# PLANTLET REGENERATION THROUGH SOMATIC EMBRYOGENESIS IN COCOA (*Theobroma cacao* L.)

By

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**THESIS**

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requirement for the degree of

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Department of Agricultural Botany
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**1994**

# DECLARATION

I hereby declare that the thesis entitled "Plantlet **Regeneration Through Somatic Embryogenesis in Cocoa** (*Theobroma Cacao* L.)" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship or other similar title, of any other University or Society.

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# CERTIFICATE

Certified that this thesis entitled **"Plantlet Regeneration Through Somatic Embryogenesis in Cocoa (*Theobroma Cacao* L.)"** is a record of research work done by **Miss Jiji Joseph**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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# CERTIFICATE

We, the undersigned members of the Advisory Committee of **Miss Jiji Joseph**, a candidate for the degree of Master of Science in Agriculture with Major in Plant Breeding and Genetics, agree that the thesis entitled "**Plantlet Regeneration Through Somatic Embryogenesis in Cocoa (*Theobroma Cacao* L.)**" may be submitted by Miss Jiji Joseph, in partial fulfilment of the requirement for the degree.

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*Dedicated to*
*My Loving Parents*

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# ABBREVIATIONS

| | | |
|---|---|---|
| ABA | - | Abscissic acid |
| AC | - | Activated charcoal |
| AMO 1618 | - | 2'-isopsopyl-4' dimethylamino-5'-methyl phenol-1' piperidine carboxylate methyl chloride |
| BA | - | Benzyle adenine |
| CCC | - | 2 Chloroethyl trimethyl ammonium chloride |
| CH | - | Casein hydrolysate |
| CW | - | Coconut water |
| 2-4-D | - | 2,4-Dichloro-phenoxy acetic acid |
| 3-4-D | - | 3,4-Dichloro phenoxy acetic acid |
| Fe EDTA | - | Ferric salt of ethelene diamine tetra acetic acid |
| GA | - | Gibberellic acid |
| IAA | - | Indole-3-acetic acid |
| IBA | - | Indole butyric acid |
| 2 ip | - | 2 Isopenenyl adenosine |
| KIN | - | Kinetin |
| KR | - | Kilo Rad |
| KV | - | Kilo Volt |
| uM | - | Micro Molar |
| NAA | - | Naphthalene acetic acid |

# Introduction

# 1. INTRODUCTION

Somatic embryogenesis is the process by which somatic cells develop through stages of embryogeny to give whole plants without gametic fusion. This process is best known as a pathway for regeneration *in vitro*. Since the first report on somatic embryogenesis in the *in vitro* cultures of carrot in 1958 (Reinert, 1958; Steward *et al.*, 1958) the process has been described in more than three hundred species (Terzi and Loschiavo, 1990). Somatic embryos have been induced from a variety of plant tissues, most frequently from zygotic embryos, germinating seedlings, shoot meristems, young inflorescences etc.

Regeneration of plants via somatic embryogenesis is considered to be an efficient approach for clonal propagation, since rapid regeneration and multiplication are possible through this process. Somatic embryogenesis also provides the basis for other biotechnological applications such as genetic modification of trees. The process has acquired unlimited importance in agriculture, horticulture, forestry and in some industries.

High volume multiplication of embryogenic propagules is the most commercially attractive application of *in vitro*

somatic embryogenesis. As commercially conceived, the system involves harvesting mature embryos from a continuously proliferating embryogenic culture of an elite genotype and converting the harvested embryos to form seedlings to transplant or synthetic seeds for direct seeding to the grower. The recurrent embryogenesis offers potential for in vitro production of embryo metabolites as lipids and seed storage proteins. Also the embryogenic system helps to evolve plants which are free of viral diseases.

In crop improvement, as current gene transfer techniques can transform single cells, the recovery of transformed embryos from chimeric embryos consisting of transformed and non-transformed tissues is virtually assured by recurrent embryogenesis. Embryogenesis appears to have an epidermal or sub-epidermal origin which allows embryogenic tissues to readily be exposed to Agrobacterium or the particle gun mediated transformation. The embryogenic cultures have been employed as source of plant protoplasts. Isolation of protoplasts from cultures that are themselves regenerable will give protoplast cultures capable of forming whole plants, also useful in intergeneric and interspecific somatic hybridisation. The embryogenic process appears to be highly sensitive to external application of chemicals. This can be made use of in in vitro screening and selection of resistant

plants to elements like iron or aluminium or fungal toxicity. Other applications include defective embryo rescue, exploitation of somaclonal/gametoclonal variation, germplasm preservation, andro or gynogenesis and induction of polyploidy.

Cocoa (Theobroma cacao L.) belongs to the family Sterculiaceae and is the most important beverage crop of the world after tea and coffee. The development of technique for somatic embryogenesis and in vitro plant regeneration of cocoa would enhance the rapid propagation of desirable genotypes and might lead to the exploitation of the likely somaclonal variation especially for disease resistance breeding (Dublin, 1984).

Although there had been a number of reports on tissue culture of cocoa since the work of Archibald (1954), those studies had for the most part emphasized the recalcitrant nature of this tree species. It has been apparent from the beginning, that there was considerable clonal variability in the growth responses in vitro. Eventhough abundant callus formation from most explants was obtained, organised development from this callus has been extremely difficult (Archibald, 1954; Hall and Collin, 1975; Prior, 1977). Limitations in using this technology for mass propagation of elite tree species are the inability to initiate embryogenic

callus from non-embryogenic tissues, low frequency of embryo formation, low germination rate, inability to control aberrant morphology, and difficulty in acclimatising germinated plantlets to ex vitro environments (Michler and Bauer, 1991). Once these limitations are overcome, regeneration by somatic embryogenesis can be used for other biotechnological applications such as genetic modification of trees.

The present investigation on plantlet regeneration through somatic embryogenesis of cocoa was done with the following objectives.

to compare the efficiency of different explants in the initiation of somatic embryos

to study the comparative efficiency of different growth regulators and compounds other than growth regulators in inducing embryogenesis

to study the genotypic differences in the efficiency of production of embryoids

to improve the developmental potential of somatic embryos by media and culture condition manipulation

to convert mature somatic embryos into acclimatized plants through techniques like micrografting.

# Review of Literature

# 2. REVIEW OF LITERATURE

Origin of plant tissue culture can be traced back to the early experiments of Haberlandt (1902) in which he tried to grow cells under aseptic conditions. The best commercial application of tissue culture has been in the production of true to type plants at a very rapid rate compared to the conventional methods (Levy, 1981). Murashige (1974, 1977) had devised three possible routes for *in vitro* propagule multiplication.

(i) enhanced release of axillary buds

(ii) production of adventitious buds through organogenesis

(iii) somatic embryogenesis

In the first route, primary meristems like shoot tips and axillary buds are cultured, which assures genetic uniformity of the progeny to a great extent (Rao and Lee, 1986). The second route which includes callus mediated somatic organogenesis, is ideal for recovering useful mutant lines to complement the existing natural variability (Hussey, 1986). The last path somatic embryogenesis, is promising conceptually as it results in the most rapid and assured array of propagation (Evans *et al*., 1981).

## 2.1 Somatic embryogenesis

The capacity of flowering plants to produce embryos is not restricted to the development of egg. By specific induction even the somatic cells can produce embryos (Evans *et al.*, 1981). Somatic embryos for the first time were detected and recognised as such in the *in vitro* cultures derived from multicellular explants of *Daucus carota* independently by Reinert and Steward *et al.* in 1958.

