AN ABSTRACT OF THE THESIS OF

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Title: *In Vitro* Culture of *Coffea* Species

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The major cultivated form of coffee, *Coffea arabica*, is susceptible to rust (*Hemileia vastatrix)* and coffee berry disease (*Colletotrichum coffeanum*). Sources of resistance have been identified in other *Coffea* species, *C. canephora, C. congensis, C. liberica,* and *C. stenophylla*. Attempts to transfer desirable traits via interspecific crosses have not been successful primarily due to low fertility of the resulting hybrids. Somatic fusion may be an alternative for gene transfer. Some prerequisites for this approach, chiefly somatic regeneration and isolation of protoplasts, were examined in the present study.

Plantlet regeneration via somatic embryogenesis was achieved on a modified Murashige and Skoog medium supplemented with kinetin (5 μM) and α-naphthaleneacetic acid (2.5 μM). The frequency of embryogenesis was highest

for *C. canephora* (27-53%), intermediate for *C. congensis* (39%), and low on *C. arabica* (0-14%). The embryogenic property of the tissues was maintained by subculturing either the sômatic embryos or callus to fresh medium supplemented with kinetin (5 μM) and indole-3-butyric acid (1 μM). Histological studies indicated that somatic embryos were anatomical similar to zygotic embryos.

Appropriate combinations of enzymes were devised to isolate protoplasts from leaves, callus and somatic embryos. Cellulase (1%), hemicellulase (1%) and pectolyase (0.03%) with glucose (0.5 M) as the osmoticum gave high yield of protoplasts from the young leaves. Cellulase (3%), driselase (1%), and pectolyase (0.3%) with mannitol (0.6 M) as osmoticum were needed for the release of protoplasts from callus tissues. Protoplasts were isolated from somatic embryos using cellulase (3%), macerozyme (1%) and pectolyase (0.3%). Cell wall regeneration was routinely observed. However, cell division occurred only in callus derived protoplasts. Protoplasts of different tissue origin can be used to develop protocols for cell fusion and selection of heterokaryons.

In Vitro Culture of *Coffea* Species

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ABBREVIATIONS

B5:	Gamborg *et al.* medium (1968)
BAP:	N^6-benzyladenine
BSA:	bovine serum albumin
2,4-D:	2,4-dichlorophenoxyacetic acid
DMSO:	dimethyl sulfoxide
GA_3:	gibberellic acid
IAA:	indole-3-acetic acid
IBA:	indole-3-butyric acid
KM8P:	Kao and Michayluk (1975)
LS:	Linsmaier and Skoog (1965)
MES:	2-(N-morpholino)ethanesulfonic acid
MS:	Murashige and Skoog medium (1962)
NAA:	α-naphthaleneacetic acid
PVP:	polyvinylpyrrolidone
SH:	Schenk and Hildebrandt (1972)

IN VITRO CULTURE OF *COFFEA* SPECIES

I. INTRODUCTION

The genus *Coffea* (family Rubiaceae) comprises over 80 species of which two species, *C. arabica* (2n=4x=44) and *C. canephora* (2n=2x=22), account for 70% and 30% of the world coffee crop respectively. *C. arabica* produces the highest quality coffee, but the primary plant breeding goal of improving yield is hindered by the narrow genetic base of *C. arabica*. *C. arabica* is the only self-pollinating and tetraploid species in the entire *Coffea* genus (Charrier and Berthaud, 1985). All *C. arabica* cultivars lack resistance to major fungal diseases, and nematode and insect pests. Therefore, transfer of desirable traits from other *Coffea* species is of major importance in coffee breeding. Sexual hybridization and somatic fusion are two possible approaches.

Useful diploid sources include *C. canephora*, a higher yielding cultivated form with less desirable flavor and which contains twice the caffeine of *C. arabica* (Van der Vossen, 1985). Therefore, *C. canephora* is used primarily for instant beverages and for blending. Wild *Coffea* species are additional sources for pest resistance, tolerance to environmental stress and varying caffeine

content (Van der Vossen, 1985). For example, resistance to the insect *Leucoptera meyricki* is found in six species of *Coffea; C. racemosa* is drought tolerant; and five species of *Eucoffea* have very low caffeine content.

Attempts to transfer useful traits via interspecific (and interploidy) crosses between *C. arabica* and *C. canephora* have been unsuccessful in general, although a few hybrids with limited economic value were recovered (Cambrony, 1988). The major barrier is the sterility of the interspecific hybrids (2n=33). Chromosome doubling of the interspecific hybrid and the synthesis of artificial tetraploid *C. canephora* are two strategies devised to improve hybrid fertility. Le Pierres and Anthony (1980) reported partially fertile amphidiploids resembling *C. arabica*. Two interspecific hybrids (2n=4x=44), Arabusta and Icatu, with desirable traits of both parents, were obtained by crossing synthetic synthetic tetraploid *C. canephora* with *C. arabica*. However, the fertility was still too low to be used for commercial cultivars.

Alternatively, somatic hybridization could be used to facilitate gene transfer between *Coffea* species. The commercial application of this approach has been demonstrated by the fusion of cultivated potato with wild *Solanum* species to form breeding lines resistant to viral and fungal diseases (Glimelius, 1988). Somatic hybrids of

Coffea should have near normal meiosis and the degree of heterozygosity will also be maintained. More importantly, cell fusion allows for the testing of large numbers of parental combinations which is not possible using sexual interspecific crosses. An ideal sequence would be the improvement or selection of both *C. arabica* and diploid *Coffea* prior to the creation of somatic hybrids.

The prerequisites for somatic hybridization include plant regeneration, protoplast isolation, cell wall regeneration and heterokaryon selection. Successful manipulations of these steps will also provide an opportunity in the future for genetic modification of targeted genes in coffee using transformation techniques. The objective of this study is to develop methodologies of regeneration and protoplast isolation applicable to *Coffea* species.

II. LITERATURE REVIEW

A. Coffee breeding objectives

Coffee is the main agricultural commodity on which many tropical and subtropical countries rely for foreign exchange (Sondahl *et al.*, 1984). *C. arabica* (2n=4x=44), grown at medium and high altitudes, is produced mainly in Latin America. Quality coffee is processed from *C. arabica* beans. *C. canephora* (2n=2x=22) (robusta coffee), adapted to low altitudes, is the main cultivated species in Africa and Asia. The higher yielding robusta beans generate a lower quality beverage, and are used primarily for instant coffee and blending with *C. arabica*. Other species, *C. liberica*, *C. dewevrei* and *C. racemosa*, are grown for local consumption only (Bettencourt and Rodrigues, 1988). *C. arabica* is the only species that is self-compatible and propagated by seed. All others are self-incompatible and must be vegetatively propagated.

Introduction of a new coffee cultivar is an expensive and long-term process (Stemmer *et al*, 1982). The primary cultivated species, *C. arabica*, lacks resistance to major fungal diseases, insects and nematodes, often resulting in severe crop losses. Diploid species are important sources of genetic resistance and potentially can be used for the

improvement of *C. arabica*. However, no interspecific hybrid has yet achieved commercial success (Cambrony, 1988) or been widely used in breeding programs. The major limitations of interspecific hybrids include low fertility, unacceptable agronomic variability, and increased homozygosity (as a result of artifical doubling of chromosomes) which leads to lower yield. *In vitro* techniques may be applied to produce fertile somatic interspecific hybrids, to improve commercial lines through directed gene transfer, and to rapidly increase promising material through micropropagation.

A very serious problem in coffee production is coffee leaf rust caused by the fungal pathogen *Hemileia vastatrix* Berk. & Br. (Van der Vossen, 1985). This destructive disease occurs in all coffee growing regions of the world. Currently, all economically important varieties of *C. arabica* are susceptible to one or more of the 30 physiological races of *Hemileia vastatrix*. Each race causes serious damage and limited control by the application fungicides is frequently uneconomical. The occurrence of coffee berry disease (*Colletotrichum coffeanum* Noack *sensu* Hindorf) is presently restricted to Africa, but the degree of destruction is greater than coffee leaf rust (Van der Graaff, 1982). Coffee berry disease affects the flower buds, flowers, and the

expanding berries. Intensive application of fungicides is necessary, and complete control is infrequent. Only a few cultivars of *C. arabica* have been reported to exhibit partial resistance to this organism (Bettencourt and Rodrigues, 1988).

Major coffee pests include several species of insects and nematodes, especially the lepidopterous leaf miner *Leucoptera myeyricki* in Africa and *L. coffeella* in Central and South America (Mitchell, 1988). Both *C. arabica* and *C. canephora* are susceptible and applications of insecticides usually result in more severe leaf miner damage due to decreases in predator populations. *C. arabica* is damaged by root-knot nematodes *Melodogyne exiqua* Goeldi, *M. incognita* Chitwood, *M. coffeicola* Lordello & Zamith, and the root-lesion nematodes *Pratylenchus brachyurus* T. Goodey and *P. coffeae* T. Goodey (Bettencourt and Rodrigues, 1988).

B. Interspecific hybridization in coffee

The more than eighty species of the *Coffea* genus provide a large reservoir of genetic diversity for breeding desirable agronomic traits, such as disease, pest and environmental stress resistance, into *C. arabica* (Charrier and Berthaud, 1985). Sources of resistance to

coffee leaf rust and coffee berry disease are found in *C. canephora* (robusta coffee). Species showing tolerance to coffee leaf rust include *C. liberica, C. congensis*, and *C. stenophylla*.

