., Muniswamy B., Naidu M. M., Dharmaraj P. S. and Ramaiah P. K. (1992) Embryo culture of three interspecific crosses in coffee. *J. Plantn. Crops* 20 (Suppl.): 243-247.

Sreenath H. L., Muniswamy B., Naidu M. M., Dharmaraj P. S. and Ramaiah P. K. (1992) Immature embryo culture in coffee from three interspecific crosses. *In*: PLACROSYM IX Nambiar K. K. N., Iyer R. D., Rao E. V. V. B., Rao W. K. Premkumar T. and Hegde M. R. (eds.) *J. Plantn. Crops* 20 (Suppl.): 243-247.

Sreenath H. L., Muniswamy B., Raghramulu Y and Ramaiah P. K. (1989) Immature embryo culture in coffee. *In*: Proc. XIII Plant Tissue Culture Conference, Shillong, Abstact p. 17.

Srinivasan C. S. (1996) Current status and future thrust areas of research on varietal improvement and horticultural aspects of coffee. *J. Coffee. Res.*, 26(1), 1-16.

Staritsky G. (1970) Embryoid formation in callus cultures of coffee. *Acta Bot. Neer.* 19: 509-514.

Staritsky G. and Van Hassel. (1980) The synchronized mass propagation of *Coffea canephora in vitro*. In: Proceed. 9th International Scientific Colloquium on Coffee. ASIC, Paris pp. 597-602.

Stautemyer V. and Cook R. (1987) Update on Triacontanol. *The Orchid Advocate* 13: 86-87.

Stewart I. (1985) Identification of caffeine in citrus flowers and leaves. *J. Agric. Food. Chem.* 33: 1163-1165.

***Suárez de Castro F.** (1960) Escogencia y tratamiento de la semilla de café. *Agric.Trop.*, Columbia 15: 715-724.

Sugiyama M., Matsuoka C. and Takagi T. (1995) Transformation of coffee with *Agrobacterium rhizogenes*. *In*: Proceed. 16th International Scientific Colloquium on Coffee, ASIC, Paris, France pp. 853-859.

Suzuki T. and Takahashi E. (1975) Biosynthesis of caffeine by tea-leaf extracts - enzymic formation of theobromine from 7-methylxanthine and of caffeine from theobromine. *Biochem. J.* 146: 87-96.

Suzuki T. and Takahashi E. (1976) Caffeine biosynthesis in *Camellia sinensis*. *Phytochem.* 15: 1235.

Suzuki T. and Waller G. R. (1984a) Biosynthesis and biodegradation of caffeine, theobromine and theophylline in *Coffea arabica* L. fruits. *J. Agric. Food Chem.* 32: 845-848.

Suzuki T. and Waller G. R. (1984b) Biodegradation of caffeine: formation of theophylline and theobromine from caffeine in mature *Coffea arabica* fruits. *J. Agric. Food Chem.* 35: 66-70.

Suzuki T. Ashihara H. and Waller G. R. (1992) Purine and purine alkaloid metabolism in *Camellia* and *Coffea* plants. *Phytochem.* 31: 2575-2584.

Swain T. (1977) Secondary compounds as protective agents. *Ann. Rev. Plant Physiol.* 28: 479-501.

Tahara M., Nakanishi T., Yasuda T. and Yamaguchi T. (1995) Histological and biological aspects in somatic embryogenesis of *Coffea arabica*. *In*: Proceed. 16th International Scientific Colloquium on Coffee. ASIC, Kyoto 860-867.

Takaki M. and Dietrich S. M. C. (1980) Effect of GA_3 and light on polysaccharide levels and metabolism in germinating coffee seeds. *J. Exp. Bot.* 31: 1643-1649.

Takaki M., Dietrich S. M. C. and Furtado J. S. (1979) Anatomical changes in hard endosperm of gibberelic acid-treated coffee seeds during germination. *Rev. Bras. Bot.* 2: 103-106.

Tantos A., Meszaros A. and Kissimon J. (1999) The effect of triacontanol on micro propagation of balm *Melissa officinalis* L. *Plant Cell Rep.* 19: 88-91.

The Wealth of India (1950) A dictionary of Indian raw materials and industrial products. Raw materials. National Institute of Science Communication, CSIR, New Delhi, India 3: 288-305.

The Wealth of India (1992) A dictionary of Indian raw materials and industrial products. First supplement series (Raw materials) National Institute of Science Communication, CSIR, New Delhi, India 2 (CI-CY): 120-149.

Thomas A S. (1944) The wild coffees of Uganda. *Empire Journal of Experimental Agriculture* 12, 1-12.