General patterns of *in vitro* embryogenesis include direct initiation from differentiated tissue, and indirect initiation via callus intermediary. Direct embryogenesis proceeds from embryogenic determined cells (Kato and Kateuchi, 1963). Indirect embryogenesis requires dedifferentiation of embryogenic-determined cells, callus proliferation, and differentiation of embryogenic cells (Sharp *et al.*, 1980).

### 2.1.1 Somatic embryogenesis in cocoa

A comprehensive review of the work carried out on the somatic embryogenesis of cocoa is presented below.

Induction of somatic embryos directly from the cotyledons was first reported by Esan (1977) and Pence *et al.* (1979). Immature embryos were found to possess the greatest

embryogenic potential (Pence et al., 1979). Esan (1977) observed somatic embryogenesis from embryonic axis as well.

Zygotic embryos of cocoa 'Amelonado' when cultured in MS medium supplemented with glycine, casein hydrolysate and sucrose, asexual embryogenesis occurred from white and white/pink zygotic embryos (Pence et al., 1979). Low frequency of embryogenesis on basal medium alone was stimulated by addition of auxins like IAA, NAA or 2,4-D and coconut water. In liquid medium the growth of zygotic embryos was found to be enhanced. Microscopic examination revealed that there are two distinct patterns of asexual embryo initiation, budding process and non budding process. In 'budding' process cells of hypocotylary epidermis develop to mimic the normal stages of embryogenesis including the development of a suspensor. In 'nonbudding' process the differentiation of embryos is from internal meristematic cotyledonary tissue. When tissues from embryogenic cultures were transferred to basal medium without auxin and coconut water, they continued to initiate asexual embryos. When embryos formed were transferred from solid medium to agitated liquid medium, these adventive embryos developed roots and primary leaf.

The initiation and growth of callus and cell suspension cultures of cocoa was studied by Tsai and

Kinsella (1981). Callus was initiated by culturing immature cotyledons from pods of 120-130 days after pollination on modified $B_5$ or MS medium. A fifteen fold increase in weight of callus occurred during four week culture at 30 $\pm$ 1°C. Coconut water improved callus growth substantially. The optimal hormonal concentration for growth of suspension was 0.1 mg $l^{-1}$ 2,4-D and 0.1 mg $l^{-1}$ KIN in MS liquid medium. The cell number and cell mass of suspensions increased twenty fold in fourteen days. No organogenesis or embryogenesis was observed.

Esan (1982 b) cultured various $F_3$ Amazon cocoa explants like embryos, cotyledons and anthers in MS medium. Asexual embryos were produced from cotyledon explant through transformation of the cotyledonary axillary bud.

Kononowicz and Janick (1984) studied the response of embryogenic callus to GA and inhibitors of GA synthesis. GA at 0.05-10 mg $l^{-1}$ stimulated asexual embryogenesis from callus of immature embryos of clone BC-5 but not from BC-36. AMO 1618 at 0.05-0.1 mg $l^{-1}$ had stimulating effect on embryogenesis in BC-5. But smaller effect was observed for BC-36. Daminozide and CCC depressed embryogenesis with both the clones.

Asexual embryogenesis via callus mediated process was first reported by Kononowicz et al. (1984). Callus derived from cotyledon explants of clones BC-5 and BC-36 were found to possess embryogenic competence. Maximum frequency and intensity of embryogenesis occurred at $10^{-3}$ to $10^{-2}$ mg $l^{-1}$ 2,4-D. Embryos originated from meristematic tissues at the periphery of callus clumps. During development, asexual embryos either remained embedded in the callus or were connected through suspensor like structures.

Tahardi (1984) could obtain the most vigorous callus growth with stem and cotyledonary segments, using explants of young stem, leaf, cotyledon, anthers and zygotic embryos cultured on modified MS medium. Presence of auxins as well as coconut water gave optimum callus proliferation. Rhizogenesis occurred in callus cultures of cotyledonary and anther explants. Asexual embryogenesis occurred only in cultures of immature zygotic embryos.

Wang and Janick (1984) reported that immature somatic embryos of cocoa rarely germinate. However, soaking spade-shaped asexual embryos in distilled water or renewing liquid medium induced radicle development in upto 40 per cent of embryos, suggesting that removal of one or more inhibitors promoted precocious germination. The leachate from asexual

embryos inhibited seed germination of lettuce and was likely to be P-coumaric acid.

Janick (1986) found that somatic embryogenesis in cocoa could be induced by culturing immature zygotic embryos in MS + 1-2 mg $l^{-1}$ 2,4-D. Somatic embryos of some clones of cocoa proliferated either by hypocotyledonary budding or indirectly from embryogenic competent callus.

Litz (1986) studied pro-embryo formation from leaf segments of cocoa. Young fully expanded flaccid leaves from budded plants of genotype Amelonado were used as explant and cultured in a medium MS + 6 per cent sucrose + 0.2 per cent activated charcoal and all possible combinations of 0, 5, 10, 15, 20 mg $l^{-1}$ of 2,4-D and 0, 20, 40, 60, 80, 100, 120, 140 mg $l^{-1}$ of BA. Callus induction from cut edges and surface was obtained in all media. Adventitious root formation was observed with 5-25 mg $l^{-1}$ 2,4-D + 0-80 mg $l^{-1}$ BA. Somatic embryogenesis was occasionally observed in media with 5-20 mg $l^{-1}$ 2,4-D and 100-140 mg $l^{-1}$ BA at very low frequency. The embryos formed were capable of development from globular to later heart shaped stage. Expansion of cotyledons has been accompanied by gradual necrosis and resulted in eventual death of somatic embryos on these media formulations as well as on a media that were free of plant hormones.

Adu-Ampomah et al. (1988) could obtain plantlets from somatic embryos developed from immature cotyledons of zygotic embryos. From 100 day old pods of Amelonado and Upper Amazon types, the cotyledons and embryo axes were cultured on MS + 4 per cent sucrose + CH 0.2 mg $l^{-1}$ + CW 10 per cent with varying levels of thiamine 0.5-2 mg $l^{-1}$ and NAA 5-20 µM. After nine weeks somatic embryos were separated and cultured in ½ MS + 2-10 per cent sucrose. Thiamine at 1 mg $l^{-1}$ and NAA at 1.8 mg $l^{-1}$ produced highest number of embryoids per cotyledon. The germination of embryoids was highest in liquid MS + 5 per cent sucrose. Removal of cotyledon and culturing on liquid medium with GA (3 µM) enhanced differentiation into shoots and leaves.

Duhem et al. (1989) cultured zygotic embryos in dark on MS + NAA 10.75 µM or BA 4.42 µM + IAA 0.57 µM + GA 0.029 µM. Somatic embryogenesis occurred on both media with immature zygotic embryos. Removal of cotyledons resulted in leaves and roots on media with zeatin or 2 ip. Percentage of plantlet obtained from somatic embryos was increased by reducing mineral salt concentration and by adding activated charcoal to the medium.

A scanning electron microscope study of Theobroma cacao L. somatic embryogenesis was conducted by Santos and Machado (1989). It revealed early developmental stages as

globular and incipient heart shaped. Morphological abnormalities such as the occurrence of three or four cotyledons and round or long forms of embryo with a long and thin or short and thick stalk like structure which seem to equate to a suspensor also was observed.

Wen and Kinsella (1992) observed somatic embryogenesis on white to white pink zygotic embryos of cultivar Amelonado, cultured on media supplemented with 1-32 μM NAA. Rate of embryogenesis was different among zygotic embryo explants from different strains of cultivar Mexico or different pods of Amelonado variety. Regeneration of plantlet via direct somatic embryogenesis from the hypocotyl surface of immature zygotic embryos was observed.