Seven *Coffea* species are sources of resistance to soil pests. Resistance to root-knot nematodes (*Meloidogyne* species) is found in *C. canephora, C. congensis, C. dewevrei, C. salvatrix, C. racemosa, C. eugenioides, C. stenophylla,* and *C. kapakata*. Immunity to leaf miners, (*Leucoptera* species) occurs in *C. stenophylla* and resistance is present in *C. racemosa, C. dewevrei, C. eugenioides, C. kapakata* and *C. salvatrix*. *C. racemosa*, a deciduous species, is drought tolerant and may also provide some cold hardiness. The quality of cultivated varieties may be varied by incorporation of genes from species of *Paracoffea* or *Mascarocoffea*, containing either high or low levels of caffeine.

Coffee breeders have focused on interspecific hybridization between *C. arabica* and *C. canephora* with the goal of improving the robusta coffee quality and introgressing vigor and disease resistance of robusta into *C. arabica* (Van der Vossen, 1985). The potential value of these hybrids has been examined since the 1940's (Carvalho, 1988). Sexual crosses between *C. arabica* and the rest of *Coffea* species produce triploid progeny which

were vigorous but usually sterile (Sondahl *et al*, 1979). Nine diploid species were artificially cross-pollinated with *C. arabica* and the percentage of plants obtained ranged from 0% with *C. stenophylla* to 2.9% with *C. kapakata*. Doubling the chromosome number of the sterile triploid resulted in improved fertility (Charrier and Berthaud, 1985).

Artifical tetraploids of *C. canephora* were produced through colchicine treatment and crossed with a doubled haploid of *C. arabica*. The progeny were backcrossed with *C. arabica*, and the Icatu hybrids (2n=44) were formed (Carvalho, 1988). These hybrids were resistant to coffee leaf rust, coffee berry disease, and nematodes *(M. exiqua* and *M. incognita*). However, fruit production of Icatu hybrids was highly variable rendering it unacceptable for cultivation (Boaventura and da Cruz, 1987). Yields range from above to significantly below that of either parent (Chambrony, 1988). In addition, selection among progeny of this hybrid often resulted in the lose of resistance to coffee leaf rust (Eskes and Costa, 1983).

Arabusta hybrids (2n=44) were selections from F_1 progeny of doubled *C. canephora* crossed with *C. arabica* (Berthaud, 1978). Chromosome doubling increased homozygosity and resulted in loss of vigor. This was often masked in the Arabusta by giant seeds and pulp. The

Arabusta was superior to robusta in beverage quality but meiosis was abnormal resulting in low pollen viability (Owuor, 1985). The reduced fertility of Icatu and Arabusta hybrids resulted in yield reduction, partly due to abnormal berries and empty shells. Artificial hexaploids (2n=66) had higher fertility than Arabusta, but were lower yielding than Arabusta when grown at target environments, i.e. low altitudes (Le Pierres and Anthony, 1980). Propagation by seed of the interspecific hybrids resulted in a heterogeneous population (due to out-crossing) of little commercial value (Rodrigues, *et al.*, 1975). Maintenance of selected clones requires vegetative propagation which is costly and time consuming.

Natural interspecific hybrids have also been identified (Bettencourt and Rodrigues, 1988). The oldest are Kalimas, Kawisari and Hibrido Piata from *C. liberica* X *C. arabica*. All show a high degree of sterility and variability. In 1914, the Bogor Prada hybrid of *C. canephora* X *C. arabica* was found but despite the vigorous vegetative growth, berry yield was low. Another natural hybrid from the same cross, Arla, exhibited some resistance to rust, but its yield was also very low. The most useful of these natural interspecific hybrids was Hibrido do Timor which has resistance to all known races of coffee rust. Unfortunately, this hybrid was highly

variable in yield. Catimor, a *C. arabica* X Hibrido do Timor hybrid was adapted to a wide range of growing conditions and has the potential for higher yield (Bettencourt and Rodrigues, 1988). However, the Catimor hybrid was not resistant to rust as the parent Hibrido do Timor.

C. Tissue culture of coffee

Tissue culture techniques may be used to facilitate interspecific hybridization and enhance clonal propagation. Essential to most of these tissue culture protocols is the ability to regenerate plants from *in vitro* tissues, cells or protoplasts either through somatic embryogenesis or organogenesis. However, these manipulations were only partially successful in *Coffea*.

1. Somatic embryogenesis

Somatic embryogenesis is the process of embryo initiation and development from cells that are not products of gametic fusion (Tisseret et al, 1979). Somatic embryos *in vivo* are confined primarily to intra-ovular structures, whereas *in vitro* asexual embryos form directly from explants, isolated cells, protoplasts, with

or without a callus phase. Somatic embryos are bipolar structures having shoot and root apices that can easily develop into whole plants (Esau, 1977). Embryogenesis of *Coffea* may be useful for regeneration of somatic hybrids. Somatic embryos may also be used in breeding schemes for rapid clonal propagation (Murashige, 1974) of the vegetatively propagated diploid species and creation of new types via somaclonal variation (Larkin and Scowcroft, 1981).

Somatic embryogenesis was first observed on orthotropic shoot explants of *C. canephora* (Staritsky, 1970). Somatic embryos appeared after several months of culture on Linsmaier and Skoog (LS) (1965) medium supplemented with cytokinin (0.1 mg/l kinetin) and auxin (0.1 mg/l 2,4-D or 1.0 mg/l NAA). *C. arabica* and *C. liberica* shoots did not produce embryogenic callus under these conditions. Embryogenic suspension cultures were obtained by transfer of callus to liquid medium (Staritsky and Van Hasselt, 1980). Stemmer *et al.*, (1982) induced callus on plagiotropic stem explants of *C. arabica* on modified Schenk and Hildebrandt (1972) medium and observed proembryos after six weeks. The proembryos developed into embryos after transfer to liquid LS medium but plant regeneration was not reported. Proembryo formation (four cell stage) was noted at 14 days on stem explant callus

from *C. canephora* cultured on MS medium with cytokinin (5 mg/l BAP) and auxin (1 mg/l IBA) (Nassuth *et al.*, 1980).

Somatic embryo formation from leaf explants has been reported in five coffee species and one interspecific hybrid. *C. arabica* leaf disks produced callus when cultured on MS medium with high levels of kinetin (up to 18.4 μM) and 2,4-D (up to 18.0 μM) for seven weeks (Sondahl and Sharp, 1977). Following two subcultures, first onto modified MS medium with the same high levels kinetin and 2,4-D for four weeks, and then onto modified MS medium, with reduced kinetin and NAA, somatic embryos developed on the callus. The proembryos first formed after 15 weeks to 25 weeks and plantlets were regenerated (Sondahl and Sharp, 1977). Somatic embryogenesis was observed in leaf disk callus of *C. dewevrei, C. canephora*, and *C. congensis* but further development into plants was not reported (Sondahl *et al.*, 1979). Garcia and Menendez (1987) increased the cytokinin and auxin levels (8 mg/l BAP and 1 mg/l 2,4-D), and shortened the time (14 weeks) of somatic embryo formation from leaf disk callus of the hybrid Catimor (Hibrido do Timor X *C. arabica*). Somatic embryos were obtained directly on leaf disks of Arabusta hybrid cultured in MS medium and high cytokinin but the frequency was lower as compared to embryogensis on callus (Dublin, 1980).

2. Organogenesis

Adventitious shoots were induced from callus tissue of *C. arabica* leaf disks, cultured on LS medium containing kinetin (0.1 mg/l) and 2,4-D (0.1 mg/l) (Herman and Haas, 1975). Roots formed after subculturing shoots on medium supplemented with NAA (0.1 mg/l). Shoot tip and node explants of *C. arabica* cultured on MS medium with cytokinin (10 mg/l BAP) and auxin (0.1 mg/l IAA) averaged 2.2 new plantlets per node and formed short, thick roots after 3-6 weeks on MS medium with high auxin (3-6 mg/l NAA) (Custers, 1979).

Multiple shoots (average 3 per explant) were initiated from shoot apical meristems of *C. arabica* cultured on MS medium with cytokinin (5 or 10 μM BAP or zeatin) and auxin (1 μM NAA) (Kartha *et al.*, 1981). Root regeneration was highest on sucrose-free modified MS medium with auxin (1 μM IBA). Nodal cuttings cultured on MS medium plus high levels of cytokinin and auxin (50 μM BAP and 170 μM IAA) were able to form shoots (Ribeiro and Carneiro, 1989).

3. Anther and ovule culture

Anther culture results in haploids, which can be utilized to produce homozygous inbreds (Nitsch and Nitsch, 1969). Anthers and microspores of *C. arabica* were cultured on LS medium supplemented with cytokinin (2 mg/l kinetin) and auxin (0.1 mg/l 2,4-D or NAA) (Sharp *et al.*, 1973). Proembryos were formed from slow growing anther-derived callus, but plantlets were not recovered. Anthers of *C. arabica* cultured on LS medium with lowered cytokinin and auxin (0.1 mg/l kinetin and 0.1 mg/l 2,4-D) produced undifferentiated callus (Sondahl and Loh, 1988). Cultured ovules of *C. canephora* produced somatic embryos and occassionally plantlets were recovered (Lanaud, 1981). The origin of the callus tissue was not known and the ploidy level of the regenerated plantlets was not determined.

4. Protoplast culture and somatic hybridization

Protoplasts of a single coffee species, *C. arabica,* were isolated from 4-5 week old callus cultures of (Sondahl *et al.*, 1980). Cell wall regeneration and callus formation was observed but these cultures failed to differentiate. A short report described the isolation of

leaf protoplasts from *C. canephora* and *C. arabica* which survived for a few weeks, with some cell wall regeneration and occasional cell division (Orozco and Schieder, 1984). Schopke *et al.* (1987) reported that several plants were regenerated 15 months after isolation of protoplasts from somatic embryos of *C. canephora*. Nevertheless, regeneration of *Coffea* plants from protoplasts remains sporadic and inconsistent.