Uefuji H., Ogita S., Yamaguchi Y., Koizumi N. and Sano H. (2003) Molecular cloning and functional characterization of three distinct *N*-methyltransferases involved in the caffeine biosynthetic pathway in coffee plants. *Plant Physiol.* 132: 372-380

Válio I. F. M. (1976) Germination of coffee seeds (*Coffea arabica* L. cv Mundo Novo) *J. Exp. Bot.* 17: 983-991.

Van Boxtel J. and Berthouly M. (1996) High frequency somatic embryogenesis from coffee leaves: Factors influencing embryogenesis, and subsequent proliferation and regeneration in liquid medium. *Plant Cell Tiss. Org. Cult.* 44: 7–17.

Van Boxtel J., Berthouly M., Carasco C., Dufour M. and Eskes A. (1995) Transient expression of *β*-glucuronidase following biolistic delivery of foreign DNA into coffee tissues. *Plant Cell Rep.* 14: 748-752.

Van Boxtel J., Berthouly M., Carasco C., Dufour M. and Eskes A. (1995) Transient expression of *β*-glucuronidase following biolistic delivery of foreign DNA into coffee tissues. *Plant Cell Rep.* 14: 748-752.

Van Boxtel J.H. (1994) Studies on genetic transformation of coffee by using electroporation and biolistic method. Abstract available from Internet: http://www.agralin.nl/wda/abstracts/ab1880.html.

Vasanthi S. and Bhat R. V. (2000) Ochratoxin in Indian coffee and its implication to international trade. *Nutrition News*, National Institute of Nutrition, Hyderabad, India Vol.21, No. 4 Oct.

Vishveswara S. and Raju K. S. (1973) Seed germination in *Arabica* coffee. II.Effect of placement of seeds on germination. *Indian Coffee* 37: 286-288.

Visser C., Aureshi J. A., Gill R., Saxena P. K., Visser C., Aureshi J. A. and Gill R. (1992) Morphoregulatory role of thidiazuron: substitution of auxin and cytokinin requirement for the induction of somatic embryogenesis in geranium hypocotyls cultures. *Plant Physiol.* 99, 1704-1707.

Waldhausser S. S. M. and Baumann T. W. (1996) Compartmentation of caffeine and related purine alkaloids depends exclusively on the physical chemistry of their vacuolar complex formation with chlorogenic acids. *Phytochem.* 42: 985-996.

Waldhausser S. S. M., Kretschmar J. A. and Baumann T. W. (1997a) *N*-methyltransferase activities in caffeine biosynthesis: biochemical characterization and time course during leaf development of *Coffea arabica*. *Phytochem.* 44: 853-859.

Waldhausser S. S. M., Gillies F. M., Crozier A. and Baumann T. W. (1997b) Separation of the *N*-7 methyltransferase, the key enzyme in caffeine biosynthesis. *Phytochem.* 45: 1407-1414.

Waller G. R., Kumari D., Friedman J. Friedman N. and Chou C. –H. (1986) Caffeine autotoxicity in *Coffea arabica* L. *In*: A.R. Putnam and C. –S. Tang (eds.) The Science of Allelopathy. John Wiley & Sons, New York pp. 243-269.

Waller G. R., MacVean C. D. and Suzuki T. (1983) High production of caffeine and related enzyme activities in callus cultures of *Coffea arabica* L. *Plant Cell Rep.* 2: 109-112.

Waller J. M. (1987) Coffee diseases current status and recent developments. *In*: S. P. Raychaudhari and J. P. Verma (eds.) Review of Tropical Plant Pathology. New Delhi, India, Today and Tomorrow's Printers and Publishers 4: 1-33.

Yamaoka-Yano D. M. and Mazzafera P. (1997) Degradation of caffeine by *Pseudomonas putida* isolated from soil. *Allelopathy J.* 5: 23-34.

Yamaoka-Yano D. M. and Mazzafera P. (1999) Catabolism of caffeine and purification of a xanthine oxidase responsible for methyluric acids production in *Pseudomonas putida* L. *Revista de Microbiologia* 30: 62-70.

Yasuda T., Fujii Y. and Yamaguchi T. (1985) Embryogenic callus induction from *Coffea arabica* leaf explants by benzyladenine. *Plant Cell Physiol.* 26(3): 595-597.

Yasuda T., Tahara M., Hatanaka T., Nishibata T. and Yamaguchi T. (1995) Clonal propagation through somatic embryogenesis of coffee species. *In*: Proceed. 16th International Scientific Colloquium on Coffee. ASIC, Kyoto 537-541.

***Zamarripa A., Ducos J. P., Tessereau H., Bollon H., Eskes A. B. and Petiard V.** (1991) Developpment d'un procéde de multiplication en masse du caféier par embryogénese somatique au milieu liquide. *In*: Proceed. 14th International Scientific Colloquium on Coffee, ASIC San Fransisco pp. 392-402.

*Originals not seen