Chatelet et al. (1992) studied the embryogenic potential of the nucellus and internal integument of immature seeds of cocoa. Initiation of embryogenic cells and early development of proembryos were obtained from both nucellar tissue and integument when cultured on a medium containing MS + 4.5 μM 2,4-D + 0.44 μM BA. Embryogenic cells differentiated early and accumulated phenolic compounds which may have been responsible for subsequently impaired development. Treatment with $AgNO_3$ upto 50 μM or replacement of 2,4-D by 3,4-D did not reduce accumulation of phenolic compounds and did not allow plantlets to develop.

Somatic embryogenesis and plant regeneration from flower parts of cocoa was studied by Lopez - Baez et al. (1993). Rate of embryogenesis was highest in explants cultured in medium containing 1 mg 2,4-D and 0.25 mg KIN $l^{-1}$ for three weeks. No somatic embryos were formed from explants maintained for more than three weeks or subcultured in the induction medium. Frequency of embryogenesis ranged from 1.3-18.7 per cent in explants from 9 genotypes. Of the 419 embryos from 7 genotypes transferred to a maturation medium for four to five weeks and then cultured under germination conditions, 313 germinated and 208 developed into plantlets with stem, roots and leaves.

Figueira and Janick (1993) reported somatic embryogenesis from nucellar tissue. The induction of nucellar embryony was confirmed and a protocol for conversion of nucellar somatic embryos into seedlings was developed that involved preculture of somatic embryos in liquid medium and transfer to semi solid medium in chambers receiving 20,000 ppm $CO_2$. Nucellar somatic embryos were successfully converted to seedlings under high $CO_2$ and could be transferred to soil and acclimatized to green house conditions.

Recent progress has been made on the development of methods for the production of somatic embryos from petals and nucellar tissue of cocoa by Sondahl et al. (1993). Somatic

embryos were produced and resulting plants have been raised to maturity. Regeneration rate was 4.3 per cent for petals, out of 9000 explants and 2 per cent for nucellar tissue (29000 explants).

### 2.1.2 Somatic embryogenesis in other tree crops

Plantlet regeneration through somatic embryogenesis had been reported in many plants using different explants. A short review of recent literature on somatic embryogenesis in tree crops are presented below.

Bhansali *et al*. (1990) were able to obtain secondary embryogenesis in cotyledon cultures of peach and nectarine. Initial phase was induced in MS + 2,4-D 5 mg $l^{-1}$ + KIN 2 mg $l^{-1}$ + BA 2 mg $l^{-1}$ + CH 500 mg $l^{-1}$ in dark. This was followed by a growth regulator-free medium with activated charcoal for adventitious and direct multiplication of somatic embryos under continuous light. Somatic embryos were originated from the epidermal layer of primary somatic embryos. Incidence of morphologically abnormal embryos was reduced by subculturing in every 20 days. These embryos multiplied continuously by repetitive embryogenesis. A third stage medium supplemented with BA 2 mg $l^{-1}$ was required for axis elongation, germination and transfer to soil.

Somatic embryos of oak (Quercus robur L.) were produced from immature zygotic embryos cultured on MS or WPM containing BA 1 mg $l^{-1}$ + GA 1 mg $l^{-1}$ or BA and IBA. Germination and conversion into plantlets was achieved on WPM containing reduced concentration of cytokinin. In Linden (Tilia cordata Mill). Somatic embryos were obtained from embryogenic tissue initiated from immature zygotic embryos cultured on MS + 2,4-D 0.3-2.0 mg $l^{-1}$. Germination and plantlet formation occurred on MS containing low concentration of IBA (Chalupa, 1990).

The effect of plant growth regulators on somatic embryogenesis in leaf cultures of Coffea canephora was studied by Hatanaka et al. (1991). Maximum number of somatic embryos were obtained on media that contained only cytokinin as a plant growth regulator. All of the auxins tested (NAA, IAA, IBA and 2,4-D) inhibited the embryogenesis. Under optimal conditions, each explant formed more than 100 embryoids with little callus and few adventitious roots. Embryoids were formed only at cut edges of leaf discs.

Michaux-Ferriere et al. (1992) studied the origin and ontogenesis of somatic embryogenesis in Hevea brasiliensis. Induction of two modes of embryogenesis in callus formed on excised portions of internal integument of immature seeds were observed. One mode is of unicellular origin and other is

multicellular. The ontogenesis of embryoids of multicellular origin bypasses the classical stages of zygotic embryogenesis. The structural abnormalities observed in most of the somatic embryos were probably responsible for the low germination rates obtained.

Goebel-Tourand et al. (1993) conducted histological studies to understand the causes of arrest of somatic embryo development in grape vine. Embryos with low conversion rate formed normal root apex but lacked a well structured shoot apex and developed a wide range of abnormal forms in the cotyledonary area like, uncontrolled cellular proliferation, formation of adventitious buds, over-growth of cotyledonary or leaf meristems etc. ABA increased conversion rate but failed to improve embryo morphology. Zeatin and BA promoted growth of somatic embryos but generated high level of abnormalities. Zeatin and BA applied in combination with ABA increased frequency of cotyledonary embryos but decreased the conversion rate.

Advances in somatic embryogenesis and plant production of black locust (Robinia pseudoacacea L.) was described by Arrillaga et al. (1994). The best embryogenic cultures were obtained when seeds collected 2-3 weeks post-anthesis were cultured for three weeks on modified Finer and Nagasawa (1988) medium containing 2,4-D 45-90 μM and BA 2.2 μM and then to

same basal medium. Fifteen days cold treatment increased the conversion rates upto 95 per cent.

Bonneau *et al.* (1994) obtained somatic embryogenesis and plant regeneration from mature embryos of European spindle tree (*Euonymus europaeus* L.) cultured on MS + 22.8 µM IAA + 0.046 µM KIN. The embryos frequently germinated on the same medium. Further development of somatic embryos into plantlets was achieved on a medium devoid of growth regulators.

## 2.2 Micrografting

Micrografting is a recent technique to produce virus-free plants by grafting shoot tips to axenic seedlings. The same technique can also be used to recover small explants like embryoid-derived plantlet. Reviewed below are a few references on this aspect.

Navardo (1990) reported shoot tip grafting (STG) *in vitro* of citrus as a means of obtaining virus-free plants. Citrus plants produced by STG maintained the same ontogenic age as the infected shoot tip source plant and in many cases showed an increase in vigour presumably due to elimination of the pathogen. The application of STG to some conifers may produce a rejuvenation, or at least a reinvigoration of mature trees.

Deogratius et al. (1991) studied the growth parameters on apricot shoot tip grafting in vitro. Actively growing shoot tips (0.5-1.0 mm) were excised from (a) dormant buds collected from November to February (b) vegetative flushes from plant growing in the field (c) in vitro derived vegetative shoots. The effect of root stocks, cultivar, temperature, composition of the medium and growth regulator treatments on the success of grafting on rootstock seedlings growing in vitro were studied. The best source of shoot tips was from in vitro derived plants.

Aguilar et al. (1992) attempted micrografting of somatic embryos of cocoa to in vitro derived seedling root stock and found that complete plant regeneration needed about 10 months. Best results were obtained using simple culture medium, three weeks old root stocks and somatic embryos without cotyledons.

The success of tissue culture in mass propagation programme depends in addition to regenerating plants, on the development of efficient methods for their transplantation to soil. From the above review it is understood that a lot of work still remains to be done in this tropical tree crop so as to raise the process of somatic embryogenesis to the status of a micropropagation technique.