Introgression through somatic hybrids of the tetraploid *C. arabica* with diploid *Coffea* species may provide a solution to some problems encountered in sexual interspecific hybridization. The most important are low fertility and variability of the sexual product. The feasibility of somatic hybridization is, to a large extent, dependent upon the design of *in vitro* protocols capable of producing high yields of viable protoplasts and regenerants.

III. MATERIALS AND METHODS

A. Plant materials

Mature seeds of *Coffea* species were obtained from Brazil (Fundacao Instituto Agronomico Do Parana, Londrina, Brazil), Hawaii (University of Hawaii, Kauai, Hawaii), and Indonesia (Research Institute for Estate Crops, Bogor, Indonesia). Seeds were received with the fleshy portion (mesocarp) of the fruit (drupe) removed and the parchment (endocarp) intact. Seeds were stored at 4°C until use.

B. Establishment of aseptic plantlets and greenhouse plants

Aseptic plantlets were obtained by culturing zygotic embryos excised from sterilized seeds. The parchment (endocarp) and most of the seed coat was removed. Seeds were surface sterilized by immersion in 70 % (v/v) ethanol for 30 sec. and in 35% (v/v) Clorox (1.8% sodium hypochlorite) containing a few drops of wetting agent (Tween 20) for 20 min *in vacuo*. Seeds were rinsed in sterile, distilled water and placed in 125 ml Erlenmeyer flasks containing a double-layer of pre-sterilized, pre-moistened filter paper.

After 6-10 days, the germinating zygotic embryos were excised from the seeds by cutting away surrounding endosperm and cultured *in vitro* by embedding the radicle in semi-solid culture medium. The culture medium (Table 1) was based on Murashige and Skoog (MS) (1962) medium. The pH was adjusted to 5.7 using 1 N NaOH or 1 N HCl prior to the addition of gelling agent (Gelrite 2 g/l). The medium was dispensed into 60 ml bottles (25 ml/bottle) or 125 ml Erlenmeyer flasks (50 ml/flask) and autoclaved at 120°C for 15 min. Plant growth regulators were dissolved in small amounts of dimethyl sulfoxide (DMSO) and added to the sterile medium. The growth regulators added were kinetin (5 μM) and α-naphthaleneacetic acid (NAA) (2.5 μM). The cultures were kept at 25°C with a photoperiod of 16 hours and the light intensity of 1,000 lux. Plantlets were recultured every 4-8 weeks in the same culture medium without growth regulators (2 plants per 125 ml Erlenmeyer or 250 ml bottle).

After secondary roots were formed, plantlets were established in soil after thoroughly washing the gelling agent from the roots. Plants were kept in 2" plastic pots with sterilized potting compost (Jiffy Mix Plus) and placed in sealed plastic bags for 2-3 weeks in a growth chamber (28°C, 12 hour/12 hour day/night, 1000 lux) and subsequently in a greenhouse (at 24°C/18°C day/night).

Table 1. Medium used for zygotic embryo culture of *Coffea* species.

Compound	Concentration (mg/l)
Inorganic nutrients:	
NH_4NO_3	825.0
H_3BO_3	3.1
$CaCl_2.2H_2O$	220.0
$Co_2.6H_2O$	0.012
$CuSO_4.5H_2O$	0.012
$FeSO_4.7H_2O$	13.9
$MgSO_4.7H_2O$	185.0
$MnSO_4.H_2O$	8.45
KI	0.42
KNO_3	1900.0
KH_2PO_4	85.0
Na_2-EDTA	18.6
$Na_2MoO_4.2H_2O$	0.12
$ZnSO_4.7H_2O$	4.3
Organic nutrients:	
cysteine	50.0
inositol	100.0
nicotinic acid	5.0
pyridoxine	0.5
sucrose	30000.0
thiamine	1.0

C. Cytological analyses

Mitosis was examined in root tips pre-treated with 0.1% (w/v) 8-hydroxylquinoline (6-8 hours), and fixed in a solution of 95% ethanol/acetic acid (3:1, v/v for 24 hours). Root tips were softened for four hours in an aqueous solution containing 1.0% (w/v) cellulase (Cellulysin, Calbiochem) and 0.2% (w/v) pectinase (Sigma). Meristematic cells were stained with acetocarmine and examined using the squash technique. Metaphase cells with good spread of chromosomes were examined using a Zeiss research microscope and a minimum of 10 cells were counted per plant.

D. Induction and maintenance of embryogenic and non-embryogenic cultures

Zygotic embryos were excised and cultured as described above. Kinetin (5 μM) and NAA (2.5 μM) were added to the initial culture medium. Details of the experiments are described in the results section. Somatic embryos and embryogenic callus were subcultured using the same medium (Table 1) supplemented with growth regulators (5 μM kinetin and 1 μM IBA or 5 μM kinetin and 2.5 μM NAA) dissolved in DMSO.

Embryogenic suspension cultures (25 ml in 125 ml Erlenmeyer) were initiated from callus tissue. The medium (pH 5.7) contained MS basal salts at half strength (2X KNO_3) supplemented with 10 mg/l thiamine, 50 mg/l L-cysteine, 100 mg/l *myo*-inositol, 30 g/l sucrose, 100 mg/l casein hydrolysate, and N^6-benzyladenine (5 μM) and IBA (1 μM) (Staritsky and Van Hasselt, 1980). The suspension cultures were placed on a rotary shaker (at 100 rpm) in the dark at 27°C.

Fast growing callus cultures were initiated on leaf disks cultured on MS medium supplemented with 30 mg/l sucrose, 100 mg/l *myo*-inositol, 50 mg/l L-cysteine, 4 mg/l thiamine, 5 μM kinetin and 2.5 μM 2,4-D (Dublin, 1984). Three to five leaf disks (approx. 0.5 cm^2) were cultured per 125 ml Erlenmeyer containing 50 ml of medium.

E. Histology of somatic embryos

Tissue sections were prepared from the embryogenic callus, somatic embryos and zygotic embyros. Morphology of the two types of embryos were compared. Samples were fixed in FAA (90 ml 70% ethylene, 5 ml glacial acetic acid, 5 ml 37% formalin) or CRAF III (5 g chromium trioxide, 50 ml glacial acetic acid, 200 ml 37% formalin, 175 ml distilled water), embedded in paraffin, and

serially sectioned with a steel knife on a standard rotary microtome at 6 μ. The sections were stained with Johansen's (1940) safranin and counter-stained with fast green (Sass, 1964).

F. Protoplast isolation and culture

Mesophyll protoplasts were isolated from leaves collected from greenhouse-grown and aseptically-maintained plants. Leaves at different developmental stages, from newly formed to fully expanded, were sampled. The sizes of leaves ranged from 1.2 cm to 6.3 cm in length for greenhouse plants and 0.5 cm to 1.5 cm from aseptic plantlets. Pre-treatment of donor plants included preconditioning in darkness or under high light (10,000 lux) for 24 or 48 hours. The effects of omitting sucrose from the culture medium of the source plantlets and shortening the subculturing time to three weeks (from 6-8 weeks) on protoplast isolation were also examined. In addition, cotyledons were used as a source of protoplasts.

Leaves were placed in plasmolysing solution (Table 2) and cut into thin slices. After one hour, the medium was replaced by isolation medium containing enzymes (Table 2). The preparations were incubated at 28°C in the dark on a rotary shaker (60 rpm) and sampled at one hour intervals.

Table 2. Media used for isolation of protoplasts of *Coffea* species.

Compound	Concentrations
Enzymes:	
Cellulases	
driselase	0.5-3%
cellulysin	0.5-4%
cellulase	0.2-4%
Hemicellulase	0.25-1%
Pectinases	
macerozyme	0.1-1%
pectolyase	0.01-0.9%
pectinase	0.2-1%
Osmoticum:	0.3-0.7 M
mannitol	
sucrose	
glucose	
Buffer:	
MES	1-3 mM
Other organics:	
myo-inositol	10-100 mg/l
nicotinic acid	1-5 mg/l
pyridoxine HCl	0.5-1 mg/l
thiamine HCl	1-10 mg/l
Inorganic nutrients:	
B-5	
MS	
KM8P	
$CaCl_2.2H_2O$	1 mM-6 mM

under a light microscope to check for release of protoplasts. The youngest leaf pair (up to 1 cm in length) from aseptic plantlets were used for protoplast isolation trials using reduced enzyme levels and for subsequent purification and culture experiments.

Leaf digests were purified to remove the enzymes, undigested cells, and other cell debris. First, the complete digest was carefully filtered through a 80 μm nylon filter and centrifuged at 50 x g for 5 min. The supernatent containing cellular fragments was discarded. The centrifugation and decanting step was repeated. The purified protoplasts were resuspended in culture medium and the density was adjusted to approximately 10^5 protoplasts per ml (estimated with a hemacytometer).

In the initial experiments protoplasts were purified by flotation on a density gradient of Percoll. After filtration, the digest was carefully mixed with a Percoll solution (final concentration 40% Percoll) and Percoll solutions of 30%, 20%, 10% were pipetted in layers on top. After centrifugation at 50 x g for 5 min the protoplasts accumulated between 40% and 30% Percoll. This step was omitted in later digests as the two washings with culture medium could not remove all Percoll before culturing the protoplasts.

The protoplasts were cultured in liquid media of MS

(1962), B-5 (Gamborg, 1968), KM8P (Kao and Michayluk, 1975), Fitter & Krikorian (1983), Schopke *et al.*, (1987), and Harris *et al.*, (1988). The compositions of the culture media are summarized in Table 3. The purified protoplasts were plated in sterile cell wells (Corning), sealed with parafilm and incubated in the dark at 27°C or in diffuse light at 25°C.