# Materials and Methods

# 3. MATERIALS AND METHODS

Studies on plantlet regeneration through somatic embryogenesis in cocoa Theobroma cacao L. were carried out at the Plant Tissue Culture Laboratory, College of Horticulture, Vellanikkara of Kerala Agricultural University during 1992-94. The materials and methods used for the study are described in the following sections.

## 3.1 Explant

The explants used for the present study included cotyledons, embryo axes and integuments from immature embryos, mature flower buds and leaf and stem segments.

### 3.1.1 Donor genotypes

The study on somatic embryogenesis from immature cotyledon explant was undertaken using the genotypes available in the germplasm collection of the Cadbury-KAU Co-operative Cocoa Research Project of College of Horticulture, Kerala Agricultural University, Vellanikkara. These included genotypes like Amelonado, Criollo, Amazon types, hybrids, plants of fertilizer trial, comparative yield trial, inbreds ($S_1$) etc. All these genotypes exhibited much variation in pod yield, pod colour, seed yield and seed size. For other

explants like leaf and stem segments and flower buds, Amelonado genotype alone was utilized. The details of genotypes included in the study are presented in Table 1.

### 3.1.2 Collection of explant

The explant source-immature pods as well as flower buds were collected from 8-10 year old plants growing in the field. The stem and leaf segments were collected from budded plants maintained in the glass house as well as *in vitro* germinated seedlings. These budded plants of genotype Amelonado were grown under good management and regularly protected with fungicides (Bavistin 0.2%) to avoid disease surveillance. Young leaves with pale green colour and slight reddish tinge were collected from new flushes in the F-1 stage (Greathouse *et al.*, 1971). Stem cuttings from immature flushes were collected at the F-2 stage from the glasshouse-grown budded plants. Explants from 3-4 weeks old seedlings from seeds germinated *in vitro* in ½MS medium containing 3 per cent sucrose were also used for the study. These included leaf segments, shoot tips, hypocotyl fragments and cotyledonary nodal fragments.

Table 1. Genotypes tested for somatic embryogenesis from embryonic tissues

| Genotypes |
|---|
| Amelonado GI 3 |
| Criollo GI 11.1 |
| Amazon GI 9.8 |
| GVI 43 |
| GVI 61 |
| Hybrids and parents |
| * SI P 1.4 |
| SI H 3.4 |
| SI H 5.3 |
| SI H 6.5 |
| ** SII H 1.2 |
| SII H 3.3 |
| SII H 3.9 |
| SII H 5.7 |
| SII H 5.8 |
| SII H 6.6 |
| SII H 7.10 |
| SII H 10.6 |
| Fertilizer trial |
| RIII 24.5 |
| RIII 26.1 |
| Comparative yield trial |
| 18.2 |
| Inbred ($S_1$) |
| M 12.21 |
| G 1.3 (Amelonado) |

* Series I ** Series II

### 3.1.3 Preparation of explant

#### 3.1.3.1 Cotyledons, embryo axes and integument of immature embryos

Pods at various stages of maturity (70-130 days post anthesis) were collected. After cutting away the stalk end of the pod, which generally contain bacterial and fungal spores, they were washed thoroughly in tap water so as to remove all the dust. The dried pod surface was then swabbed with cotton dipped in 70 per cent alcohol and disinfected by immersing in 0.1 per cent mercuric chloride solution for one hour.

The pods were cut open in the laminar air flow cabinet and seeds were extracted. The cotyledons were taken out aseptically after removing the mucilaginous seed coat. The axis of the embryo also was separated. The integument was taken after removal of mucilaginous seed coat and cut into small pieces of size 5 mm x 5 mm.

#### 3.1.3.2 Leaf and stem segments

Leaf and stem segments as well as flower buds were brought to the laboratory, washed thoroughly in tap water and allowed to drain on a blotting paper. Surface sterilization was carried out under aseptic conditions as suggested by Antony (1993).

### 3.1.3.3 Flower buds

In order to standardise the treatment time and sterilant for surface sterilization of flower buds an experiment was conducted using chlorine water and mercuric chloride (0.1%) for 1, 3, 5, 7 and 10 minutes. The washed and drained flower buds were brought to the laminar flow and kept immersed in the sterilant for the required time, shaking at intervals. The sterilant was drained off and the buds rinsed thrice with sterile distilled water to remove the traces of sterilant and allowed to air dry on a sterilized blotting paper in the laminar flow air cabinet. The flower buds were destalked and sepals were removed so as to expose the petals and gynoecium for culturing.

## 3.2 Nutrient media

The nutrient media used in the present study were the MS medium (Murashige and Skoog, 1962) $B_5$ medium (Gamborg et al., 1968) and Woody Plant Medium (Lloyd and McCown, 1980). The compositions of the different media used are given in Table 2.

### 3.2.1 Preparation of nutrient media

One litre each of the stock solutions of macro and micro elements including iron and vitamins were first prepared

Table 2. Chemical composition of various culture media used for somatic embryogenesis in Theobroma cacao L.

| Compound | Amount mg $l^{-1}$ | | |
|---|---|---|---|
| | Murashige and Skoog | Woody plant medium (WPM) | $B_5$ medium |
| Inorganic | | | |
| Ammonium nitrate | 1650.000 | 400.000 | - |
| Ammonium sulphate | - | - | 134.000 |
| Boric acid | 6.200 | 6.200 | 3.000 |
| Calcium chloride-2 hydrate | 440.000 | 96.000 | 150.000 |
| Calcium nitrate-4 hydrate | - | 556.000 | - |
| Cobalt chloride-6 hydrate | 0.025 | - | 0.025 |
| Copper sulphate-5 hydrate | 0.025 | 0.250 | 0.025 |
| Ferrous sulphate-7 hydrate | 27.800 | 27.800 | 27.800 |
| Manganese sulphate-1 hydrate | 22.300 | 22.300 | 10.000 |
| Magnesium sulphate-7 hydrate | 370.000 | 370.000 | 250.000 |
| $Na_2$-EDTA-2 hydrate | 37.300 | 37.300 | 33.600 |
| Potassium iodide | 0.830 | - | 0.750 |
| Potassium nitrate | 1900.000 | - | 2500.000 |
| Potassium sulphate | - | 990.000 | - |
| Potassium dihydrogen phosphate | 170.000 | 170.000 | - |
| Sodium dihydrogen phosphate | - | | 150.000 |
| Sodium molybdate-2 hydrate | 0.250 | 0.250 | 0.250 |
| Zinc sulphate-2 hydrate | 8.600 | 8.600 | 2.000 |

Contd.

Table 2 (Contd.)

| Compound | Amount mg $l^{-1}$ | | |
|---|---|---|---|
| | Murashige and Skoog | Woody plant medium (WPM) | $B_5$ medium |
| Organic | | | |
| Inositol | 100.000 | 100.000 | 100.000 |
| Nicotinic acid | 0.500 | 0.500 | 1.000 |
| Thiamine HCl | 0.100 | 1.000 | 10.000 |
| Pyridoxine HCl | 0.500 | 0.500 | 1.000 |
| Glycine | 2.000 | 2.000 | - |
| Others | | | |
| Sucrose W/V | 3.0% | 3.0% | 3.0% |
| Agar W/V | 0.8% | 0.8% | 0.8% |

by dissolving adequate quantities of each element. The chemicals used for preparing various media were of analytical grade from British Drug House (BDH), SISCO Research Laboratories (SRL), Qualigens, Merck or Sigma. Growth regulators as well as amino acids also were prepared as stock solutions and made up using glass distilled water and stored under refrigerated conditions. Standard procedures (Gamborg and Shyluk, 1981) were followed for preparation of the media.