Callus tissues were plasmolysed for 1 hour (0.6 M osmoticum) and placed in isolation medium with enzyme mixtures (Table 2) for up to 12 hours. Protoplasts were purified by filtration (130 μm and 80 μm nylon filters) and washed twice with culture medium by centrifugation (5 min at 50 x g). The protoplast density was adjusted to 10^5 protoplasts per ml (Table 3). The protoplasts were then plated in sterile cell wells and incubated in the dark at 27°C. After 2 weeks, cultures were diluted with culture medium reducing the osmolarity to approximately 0.37 M.

Somatic embryos were macerated and plasmolysed for 1 hour in 0.6 M mannitol solution. The mixture was washed with plasmolysing solution and then incubated in isolation medium (Table 2) for up to 12 hour in the dark at 27°C. Protoplasts were purified by filtration (130 μm and 80 μm nylon filters) and washed twice with culture medium by centrifugation (5 min at 50 x g).

Table 3. Media variations used for culture of isolated protoplasts.

Compound	Concentration (mg/l)
Mineral salts:	
NH_4NO_3	250-1650
NH_4 citrate	100
$(NH_4)2SO_4$	134
H_3BO_3	3-6.2
$CaCl_2.2H_2O$	400-600
$Co_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
$FeSO_4.7H_2O$	27.8
$MgSO_4.7H_2O$	250-370
$MnSO_4.H_2O$	10-16.9
KI	0.75-0.83
KNO_3	1900-2500
KH_2PO_4	100-170
Na_2-EDTA	37.3
$Na_2MoO_4.2H_2O$	0.25
$NaH_2PO_{4H}O$	150
$ZnSO_4.7H_2O$	2-3.6
urea	100
Vitamins:	
myo-inositol	100-200
nicotinic acid	0.5-1
pyridoxine HCl	0.5-1
thiamine HCl	0.1-10
glycine	2
D-Ca pantothenate	0.5-1
folic acid	0.2-0.4
p-aminobenzoic acid	0.01-0.02
biotin	0.005-0.01
choline chloride	0.05-1
riboflavin	0.01-0.2
ascorbic acid	0.01
vitamin A	0.01
vitamin B12	0.01-0.02
vitamin D3	0.01

Table 3 continued.

Sugars:	
sucrose	0.1-0.3 M
glucose	0.1-0.6 M
mannitol	0.1-0.7 M
fructose	125-250
ribose	125-500
xylose	125-500
mannose	125-250
rhamnose	125-250
cellobiose	125-250
sorbitol	125-250
Organic acids:	
citric acid	10-40
malic acid	10-40
fumaric acid	10-40
Na pyruvate	5-20
Amino acids:	
L-cysteine	0.2-10
L-glutamine	5.6-100
L-glycine	10
L-tryptophan	10
L-methioine	10
L-arginine	210
Natural complexes:	
casein hydrolysate	250
coconut water	10-20 ml
malt extract	100
yeast extract	100
Growth regulators:	
kinetin	1
2,4-D	0.5-1.1
NAA	1
BAP	1
Other:	
MES	585-975
Ficoll	5000

IV. RESULTS

A. Occurrence of twin and triplet embryos in *C. canephora, C. congensis, C. dewevrei* and *C. arabica*

Germinating embryos were excised from seeds of *Coffea* species. A small percentage of the seeds were found to contain more than one embryo. Fourteen seeds had twin embryos and one seed of *C. arabica* had triplets (Table 4). This seems to be the first time that triplet polyembryogeny has been found in *Coffea* species. The morphology of the twins and triplets appeared normal and comparable to other zygotic embryos excised from the same species. The two pairs of twins of *C. canephora* were also mature, but were brown without healthy tissue like many embryos of this line (1988 harvest) and when cultured, failed to germinate. Four pairs of twins, the set of triplets, and one embryo of each of three twin sets germinated in culture medium supplemented with auxin and cytokinin. The other twin of those three pairs as well as seven sets of twins were lost to contamination or failed to germinate. The total number of seeds excised was too small to determine the relationship between the *C. arabica* or *C. canephora* genotype and frequency of polyembryogeny.

The potential of haploid (n=2x=22) plantlet formation

Table 4. Genotypes, total seeds, and number of polyembryonic seeds used for establishing aseptic plantlets.

Species	Genotype	Ploidy Level	Total Seeds	Twins or Triplet
C. canephora	69-14-cv5	diploid	356	2 twins
C. congensis	6623	diploid	34	0
C. dewevrei	6622	diploid	26	0
(C. canephora X *C. arabica)*	Icatu hybrid PR78041-2	tetraploid	27	0
C. arabica	376-4	tetraploid	431	3 twins 1 triplet
C. arabica	Mundo Novo	tetraploid	46	1 twin
C. arabica	Medio Cuerpo	tetraploid	93	4 twins
C. arabica	Columaris	tetraploid	45	0
C. arabica	Cumbaya 6634	tetraploid	41	0
C. arabica	Pache 6788	tetraploid	38	3 twins
C. arabica	6843	tetraploid	29	1 twin
C. arabica	AB-7	tetraploid	18	0
C. arabica	Yellow Bourbon	tetraploid	16	0
C. arabica	USDA230762	tetraploid	14	0
C. arabica	S 705	tetraploid	14	0
C. arabica	San Ramon 6444	tetraploid	13	0
C. arabica	Bourbon Select	tetraploid	8	0
C. arabica	Catura Red	tetraploid	2	0

from polyembryonic seeds was investigated by determining the chromosome number from dividing (metaphase) cells of root tips. Attempts to count root tip chromosomes of plants in culture were not successful. The *C. arabica* twins and triplets were transferred to soil when good root systems were established. None of the plants had characteristics indicative of haploidy, such as reduced leaf size and yellow-green color (Dublin and Parvais 1976). The chromosomes were counted after the plants were established in soil and the *C. arabica* triplets and twins were found to be tetraploid (2n=44).

B. Somatic embryogenesis directly from *in vitro* plantlets of *Coffea* species.

Embryos (Figure 1) were excised from aseptically germinated seeds and cultured on modified MS medium supplemented with 5 μM kinetin and 2.5 μM NAA to establish *in vitro* plantlets of tetraploid, diploid, and interspecific hybrid genotypes (*C. arabica* LCMD 376-4, *C. canephora* 69-14-cv5, and Icatu PR78041-2, respectively) from the 1987 crop of Brazil. The embryos germinated *in vitro* and developed into complete plantlets. Unexpectedly, somatic embryogenesis was observed directly on cultured plantlets. Callus was formed on the

Figure 1. Zygotic embryo excised from aseptically germinated seed of *C. arabica*.

hypocotyls of *C. canephora* within the first few weeks of culture. After 5 weeks, somatic embryos became visible on the hypocotyl callus of approximately 30% of the *C. canephora* plantlets (Figure 2). All stages of somatic embryo development were found on the embryogenic plantlets (Figure 3). Many of the somatic embryos had normal morphology comparable to that of zygotic embryos (Figures 4 and 5). Somatic embryos were also formed on plantlets of Icatu hybrid (*C. canephora* X *C. arabica*) after three months in culture. These formed in the same transition region as *C. canephora*, but with no or minimal callus formation. Callus was formed on *C. arabica* LCMD 376-4 plantlets similarly to *C. canephora*, however, somatic embryos appeared on only a few plantlets after a long time (nine months) in culture. Selections of eight other *C. arabica* genotypes (Indonesian and Hawaiian) cultured on kinetin (5 μM) and NAA (2.5 μM) produced prolific but mostly non-embryogenic callus on the basal portion of the hypocotyl. Callus proliferated, but growth of the radicle and cotyledons was inhibited on many of the developing seedlings. Only a few of these genotypes formed somatic embryos. The responses of *Coffea* genotypes that were cultured on kinetin (5 μM) and NAA (2.5 μM) are summarized in Table 5.

To determine whether somatic embryo formation could be

Figure 2. Somatic embryogenesis on callus formed on the hypocotyl of *C.canephora* 69-14-cv5 plantlet cultured on medium supplemented with 5 μM kinetin and 2.5 μM NAA.

Figure 3. Various developmental stages of somatic embryos from plantlets of *C. canephora*.