Specific quantities of stock solutions of chemicals were pipetted out and sucrose, inositol and filtered coconut water were added fresh. Volume was made up by adding glass distilled water. pH of the solution was adjusted to 5.8 using 1 per cent NaOH/HCl. To prepare semi solid medium, agar (0.7%) was added and melted by heating the solution. Medium was then poured to culture vessels which were washed thoroughly, rinsed with distilled water and dried. These were plugged with non-absorbent cotton and sterilized at 15 psi and 121°C for 15-20 minutes (Monaco et al., 1977). In case of liquid medium strips of No.1 Whatman filter paper sheets were used to support the explants. The filter paper strips made in the form of M shaped bridge were inserted into the tubes and the medium was poured into these tubes. After sterilization the culture tubes were kept in an air conditioned culture room.

### 3.3 Inoculation and culturing of explants

The transfer of explants to culture tubes was done in a Klenzaids laminar air flow chamber under aseptic conditions. The chamber was first sterilized with absolute alcohol and then with ultraviolet radiation for 20 minutes.

To inoculate the explants to the culture medium, the cotton plug of the culture vessel was removed, the vessel neck first flamed and sterile explants were quickly transferred to the medium using sterile forceps. The neck of the culture vessel was once again flamed and cotton plug replaced.

The culture vessels were then kept in the culture room where they were incubated at 30 $\pm$ 2°C. To induce callus and embryoids, cultures were incubated in the dark and for embryoid germination incubation was done under light. Artificial illumination at an intensity of 2000 lux was provided using cool white fluorescent lamps for 12-14 hours per day.

A pilot study was conducted initially using three basal media to screen suitable medium for the induction of somatic embryogenesis. The basal media were MS, $B_5$ and WPM supplemented with NAA 1.8, thiamine 1.0, CH 200 mg $l^{-1}$, CW 10 per cent and sucrose 4 per cent. The genotypes tried included Amelonado and Criollo.

In order to further improve the efficiency of embryogenesis the reported media ingredients for somtic embryogenesis (Adu Ampomah et al., 1988) were slightly altered and response observed.

## 3.4 Direct embryogenesis

### 3.4.1 Relation between pod maturity and degree of embryogenesis

Zygotic embryos of varying maturity were cultured on a medium containing MS basal salts, NAA 1.8, thiamine 1.0 mg $l^{-1}$, CW 15 per cent and sucrose 4 per cent in order to study the relationship between pod maturity and embryogenesis. Details of the experiment are presented in Table 3 and Plate 1a.

### 3.4.2 Standardisation of physical conditions

An experiment was conducted to find out the effects of temperature and light on somatic embryogenesis from immature cotyledon explant. Two temperatures tried were 26 $\pm$ 2°C and 30 $\pm$ 2°C and two light intensities namely, 500 and 2000 lux ans dark condition. The genotype used was from fertilizer trial-R III 19.3 in a medium containing MS basal salts, NAA 2.0, ABA 0.5 mg $l^{-1}$ and sucrose 4 per cent.

Table 3. Relation between pod maturity and somatic embryogenesis in cocoa

Explant - Cotyledon

| Standard medium | Pod maturity in days | Colour of cotyledon |
|---|---|---|
| (Modified Adu-Ampomah medium) | | |
| MS + NAA 1.8 + thiamine-1.0 mg $l^{-1}$ + CW 15 + sucrose 4 per cent | 70 | White |
| | 80 | White |
| | 90 | Pinkish white |
| | 100 | Light pink |
| | 110 | Pink |
| | 120 | Purple |
| | 130 | Deep purple |

### 3.4.3 Growth regulators and media additives

In order to standardise the growth regulator and media supplements for induction of somatic embryos from immature cotyledon, a trial was conducted. The growth regulators used were ABA, NAA and 2,4-D at four different levels of 0.5, 1.0, 1.5 and 2.0 mg $l^{-1}$ with and without coconut water 10 per cent. Sucrose at 4 per cent, amino acid stock 10 ml $l^{-1}$ (lysine 40 ml $l^{-1}$, tryptophan 20 ml $l^{-1}$, leucine 40 ml $l^{-1}$, arginine 40 ml $l^{-1}$ and glycine 200 ml $l^{-1}$) were also added to the MS basal medium. The genotype used for this study was Amelonado.

### 3.4.4 Induction of embryogenesis from explants other than zygotic embryo

A trial was conducted for embryoid induction using the vegetative and floral parts like leaf and stem segments, integument, petals and gynoecium as explants. These were cultured in MS medium containing varying levels of ABA, 2,4-D and NAA at levels 0.5, 1.0, 1.5 and 2.0 mg $l^{-1}$ with and without CW 10 per cent, sucrose 4 per cent and aminoacids stock 10 ml $l^{-1}$.

Induction of callus from leaf and stem segments and petals were tried with MS medium containing 2,4-D 1.0 mg $l^{-1}$, sucrose 4 per cent and cytokinins BA, 2 ip or KIN at the levels 1, 2, 4, 6 and 8 mg $l^{-1}$.

An experiment was conducted using the explants, leaf and stem segments for induction of embryoids. The treatments tried were MS media containing 6 per cent sucrose, 1 per cent charcoal and 4x5 combinations of 2,4-D 5, 10, 15 and 20 mg $l^{-1}$ and BA 100, 110, 120, 130 and 140 mg $l^{-1}$.

### 3.5 Indirect embryogenesis via callus and suspension culture

Indirect embryogenesis was tried using calli from leaf, stem and petals obtained from 2,4-D containing media. These calli were later sub-cultured to various concentrations of cytokinins like BA, KIN and 2 ip at 1, 2, 4, 6 and 8 mg $l^{-1}$ each. The calli of leaf, stem and petals induced in the media containing MS basal salts, sucrose 4 per cent, 2,4-D 1 mg $l^{-1}$ and various levels of BA, KIN, 2 ip (1, 2, 6, and 8 mg $l^{-1}$) were used for indirect induction of embryogenesis. The calli were sub-cultured to the 3x2 combinations of ABA, 2,4-D, NAA and BA or 2 ip. The levels of ABA, 2,4-D or NAA were 1, 2 and 3 mg $l^{-1}$ and the levels of BA or 2 ip were 1.0, 2.5 and 5.0 mg $l^{-1}$. Basal medium was MS with sucrose 4 per cent.

The same media with 3x2 combination of ABA, 2,4-D or NAA 1, 2, 3 mg $l^{-1}$ and BA or 2 ip 2.0, 2.5 and 5.0 mg $l^{-1}$ in MS basal salts and 4 per cent sucrose were used for suspension culture. Cell suspension cultures were initiated from callus produced on immature cotyledons, stem, leaf segments and

petals. Friable creamy callus of approximately 1 g was harvested and inoculated into 100 ml flasks with 30 ml of liquid medium. Cultures were agitated continuously in dark at 80-100 rpm at 28°C. These were subcultured every seven days.

### 3.5.1 Irradiation studies

Calli derived from various explants - leaf, stem, petal and cotyledon were irradiated using gamma rays from Gamma chamber 400 with $^{60}Co$ as the source. The radiation doses were 1 to 10 KR. The irradiated calli were sub-cultured to a medium containing MS basal salts, amino acids stock 10 ml $l^{-1}$, NAA 2.0 mg $l^{-1}$ and sucrose 4 per cent.

## 3.6 Morphological studies of embryoids

### 3.6.1 Scanning electron microscopy

In order to study the development of somatic embryos from immature cotyledon explant scanning electron microscopic observations were conducted (Plates 7a and b). Cultures with somatic embryos at varying stages of development were fixed in FAA (Formalin-acetic acid-ethyl alcohol). These were dehydrated by passing through graded alcohol series - 50 per cent to absolute and critical point dried in $CO_2$. These were then mounted on SEM supports with conductive paint, covered with a thin layer of gold, examined and photographed at 15 KV.

### 3.6.2 Stereomicroscopy

Since the scanning electron microscopic studies did not yield expected results, for want of proper refinement of the technology for cocoa, the embryoids were examined under the stereoscopic microscope for their detailed morphology and photographs were taken using Getner GMX camera.

## 3.7 Regeneration of plantlets from embryoids

### 3.7.1 Germination of embryoids

Embryoids were cultured on liquid MS (full or half strength) and WPM, with 3-5 per cent sucrose to standardise the media for germination.

In order to enhance the plantlet regeneration from embryoids an experiment was conducted. The treatments included rinsing the embryoids in sterile distilled water for one to three minutes or rinsing for one to three minutes followed by desiccation for three and five minutes or for longer durations of 12, 24 and 48 hours.

An experiment was conducted using growth regulators, GA 1.0, NAA 0.18 mg $l^{-1}$ and CW 15 per cent along with the basal media like ½MS, MS or WPM. Coconut water alone in basal ½MS, MS or WPM also was tried to standardise media supplements for regeneration of plantlets from embryoids.

### 3.7.2 Micrografting

Micrografting was attempted to recover plantlets from embryoids by grafting the germinating embryoids to *in vitro* seedlings. *In vitro* seedlings were raised in culture tubes containing the medium MS + 3 per cent sucrose. Three to four week old seedlings were used for micrografting.

The axenic seedling was taken out and the stock was cut into a length of about 5 cm retaining part of the root and shoot. A wedge shaped incision was made at the shoot end and also at the radicle end of the embryoid. The embryoid was placed in the wedge and tied firmly in position using a thin copper wire. The micrografted stock was transferred back to the medium.

Grafting was also attempted on *ex vitro* seedlings raised in the nursery in polybags. Three to four week old seedlings were topped retaining 4-5 cm stem above the cotyledons. The method of grafting followed was the same as in the earlier case. Hundred per cent humidity was maintained by covering the grafted plants with polythene bags. The whole seedlings were watered twice weekly for the first seven days.

The grafted embryoids were hardened gradually by periodical exposure of the plant for definite time intervals.

The bags were opened for about 1 hour from 9-10 am in the second week, for 2 hours from 9-11 am in the third week and 3 hours from 9-12 noon in the fourth week. Later the plantlets were completely exposed to ambient conditions.

# Results

# 4. RESULTS

The results of various experiments conducted on somatic embryogenesis from immature cotyledon explant of cocoa (Theobroma cacao L.), its regeneration into plantlet and induction of callus from various explants like vegetative and floral parts for indirect embryogenesis through in vitro techniques are presented in the sections to follow.

## 4.1 Surface sterilization of explants

Special care was taken for surface sterilization of explants as the field grown material is known to be heavily infested with microbes and their spores. An experiment was conducted to standardise the sterilization procedure for the flower buds. The results of this experiment are presented in Table 4. Mercuric chloride treatment at 0.1 per cent for five minutes gave the maximum percentage of culture establishment (80%). Freshly prepared chlorine water diluted with equal volume of sterile distilled water treated for five minutes gave 40 per cent culture establishment which was equal to mercuric chloride 0.1 per cent treated for three minutes. The chlorine water and mercuric chloride treatments for lesser time led to greater extent of contamination. Treatments

Table 4. Standardisation of sterilization for flower buds of cocoa

| Sterilant | Time (minute) | Number of explants inoculated | Number of explants infected | Number of explants not infected | |
|---|---|---|---|---|---|
| | | | | Living | Dead |
| Chlorine water | 1 | 50 | 50 | 0 | 0 |
| | 3 | 50 | 45 | 5 | 0 |
| | 5 | 50 | 30 | 20 | 0 |
| | 7 | 50 | 0 | 0 | 50 |
| | 10 | 50 | 0 | 0 | 50 |
| Mercuric chloride (0.1%) | 1 | 50 | 50 | 0 | 0 |
| | 3 | 50 | 30 | 20 | 0 |
| | 5 | 50 | 5 | 40 | 5 |
| | 7 | 50 | 0 | 5 | 45 |
| | 10 | 50 | 0 | 0 | 50 |

involving longer duration of sterilization led to death of explants.

## 4.2 Direct embryogenesis

### 4.2.1 Screening of basal media for embryogenesis

In order to screen the basal media for induction of embryogenesis from immature cotyledon explants a preliminary study was conducted. The results of the study are presented in Table 5a. The basal media used were $B_5$, WPM and MS and the genotypes tried were Amelonado and Criollo. The results showed that, the three basal media used were having varying effects on callus induction of both the genotypes. For embryogenesis MS medium was found to be more effective compared to others. The percentage of cultures were showing embryogenesis in Amelonado and Criollo were 65.5 and 79.8, respectively. This also showed that the genotype Criollo had greater response to embryogenic pattern of development in this particular media. The basal WPM also had some positive influence on embryogenesis. This induced embryogenesis in 38.1 and 42.8 per cent of cultures, respectively. Genotypes were not having much difference in response. Using $B_5$ as basal medium Amelonado was responding at a rate of 38.9 per cent. Response of Criollo in $B_5$ medium was found to be very poor (3.4%).

Table 5a Effect of different basal media for induction of somatic embryos from immature cotyledon explants of cocoa

| Basal medium | Genotype | Percentage of cultures showing | | |
|---|---|---|---|---|
| | | Simple expansion of cotyledon | Callusing alone | Callus and embryoids |
| $B_5$ | Amelonado | 14.3 | 46.7 | 38.9 |
| | Criollo | 12.5 | 84.1 | 3.4 |
| WPM | Amelonado | 23.7 | 38.1 | 38.1 |
| | Criollo | 8.3 | 48.8 | 42.8 |
| MS | Amelonado | 11.9 | 22.6 | 65.5 |
| | Criollo | 10.7 | 11.3 | 79.8 |

Addition to basal medium - NAA 1.8 + thiamine 1.0 + CH 200 mg $l^{-1}$ + CW 10% + sucrose 4%

In the media proposed by Adu-Ampomah et al. (1988) for somatic embryogenesis in cocoa, immature cotyledon explants of genotype Amelonado were cultured. The embryogenic response observed was 62.5 per cent with an intensity of 14.5. Normal embryoids of size more than 4 mm was 15.8 per cent. In order to further improve the embryogenic efficiency of this medium, an experiment was conducted by altering the levels of CW and CH. The results of the experiment are presented in Table 5b.

Elimination of the complex organic nutrients resulted in low frequency and intensity of embryogenesis (58.2 % and 6.2). When one of these was removed from the medium a reduction in embryogenesis was observed. The effect was more when CW was removed from the medium (frequency 59.8 per cent and intensity 7.4). But elimination of CH and addition of CW at 15 per cent resulted in increase in frequency, intensity as well as percentage of larger sized embryoids. These were 72.5, 18.3 and 20.4, respectively. Hence the following medium was found to be the best to promote embryogenesis in cocoa, viz. MS + NAA 1.8 + thiamine 1 mg $l^{-1}$ + CW 15 per cent + Sucrose 4 per cent. This medium was further used in most other studies on embryogenesis and hence referred to as the standard medium in the present study.