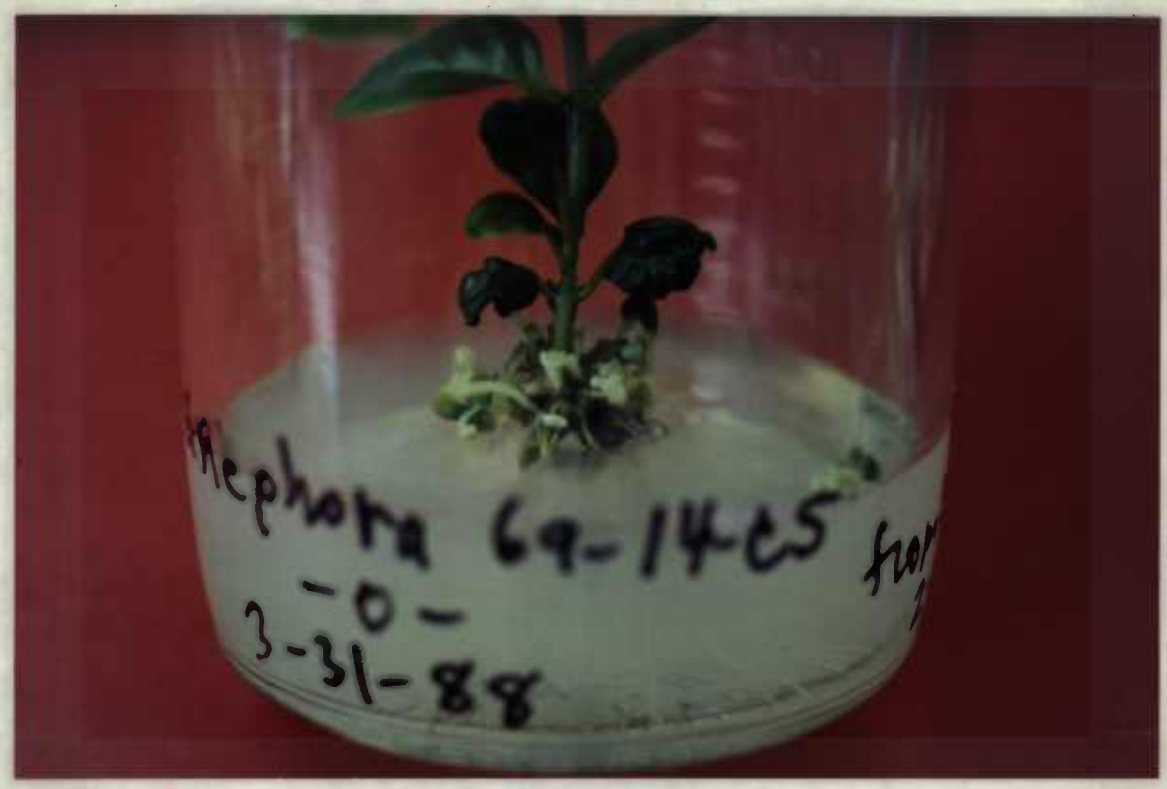

Figure 2.

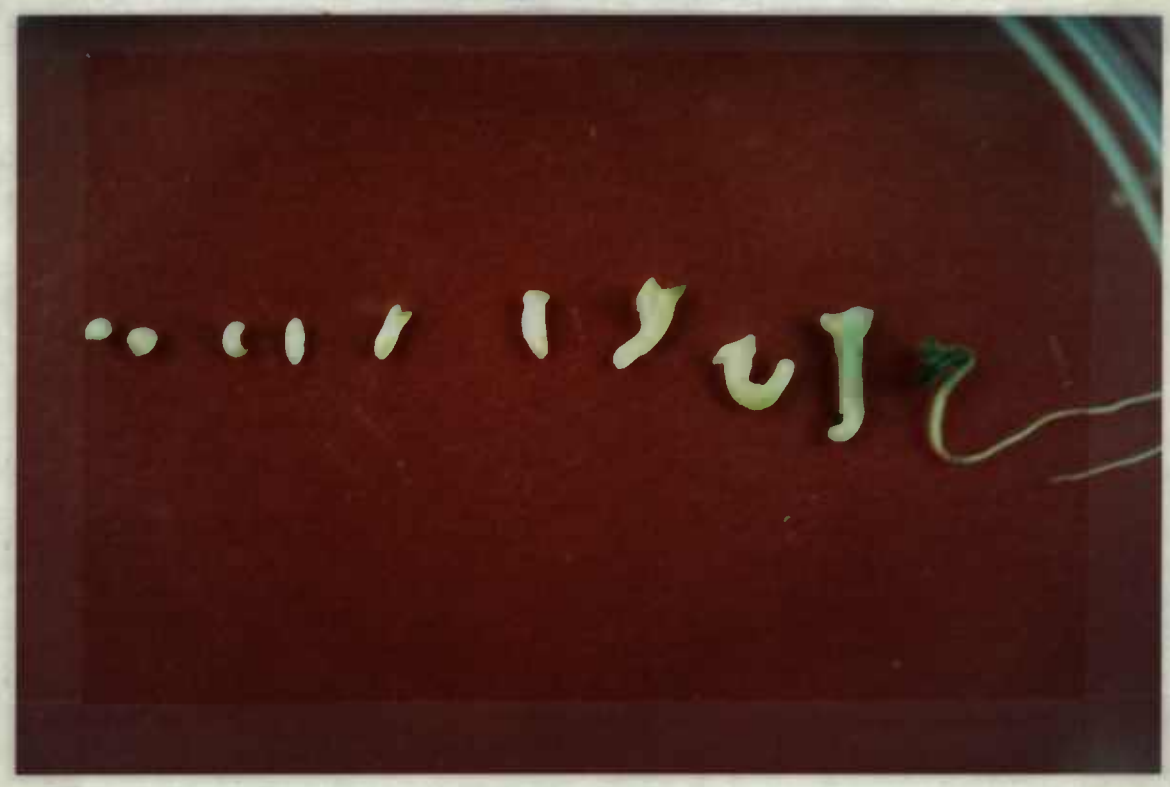

Figure 3.

Figure 4. Proembryos and somatic embryos formed on the hypocotyl on *C. canephora* 69-14-cv5 plantlet.

Figure 5. Mature somatic embryo of *C. canephora* 69-14-cv5 formed on the hypocotyl of plantlet.

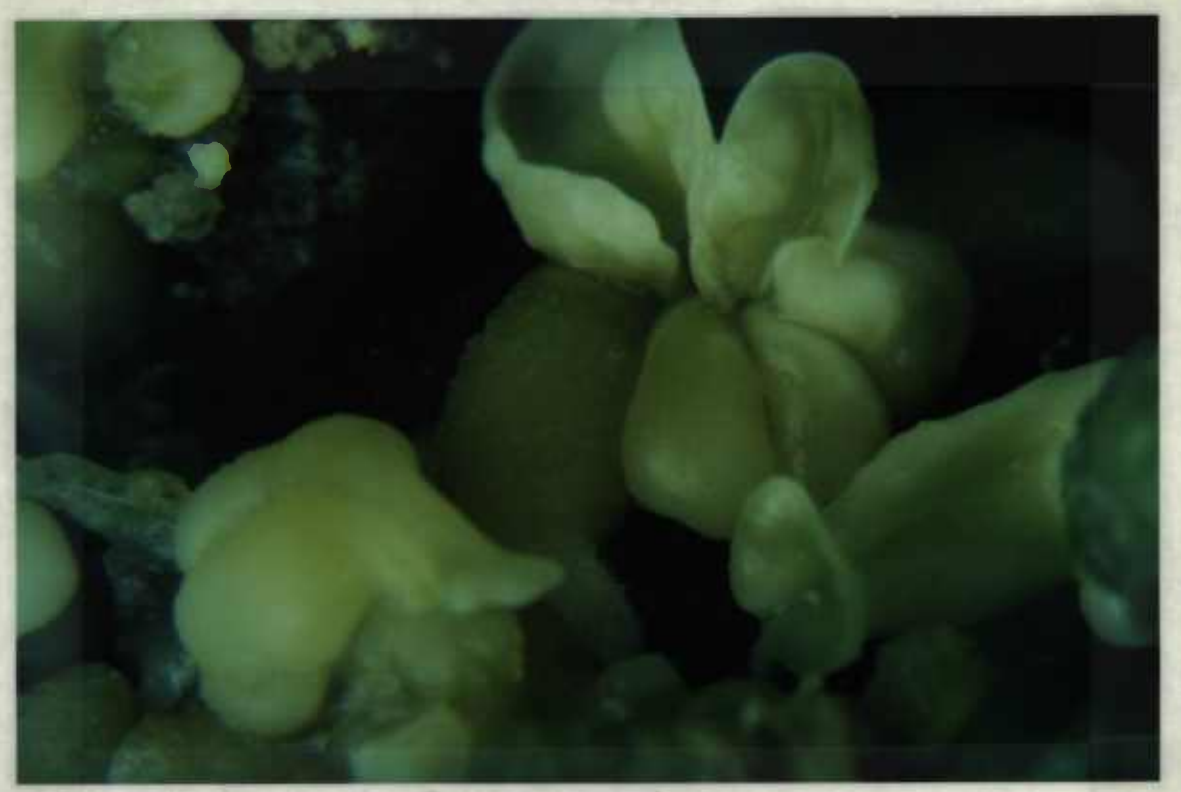

Figure 4.

Figure 5.

Table 5. Effect of 5 μM kinetin and 2.5 μM NAA on somatic embryogenesis of *Coffea* species.

Species	Genotype	No. of plants	Percent embryo-genic	No. of embryos/ plant
Brazilian genotypes				
C. canephora	69-14-cv 5	15	27 %	30
(*C. arabica* X *C. canephora*)	Icatu hybrid PR78041-2	25	16 %	12
C. arabica	LMCD 376-4	55	7 %	14
Indonesian and Hawaiian genotypes				
C. arabica	Medio Cuerpo	25	0 %	0
C. arabica	S705	12	8 %	1
C. arabica	San Ramon 6444	12	0 %	0
C. arabica	USDA 78041-2	10	0 %	0
C. arabica	Mundo Novo	8	0 %	0
C. arabica	AB-7	7	14 %	1
C. arabica	Yel Bourbon	6	0 %	0
C. arabica	Columaris 6435	6	0 %	0

enhanced by changes in growth regulator levels, *C. canephora* 69-14-cv5 and *C. arabica* LCMD 376-4 were cultured in various cytokinin concentrations (0, 0.2, 1, 5, 25 μM kinetin) in combination with various levels of auxin (0, 0.1, 0.5, 2.5, 12.5 μM NAA). The results indicate that somatic embryogenesis was higher on *C. canephora* than on *C. arabica* LCMD 376-4. Somatic embryos formed both on the basal end of the hypocotyl and on the margins and lower surface of *C. canephora* cotyledons. These plantlets required high cytokinin concentrations (5 μM and 25 μM kinetin) for somatic embryo formation (Figure 6). High auxin concentrations (2.5 μM NAA and 12.5 μM NAA) when combined with kinetin (25 μM) enhanced the frequency of plantlets of *C. canephora* forming somatic embryos. Although somatic embryogenesis increased with higher cytokinin and auxin concentrations, embryo and plantlet abnormalities also increased. These abnormalities included proliferation of callus, abnormal or no cotyledon growth, and stunted or no root growth. The growth regulator concentration that resulted in both somatic embryogenesis and healthy aseptic plantlets of *C. canephora* was 5 μM kinetin and 2.5 μM NAA.

Somatic embryogenesis was low overall in *C. arabica* LCMD 376-4 at all kinetin and NAA levels. A few somatic embryos formed only on the hypocotyl callus of *C. arabica*

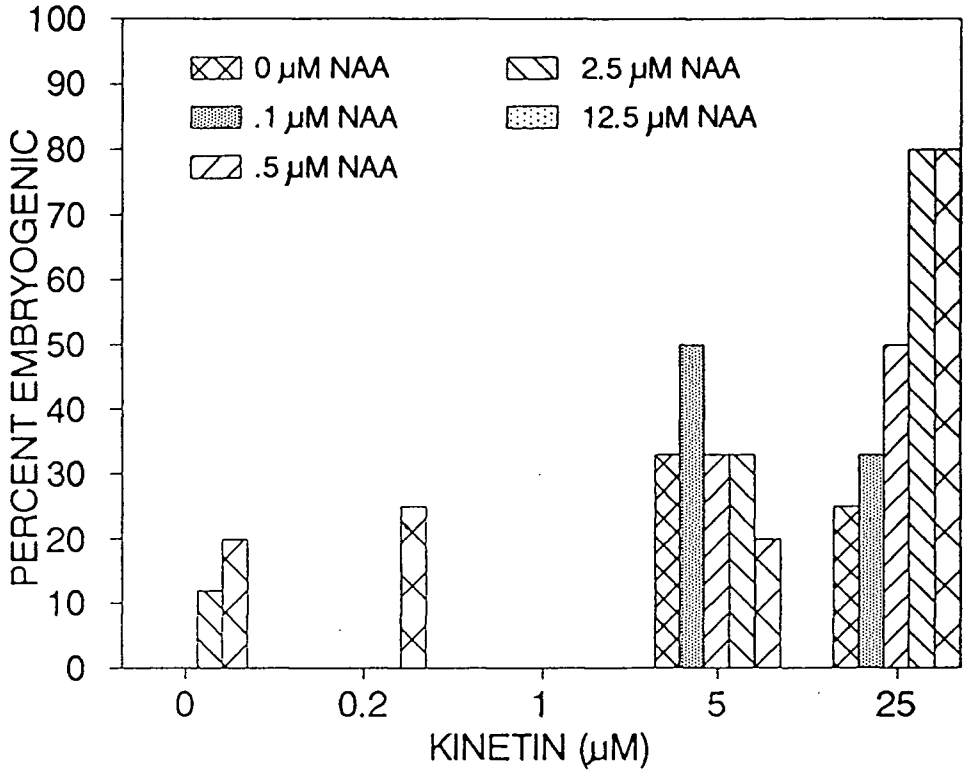

Figure 6. The effects of various concentrations of kinetin and NAA on somatic embryogenesis from zygotic embryos of *C. canephora 69-14-cv5*.

LCMD 376-4. Zygotic embryos of eight other genotypes of *C. arabica* (Medio Cuerpo, S 705, San Ramon 6444, USDA 78041-2, Mundo Novo, AB-7, Yellow Bourbon and Columnaris 6435) were cultured in various combinations of cytokinin (0.5-5 μM kinetin) and auxin (0, 0.25, 2.5 μM NAA). Somatic embryogenesis was also low (0 to 14%) also on these *C. arabica* genotypes. Several of the somatic embryos formed only after many months of culture.

The potential for formation of somatic embryos on *C. arabica* was further examined using other plant growth regulators. *C. arabica* 376-4 was cultured on kinetin (5 μM) in combination with IBA (0, 0.2, 1, 5, 25 μM) and embryos of five other genotypes of *C. arabica* (Medio Cuerpo, Cumbaya 6634, Mundo Novo, Columnaris 6435, and Pache 6788) were screened for embryogenic response in medium with various combinations of cytokinin (0.5-5 μM kinetin) and auxin (0-1 μM IBA). For 'Pache 6788' growth regulator combinations included gibberellic acid (0.1 μM GA_3). 'Medio Cuerpo' cultures did not exhibit any tendency to become embryogenic on medium containing NAA but showed some response to IBA, although only after five months in culture.

To test whether inclusion of IBA in the medium could also increase somatic embryogenesis on *C. canephora*, zygotic embryos of this species were cultured in kinetin

(5 μM) in combination with various IBA concentrations (0, 0.2, 1, 5, 25 μM). The embryogenic response was higher than with NAA. Another diploid species, *C. congensis* 6623, cultured on medium with kinetin (5 μM) and IBA (1 μM) was also highly embryogenic. The results of this test are summarized in Table 6. Healthy plantlets that could be used for further experiments were also obtained on this medium.

Subculturing of somatic embryos and embryogenic callus on medium supplemented with cytokinin (5 μM kinetin) and auxin (2.5 μM NAA or 1 μM IBA) resulted in germination of the embryos and formation of hundreds of new somatic embryos. The subcultured somatic embryos formed new somatic embryos directly on the somatic embryo, or germinated and formed somatic embryos on the basal portion of the hypocotyl (Figures 7 and 8) as on the plantlets from zygotic embryo culture. New somatic embryos and embryogenic tissue were obtained by repeated subculturing of the somatic embryos onto fresh medium.

Histological comparison of longitudinal sections of somatic embryos and zygotic embryos of *C. canephora* revealed similarities in structure and morphology. All stages of embryo development were found in serial sections of somatic embryo-forming tissues. Cross-sections of the colyledons showed similar development of the cotyledons

Table 6. Effect of 5 μM kinetin and 1 μM IBA on somatic embryogenesis of plantlets of *Coffea* species.

Species	Genotype	No. of plants	Percent embryo-genic	No. of embryo/plant
C. canephora	69-14-cv 5	17	53 %	42
C. congensis	6623	34	39 %	26
C. arabica	LCMD 376-4	219	2 %	13
C. arabica	Medio Cuerpo	22	9 %	4
C. arabica	Cumbaya 6634	16	0 %	0

Figure 7. Plantlet from somatic embryo of *C. canephora* 69-14-cv5 with somatic embryos on hypocotyl.

Figure 8. Repetitive somatic embryogenesis of somatic embryos subcultured on medium supplemented with 5 μM kinetin and 1 μM IBA.

Figure 7.

Figure 8.

and their vascular bundles in both the zygotic and somatic embryos. The rough edges of the somatic embryo sections seemed to be due to proembryo formation adjacently. The formation and development of proembryos from cotyledonary callus can be seen on cross sections of zygotic embryos of *C. canephora* cultured at cytokinin and auxin concentrations of 25 μM kinetin plus 12.5 μM NAA (Figure 9) and 25 μM kinetin plus 0.5 μM NAA (Figure 10).

Suspension cultures were initiated by subculturing embryogenic *C. arabica* tissue in liquid MS medium (Figure 11). Somatic embryos formed slowly over the next two months and were subcultured as they matured onto semi-solid MS medium with 5 μM kinetin and 1 μM IBA. The subcultured somatic embryos of *C. arabica* continued to form new somatic embryos on the surface of the radicle.

Fast growing callus was induced on leaf disks of *C. canephora*, the Icatu hybrid, and most *C. arabica* genotypes cultured on MS medium containing 5 μM kinetin and 2.5 μM 2,4-D (Figure 12). However, this fast growing, light colored, and watery callus had lost its embryogenic potential. The rapidly growing callus cultures were used for the preparation of protoplasts.

Figure 9. Cross sections of embryogenic tissue with golbular somatic embryos of *C. canephora* 69-14-cv5 cultured on 25 μM kinetin and 12.5 μM NAA.

Figure 10. Cross sections of tissue with early somatic embryos of *C. canephora* 69-14-cv5 cultured on medium containing 25 μM kinetin and 0.5 μM NAA.

Figure 9.