Table 5b. Response of cocoa cotyledons for embryogenesis in media supplemented with different levels of complex organic nutrients

Genotype - Amelonado

Explant - Immature cotyledon

Basal medium - MS + NAA 1.8 + thiamine 1.0 mg $l^{-1}$ + sucrose 4%

| Addition to basal medium | Frequency of embryo-genesis* | Intensity of embryo-genesis** | percentage of normal embryoids of classes | | | |
|---|---|---|---|---|---|---|
| | | | <1 mm | 1-2 mm | 2-4 mm | >4 mm |
| Nil | 58.2 | 6.2 | 34.5 | 32.4 | 26.1 | 7.0 |
| CH 200 mg $l^{-1}$ + CW 10% | 62.5 | 14.5 | 11.3 | 32.7 | 40.2 | 15.8 |
| CH 200 mg $l^{-1}$ | 59.8 | 7.4 | 7.3 | 40.8 | 38.4 | 13.5 |
| CW 10% | 60.2 | 10.2 | 14.8 | 59.8 | 18.5 | 6.9 |
| CW 15% | 72.5 | 18.3 | 11.3 | 25.8 | 32.5 | 20.4 |

* Percentge of cultures showing embryogenesis

** Number of embryoids formed per explant

### 4.2.2 Pod maturity and embryogenesis

A study was conducted using the cotyledon explants of genotype Amelonado, to find out the relation between pod maturity and degree of embryogenesis. The medium used was modified Adu-Ampomah (1988). Cotyledons from pods of different stages of maturity were used as explants (Plate 1a) the results of the study are presented in Table 6.

The embryogenic response was highest in cotyledons of 100 days maturity, indicated by light pink colour. When explants were collected from pods of maturity less than 70 days or more than 120 days no embryogenesis was observed. Cotyledons of 90 days (pinkish white) and 110 days (pink) old pods were showing embryogenic response in 40.1 and 37.8 per cent of cultures. In 10.3 per cent of cultures embryogenesis was induced when cotyledons were of 80 days maturity. In older cotyledons roots were produced instead of embryoids. In 90 and 100 day old cotyledons, rhizogenesis was observed in 2.6 and 11.4 per cent of cultures, respectively. A higher degree of rhizogenesis 22.3 and 30.9 per cent, respectively, was observed with 110 and 120 days old cotyledons.

### 4.2.3 Physical conditions of culture

Using immature cotyledon explants, an experiment was conducted to standardise the physical conditions for somatic

Table 6. Effect of development stage of zygotic embryo on somatic embryogenesis

Explant - Cotyledon Genotype - Amelonado

Medium - MS + NAA 1.8 + thiamine 1.0 mg $l^{-1}$ + CW 15% + Sucrose 4%

| Pod maturity in days | Colour of cotyledon | Percentage of cultures showing | |
|---|---|---|---|
| | | Somatic embryogenesis | Rhizogenesis |
| 70 | White | 0.1 | 0.2 |
| 80 | White | 10.3 | 0.5 |
| 90 | Pinkish white | 40.1 | 2.6 |
| 100 | Light pink | 76.2 | 11.4 |
| 110 | Pink | 37.8 | 22.3 |
| 120 | Purple | 0.3 | 30.9 |
| 130 | Deep purple | 0.0 | 15.0 |

embryogenesis in cocoa. Pods were collected from the plant fertilizer trial R III 19.3. Medium used was MS with NAA 2.0, ABA 0.5 mg $l^{-1}$ and sucrose 4 per cent. Results are presented in Table 7.

The cultures were kept in two different culture rooms maintained at two temperatures namely 26 $\pm$ 2°C and 30 $\pm$ 2°C and two light intensities 500 and 2000 lux and in dark. When incubated in the dark at 26 $\pm$ 2°C the response in callusing and embryogenesis was zero. Only expansion of cotyledons were noted. When light intensity was 500 lux, 14.3 per cent of the cultures were showing embryogenesis. Callusing was noted in 25.1 per cent of the cultures. At intensity of light 2000 lux 11.6 per cent of cultures showed embryogenesis and 19.8 per cent callusing. At both the light intensities only one embryoid was formed per explant. Greening of cotyledons was noted when cultures were incubated under light.

When the temperature of incubation was 30 $\pm$ 2°C the response of immature cotyledons to embryogenesis was found to be much more compared to that at 26 $\pm$ 2°C. Under complete darkness 75.2 per cent of cultures showed callusing and embryogenesis. The intensity of embryogenesis was higher (10) and 24.9 per cent of the normal embryoids were of more than 2 mm size. When light intensity was increased to 500 lux, 70.4 per cent of cultures showed callusing, while 50.3 per cent

Table 7. Effect of physical conditions on somatic embryogenesis in cocoa

Explant - Immature cotyledon Genotype - Fertilizer trial R III 19.3

Media - MS + NAA 2.0 + ABA 0.5 mg $l^{-1}$ + Sucrose 4%

| Physical conditions | | Response of cultures | | | | | |
|---|---|---|---|---|---|---|---|
| Temperature | Light intensity (lux) | Callusing % | Frequency of embryogenesis | Intensity of embryogenesis | % of embryoids of sizes | | |
| | | | | | <1 mm | 1-2 mm | 2-4 mm |
| | 0 | 0.0 | 0.0 | 0 | - | - | - |
| 26 ± 2°C | 500 | 25.1 | 14.3 | 1 | - | 100.0 | - |
| | 2000 | 19.8 | 11.6 | 1 | - | 100.0 | - |
| | 0 | 75.1 | 75.2 | 10 | 18.9 | 56.2 | 24.9 |
| 30 ± 2°C | 500 | 70.4 | 50.3 | 5 | 20.1 | 62.0 | 17.9 |
| | 2000 | 77.6 | 42.4 | 3 | 12.3 | 88.1 | - |

showed embryogenesis at an intensity of 5 and 17.9 per cent of normal embryoids were more than 2 mm size. At 2000 lux 77.6 per cent of cultures showed callusing and 42.4 per cent had somatic embryogenesis. Intensity of embryogenesis was 3 and no embryoids of more than 2 mm was observed.

### 4.2.4 Genotype and somatic embryogenesis

Immature cotyledons of different genotypes were cultured on the standard (modified Adu-Ampomah) medium. The results are presented in Table 8.

Frequency and intensity of embryogenesis as well as morphology of embryoids varied with genotype. Out of 22 genotypes tested, the genotype G VI 61 recorded the maximum frequency of embryogenesis. The hybrids H 3.4, and 6.5 of series I and H 3.3, 5.7, 6.6, 10.6 of Series II, plant number 18.2 of CYT and the types Amelonado and Criollo responded to embryogenic pattern of development at a higher frequency ranging from 59.2 to 75.2 per cent. Others showed a moderate embryogenic potential of 30.0-53.6 per cent. Inbred $S_1$ of M 12.21 recorded the lowest response of 17.5 per cent.