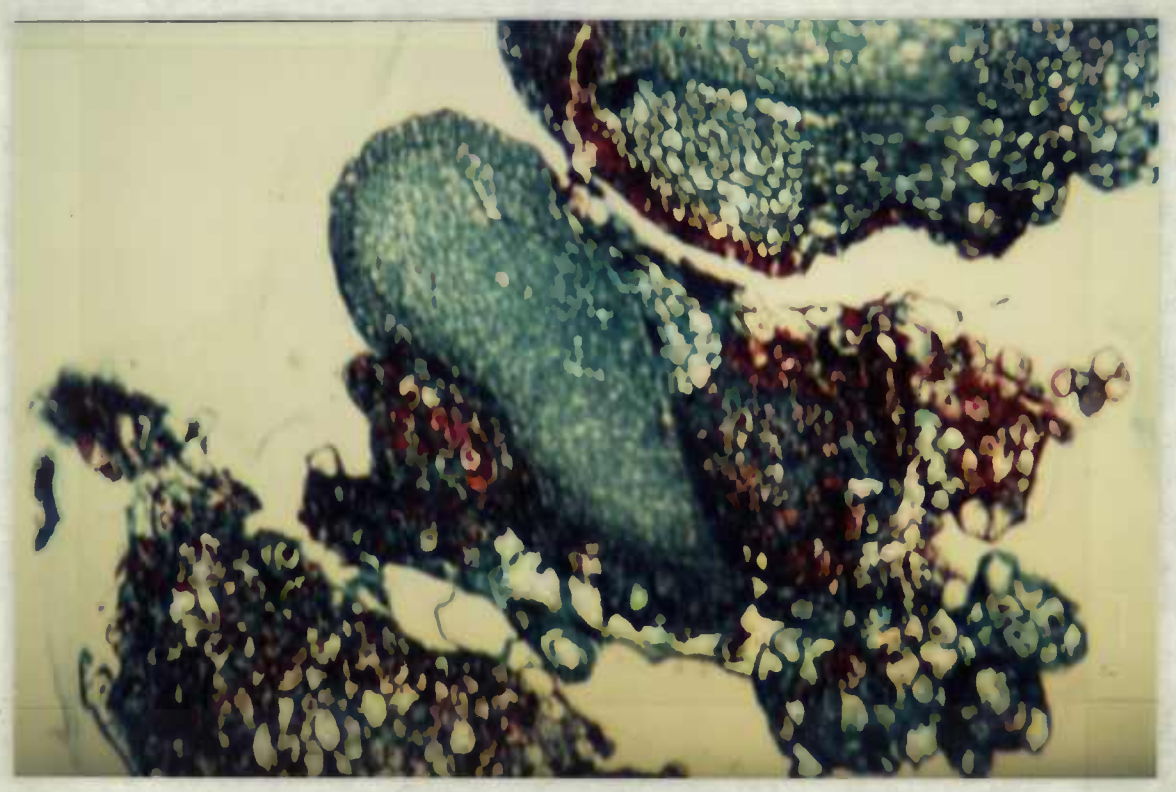

Figure 10.

Figure 11. Embryogenic suspension culture of *C. arabica* 376-4 in the presence of 25 μM BAP and 5 μM IBA.

Figure 12. Fast growing but non-embryogenic callus of *C. arabica* Medio Cuerpo cultured on medium containing 5 μM kinetin and 2.5 μM 2,4-D.

C. Isolation and culture of protoplasts from leaves, callus and somatic embryos of *Coffea* species

1. Leaf mesophyll protoplast isolation and culture of *C. arabica*, *C. canephora* and *C. congensis*

The medium for mesophyll protoplast isolation described by Orozco and Schieder (1984) was used on leaves collected from greenhouse grown plants of *C. arabica* and *C. canephora* as well as aseptic plantlets of *C. canephora*. The enzyme mixture contained cellulase (3%) and pectolyase (0.5%) dissolved in mannitol (0.6 M). After incubation for four hours on a rotary shaker (60 rpm), the digest consisted of shrunken cells and free chloroplasts from ruptured cells or protoplasts, but no intact protoplasts were found.

Other combinations of enzymes were systematically screened using the youngest leaves obtained from greenhouse grown plantlets. Fully expanded leaves did not yield protoplasts. Protoplasts were isolated using an enzyme mixture of cellulase (3%), macerozyme (1%), hemicellulase (1%), and pectolyase (0.3%) dissolved in isolation medium containing mannitol (0.3 M) and sucrose (0.3 M) as osmoticum. Surface sterilization was necessary for greenhouse leaves but the disinfestant treatment

resulted in discolored and damaged cells and produced protoplasts with an excessive amount of cellular debris. This enzyme mixture also released protoplasts from *in vitro* plantlets which were already aseptic. Thus for all subsequent experiments the newest leaf pair from cultured plantlets was used. High yields of viable protoplasts were isolated and purified. However, the protoplasts appeared shrunken and discolored after 24 hours in culture medium.

Efforts to optimize the isolation protocol included changing the growth environment of the donor plantlet and reductions in the enzyme concentrations of the isolation medium. Pretreatments of the donor plant, such as darkness or 24 hours under high light (10,000 lux), were not effective in improving the yield and health of the protoplasts isolated and cultured. Stepwise reductions were made in the concentration of each enzyme to improve protoplast yield and viabilty. The optimized isolation medium contained cellulase (1%), hemicellulase (1%) and pectolyase (0.03%) (Table 7). This enzyme mixture consistently produced high numbers of viable protoplasts from three species, *C. congensis*, *C. canephora*, and *C. arabica*, after eight hour of incubation (Figure 13). Lowering the concentrations of enzymes any further decreased the release of completely digested protoplasts

Table 7. Optimal media for isolation of protoplasts of *Coffea* species.

Compound	Concentrations		
	Leaf	Callus	Somatic Embryos
Enzymes:			
Cellulases			
driselase		1%	1%
cellulase	1%	3%	2%
Hemicellulase	1%		
Pectinases			
pectolyase	0.03%	0.3%	
pectinase			1%
Osmoticum:			
mannitol		0.6 M	0.6 M
glucose	0.5 M		
Buffer:			
MES	3 mM	3 mM	3 mM
Other organics:	mg/l		
myo-inositol	100	100	100
nicotinic acid	1	1	1
pyridoxine HCl	1	1	1
thiamine HCl	10	10	10
Inorganic nutrients:			
B-5	X	X	X

Figure 13. Mesophyll protoplasts from young leaves of aseptic plantlet of *C. arabica* LCMD 376-4.

from the leaf matrix.

C. canephora, *C. congensis*, and the *C. arabica* genotypes Cumbaya 6634, LCMD 376-4, Mundo Novo, Medio Cuerpo and Pache 6788 all produced a high yield of viable protoplasts for purification and culture. The purification procedure of filtration and centrifugation removed undigested tissue and the isolation medium. Cell wall regeneration was inhibited unless the osmoticum was reduced to 0.5 M for plasmolysis, isolation, and initial protoplast culture. Protoplasts remained viable for up to three weeks, formed cell walls and occasionally divided when cultured in liquid medium (modified B-5) supplemented with cytokinin and auxin (5 μM kinetin and 2.5 μM 2,4-D).

More protoplasts regenerated cell walls and some divided in liquid medium which included additional vitamins, sugars, and the natural complex casein hydrolysate plus inorganic salts (modified MS, B-5, and KM8P). The addition of amino acids individually increased browning in cultured protoplasts. The optimized leaf protoplast culture medium for *Coffea* species from this research is given in Table 8. Further modifications of the culture medium did not increase cell division. These included changes in inorganic nutrients, vitamins, organic acids, amino acids, sugars, growth regulators, and natural complexes listed in Table 2.

Table 8. Optimal media for culture of leaf and callus protoplasts.

Compound	Leaf	Callus
Mineral salts:		
Modified MS	+	N.T.
B-5	+	++
KM8P	+	+
Vitamins:		
myo-inositol	100	100
nicotinic acid	10	10
pyridoxine HCl	1	1
thiamine HCl	1	1
glycine	2	
D-Ca pantothenate	0.5	
folic acid	0.1	
p-aminobenzoic acid	0.1	
biotin	1	
choline chloride	0.5	
riboflavin	0.1	
ascorbic acid	1	
vitamin B12	0.02	
vitamin D3	0.01	
Sugars:		
sucrose	125	
glucose	9000	9000
mannitol	125	
fructose	125	
ribose	125	
xylose	125	
mannose	125	
rhamnose	125	
cellobiose	125	
sorbitol	125	
Natural complexes:		
casein hydrolysate	250	250
coconut water		20 ml
Growth regulators:		
kinetin	1	1
2,4-D	0.5	0.5

++ Tested, protoplasts healthy, cell division.
+ Tested, protoplasts healthy, cell wall regenerated.
N.T. Not tested.

2. Protoplast isolation and culture from callus of *C. canephora* and *C. arabica*

High numbers of viable protoplasts were isolated from callus tissue formed on four-week-old leaf disk cultures of *C. canephora* and *C. arabica*. Protoplasts were released after five hours in isolation medium with the enzyme mixture of cellulase (3%), driselase (1%), and pectolyase (0.3%) and mannitol (0.6 M) as osmoticum. Other combinations of enzymes and concentrations of osmoticum did not release protoplasts as well, leaving most of the callus undigested. The high enzyme concentrations (2.5% pectinase and 3.5% driselase) reported by Sondahl (1980) resulted in a yield of protoplasts too low for purification and culture. Cell wall regeneration occurred within 7 days after purification and culture in modified B-5 medium (Table 8) and cell division was observed by nine days after culture (Figure 14).

3. Protoplast isolation of somatic embryos of *C. arabica* and *C. canephora*

Application of the enzyme mixture of Schopke (1987) of cellulase (2%), pectinase (1%), and driselase (1%) resulted in protoplast isolation from somatic embryos of

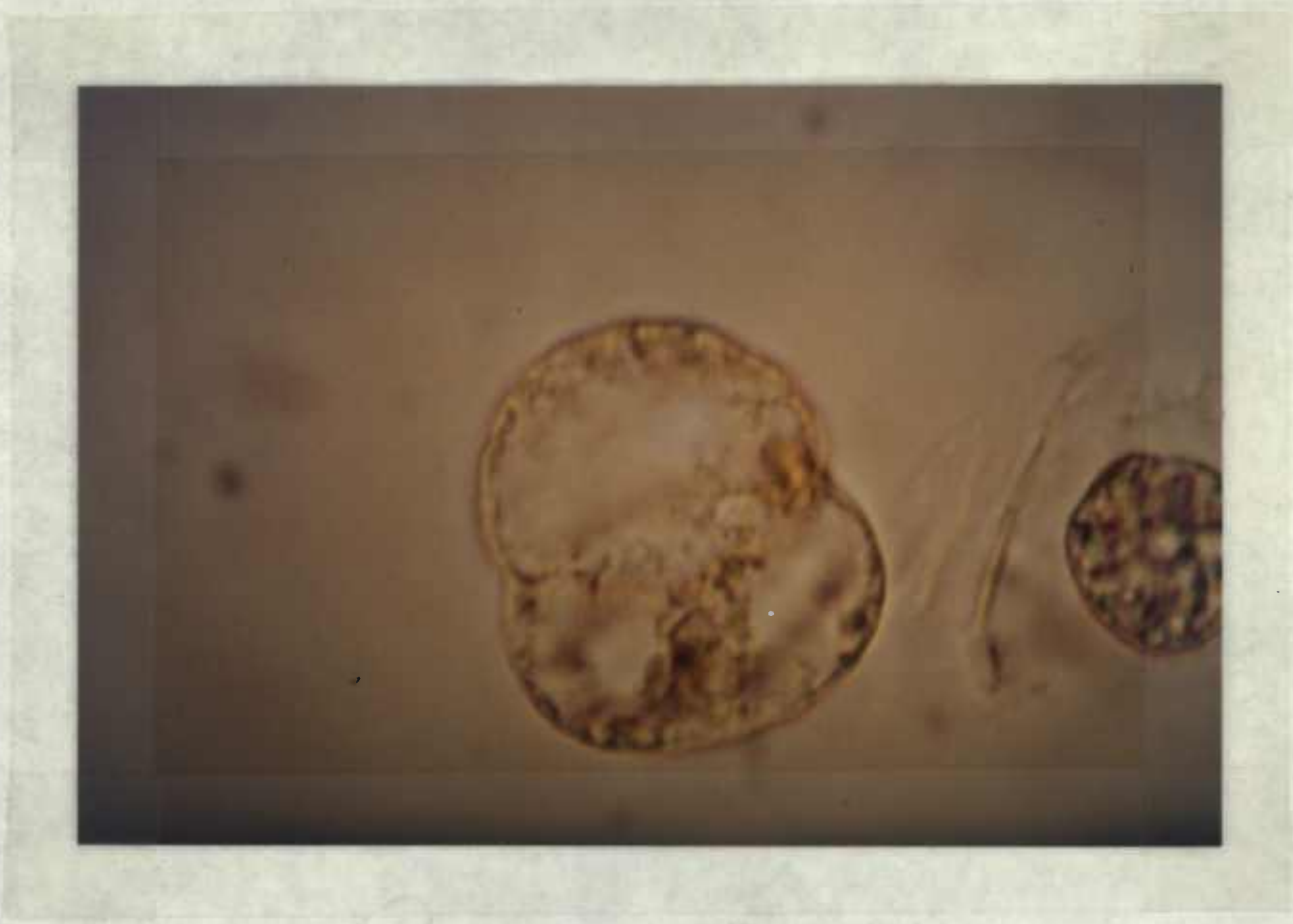

Figure 14. Cell division in culture derived from callus protoplasts of *C. arabica* LCMD 376-4.

C. canephora but not *C. arabica*. Poor results were obtained when this enzyme combination was dissolved in the isolation medium reported by Schopke (1987). However, these enzymes were effective when used with an isolation medium of B-5 salts and mannitol (0.6 M) as osmoticum. Protoplasts were isolated from somatic embryos of both *C. arabica* and *C. canephora* using cellulase (3%), macerozyme (1%) and pectolyase (0.3%) after 4 hours of incubation.

V. DISCUSSION

In vitro methodologies were developed from this study to facilitate introgression between *Coffea* species via somatic cell fusion. A protocol has been established for induction of direct somatic embryogenesis on plantlets. Zygotic embryos of *C. canephora* gave rise to somatic embryos in culture when supplied with exogenous cytokinin and auxin. The embryogenic capacity of the tissues could be maintained; subcultures of somatic embryos and embryogenic callus continued to form mature somatic embryos. In addition, methodologies for isolation of protoplasts from these repetitively embryogenic tissues, as well as mesophyll tissues of a number of *Coffea* genotypes have been described. The use of protoplasts derived from different tissues may facilitate development of protocols for somatic fusions and selection of heterokaryons.

Various stages of somatic embryogenesis could be recognized on embryogenic plants. The histological sections of somatic embryos showed close resemblance to zygotic embryos. Although somatic embryos were obtained from all three species used for these studies, there were clear genetic differences. *C. canephora* was highly embryogenic while *C. arabica* exhibited low embryogenic

potential. *C. congensis* gave rise readily to somatic embryos. Somatic embryogenesis seems to be influenced by the genotype (Tisserat *et al.*, 1978). Genotypic differences in embryogenic competence have been demonstrated in crop species like maize, barley, wheat and soybean (Ou *et al.*, 1989, Komatsuda and Ohyama, 1988). For example, *in vitro* induction of somatic embryogenisis was found to be a heritable trait in maize (Petolino *et al.*, 1988). The embryogenic response of *Coffea* species to growth regulators was also dependent on genotype. Somatic embryogenesis of *C. canephora was* enhanced by growth regulator additions while embryogenesis seemed independent of the growth regulator concentrations in *C. arabica*.

Growth regulator regimes which induced somatic embryogenesis on plantlets of *Coffea* were dissimilar to those for carrot embryogenesis, the classical system first described by Reinert and Steward (Tisserat, 1978). In carrots and many other species, explants cultured on medium containing auxin form embryogenic callus, and upon subculture to medium without auxin, somatic embryos are induced. Other crop species like soybean (Komatsuda and Ohyama, 1988) and pea (Kysely *et al*, 1987) require a three step sequence of different media for somatic embryo formation, maturation and germination. Somatic embryos of *Coffea* plantlets are initiated on medium supplemented with

both cytokinin and auxin and develop into plantlets without changes in plant growth regulators.

Some similarities exist in the growth regulator requirements for somatic embryogenesis in *Coffea* and other woody species. Mature and/or immature zygotic embryos of grape, chestnut, walnut, Sequoia, Norway spruce, and Douglas fir cultured on media supplemented with cytokinin and auxin also produce somatic embryos (Stamp and Meredith, 1988; Radojević, 1988; Polito *et al.*, 1989; Bourgkard and Favre, 1988; Durzan and Gupta, 1987; and Hakman and Fowke, 1987). Interestingly, somatic embryos of Norway spruce subcultured to germination medium form new somatic embryos on hypocotyl callus (Verhagen and Wann, 1989) which may be similar to coffee. However, a generalization cannot be made for woody species that zygotic embryos cultured on medium supplemented with auxin and cytokinin results in somatic embryo formation. Other species, for example cacao, redbud, larch, and avocado produce embryogenic tissues from immature zygotic embryos exposed sequentially to media with and without auxin (Duhem *et al.*, 1989; Trigiano *et al.*, 1988; Klimaszewska, 1989; and Mooney and Van Staden, 1987).

Haploidy linked to polyembryony of *C. arabica* was investigated by Dublin and Parvais (1976) who found that 19 out of 2000 plants derived from twins were haploid. A

cytolgical study (Berthaud, 1976) of one set of twins from *C. arabica* indicated that one embryo had normal ploidy (2n=44) and the second was haploid (n=22). Since embryos from polyembryonic seeds can be cultured, immature or developmentally arrested haploid embryos can potentially be rescued (Raghavan, 1980). Alternatively, embryos can be grafted onto *in vitro* or *in vivo* shoots (Couturon and Berthaud, 1979). This grafting method was used on coffee to obtain 82 haploids of *C. canephora* from 623 polyembryos (Courturon, 1982). The somatic embryogenesis protocol presented here could be used to multiply haploid plantlets obtained from polyembryonic seed. Hundreds of haploid plants could be formed and used for production of completely homozygous plants. In this study, the few plants of *C. canephora* and *C. arabica* that originated from twin embryos and were analyzed cytologically and found not to be haploid.

Protoplasts were successfully isolated and purified from *in vitro* tissue sources of *C. arabica* and *C. canephora* species. Large numbers of viable protoplasts were isolated and cultured from the young leaves of eight genotypes belonging to three species. The optimized enzyme mixture of cellulase (1%), hemicellulase (1%) and pectolyase (0.03%) appears effective for isolating mesophyll protoplasts from a wide range of *Coffea* species

and genotypes. Isolation of mesophyll protoplasts from *Coffea* leaves required the addition of hemicellulase to the standard mixture of cellulases and pectinases. Hemicellulase was also required for protoplast isolation from leaves of some other woody species such as apple (Patat-Ochatt *et al.*, 1988). The fact that of young leaves obtained from *in vitro* cultures are good for mesophyll protoplast isolation was also noted in another woody species (Wallin and Welander, 1985). No pretreatment regime of plantlets was needed before protoplast isolation. *In vitro* plantlets offer the additional advantage that leaf sterilization procedures are not required. Protoplasts were also isolated from callus cultures and somatic embryos of *C. canephora* and *C. arabica*. However, callus and somatic embryos each require a different enzyme mixture than leaves, cellulase (3%), driselase (1%), pectolyase (0.3%) and cellulase (2%), driselase (1%), pectinase (1%), respectively, for consistent release of protoplasts.

Somatic hybridization requires a method to select fusion products of the two donor parents. Protoplasts isolated from leaf tissue fused with callus or somatic embryo protoplasts from the other parent allows for visual selection of heterokaryons. The mesophyll protoplasts with chloroplasts and fusion products can be distinquished

from parental types within the first few hours after fusion. The ability to regenerate plants from one parent will probably be sufficient to allow for regeneration of the somatic hybrid. For example, somatic hybridization between sexually incompatiable species of citrus was successful when only one species had been previously regenerated (Kobayashi *et al.*, 1988).

Somatic hybridization between the tetraploid *C. arabica* and diploid *Coffea* species may result in improvement of yield, disease and pest resistance, and beverage characteristics. Now that consistent isolation of high yields of viable protoplasts has been obtained, plant regeneration protocols must next be developed for hybrids from protoplast fusion. Once plantlets are regenerated from protoplasts, genetic transformation via vector mediated or direct DNA transfer may also be possible.

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