Intensity of embryogenesis was found to be maximum in Series I hybrid 6.5 and Series II hybrid 5.7. On an average nearly 30 embryoids were produced per cotyledon. In Series II hybrids H 3.9 and H 6.6 the mean number of embryoids produced

Table 8. Effect of genotype on somatic embryogenesis of cocoa

Explant-immature cotyledon

| Medium | Genotype | Frequency of embryo-genesis | Intensity of embryo-genesis | Frequency of abnor-malities | Percentage of normal embryoids of sizes | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | <1 mm | 1-2 mm | 2-4 mm | >4 mm |
| MS + NAA 1.8 + thiamine-1.0 mg $l^{-1}$ + CW 15% + sucrose 4% | Germplasm I | | | | | | | |
| | Amelonado GI 3 | 67.3 | 7.1 | 49.3 | 26.2 | 44.8 | 20.3 | 8.7 |
| | Criollo GI 11.1 | 59.2 | 5.2 | 52.1 | 35.4 | 47.4 | 17.2 | |
| | Amazon GI 9.8 | 30.0 | 5.6 | 40.4 | | 46.5 | 53.5 | |
| | Germplasm VI | | | | | | | |
| | GVI 43 | 51.2 | 10.2 | 43.3 | 66.4 | 22.6 | 7.8 | 2.2 |
| | GVI 61 | 80.3 | 4.6 | 61.8 | 50.2 | 40.4 | 7.6 | 1.8 |
| | Series I hybrids and parents | | | | | | | |
| | H 3.4 | 59.8 | 4.1 | 40.9 | 18.8 | 63.2 | 5.9 | 9.9 |
| | H 5.3 | 35.4 | 5.2 | 65.2 | 65.4 | 34.6 | | |
| | H 6.5 | 75.0 | 30.4 | 43.6 | 3.2 | 9.4 | 58.4 | 29.4 |
| | P 1.4 | 33.2 | 9.5 | 48.7 | 31.6 | 34.1 | 29.3 | 12.0 |
| | Series II hybrids | | | | | | | |
| | H 1.2 | 53.6 | 5.4 | 40.7 | 34.2 | 33.5 | 24.2 | 6.7 |
| | H 3.3 | 60.1 | 11.3 | 42.4 | 12.5 | 6.2 | 61.4 | 8.9 |
| | H 3.9 | 40.3 | 22.5 | 45.3 | 8.4 | 52.6 | 11.7 | 19.3 |
| | H 5.7 | 70.4 | 30.2 | 47.1 | 28.2 | 62.7 | 9.3 | |
| | H 5.8 | 33.5 | 4.1 | 24.6 | 6.2 | 24.3 | 8.3 | 36.2 |
| | H 6.6 | 75.2 | 20.0 | 42.3 | 1.0 | 19.3 | 74.9 | 4.8 |
| | H 7.10 | 33.2 | 10.4 | 50.2 | 10.4 | 74.8 | 10.2 | 4.6 |
| | H 10.6 | 65.1 | 13.2 | 40.5 | 11.9 | 56.6 | 21.6 | 10.1 |
| | Fertilizer trial R III 26.1 | 50.2 | 5.7 | 52.4 | - | 45.3 | 44.7 | 10.0 |
| | Inbred ($S_1$) M 12.21 | 17.5 | 2.3 | 42.2 | 18.9 | 72.2 | 8.9 | |
| | Comparative yield trial 18.2 | 59.8 | 4.2 | 45.6 | 49.2 | 24.7 | 22.1 | 4.0 |
| MS + ABA 1.5 mg $l^{-1}$ + sucrose 4% | Series I Hybrid H 2.4 | 20.6 | 5.3 | | | 28.7 | 71.3 | |
| | Fertilizer trial 24.1 | 18.4 | 5.5 | | | 70.6 | 29.4 | |
| | Criollo GI 11.1 | 18.2 | 5.4 | | | 20.2 | 79.8 | |
| | Amelonado | 25.3 | 4.2 | | | 12.3 | 87.5 | |
| | GI 3 (Amelonado $S_1$) | 8.2 | 2.1 | | | 20.4 | 79.6 | |

per explant was nearly 20. Others produced 4 to 13 embryoids per culture. The number of embryoids produced was minimum in the $S_1$ inbred of M 12.21.

Cocoa somatic embryoid usually has a short axis and two well developed cotyledons. Often the embryoids formed showed abnormalities in the form of variable number of cotyledons. Somatic embryos with 3 or 4 cotyledons were not rare. Also differences were found in the length of the embryonal axis. Two or more axes were often found fused. In some cases the axes were completely suppressed and the cotyledons alone were present.

The frequency of formation of such abnormal embryos was higher in genotypes Series I hybrid H 5.3 and G VI 61 where the percentage of abnormal embryoid were 65.2 and 61.8, respectively. In others nearly around half of the embryoids formed were abnormal. In Series II hybrid H 5.8 abnormality noted was less (24.6). But here frequency and intensity of emryogenesis also were less 33.5 per cent and 4.1.

In this experiment the Series II hybrid H 5.8 produced maximum number of embryoids having a size more than 4 mm (36.2%). The Series I hybrid H 6.5 produced 29.4 per cent embryoids of this group. Others P 1.4, Series II hybrids 3.9 and 10.6 produced more than 10 per cent of embryoids of

size above 4 mm. In all the cases a large proportion of embryoids formed were of lesser size.

When another medium having MS basal salts supplemented with ABA 1.5 mg $l^{-1}$ and sucrose 4 per cent was used the response was a reduced frequency and intensity of embryogenesis. However no abnormal formation of embryos was noticed and the embryoids formed were of larger size. Among the 6 genotypes tested Amelonado was having the highest frequency (25.3%) others, viz., H 2.4 of Series I, 24.1 of fertilizer trial and GI 11.1 (Criollo) showed an almost similar frequency of embryogenesis (18.2-20.6%). Here also $S_1$ progeny of Amelonado ($S_1$ Gl 3) produced embryoids at a lesser frequency and intensity (8.2% and 2.1).

4.2.5 Media supplements and somatic embryogenesis

The immature cotyledons of genotype Amelonado were cultured in media containing MS salts supplemented with 4 per cent sucrose, amino acids stock 10 ml $l^{-1}$ (Flynn et al, 1991) and varying concentrations of ABA, NAA and 2,4-D with and without 10 per cent CW. The results are presented in Table 9.

It was seen that there was not much variation in the frequency and intensity of embryogenesis at 1-2 mg $l^{-1}$ of NAA (60.8-70.3% and 5.1-6.8). However, with 0.5 mg $l^{-1}$ of NAA response was much less (8.2% and 1.1). At higher levels of

Table 9. Effect of media supplements on induction of embryoids from immature cotyledon explant

Genotype - Amelonado
Basal medium - MS + Amino acids stock 10 ml $l^{-1}$ + Sucrose 4%

| Growth regulator mg $l^{-1}$ | | Without coconut water | | | | | | With coconut water | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Frequency of embryo-genesis | Intensity of embryo-genesis | Percentage of normal embryoids of sizes | | | | Frequency of embryo-genesis | Intensity of embryo-genesis | Percentage of normal embryoids of sizes | | | |
| | | | | <1 mm | 1-2 mm | 2-4 mm | >4 mm | | | <1 mm | 1-2 mm | 2-4 mm | >4 mm |
| NAA | 0.5 | 8.2 | 1.1 | | | 100.0 | | 56.4 | 3.1 | 40.3 | 60.4 | | |
| | 1 | 60.8 | 5.2 | 27.2 | 28.5 | 35.3 | 9.0 | 54.1 | 9.3 | 58.2 | 42.1 | | |
| | 1.5 | 68.1 | 6.8 | 31.8 | 27.1 | 35.4 | 5.6 | 55.2 | 6.9 | 28.1 | 50.2 | 13.6 | 7.1 |
| | 2 | 70.3 | 5.1 | 34.7 | 26.1 | 32.4 | 6.9 | 56.6 | 11.2 | 18.5 | 59.8 | 13.9 | 6.9 |
| 2,4-D | 0.5 | 7.4 | 1.2 | | 100.0 | | | 40.1 | 3.2 | 17.5 | 82.5 | | |
| | 1 | 21.2 | 5.4 | 55.3 | 44.7 | | | 41.9 | 4.4 | 33.2 | 66.8 | | |
| | 1.5 | 41.2 | 10.3 | 29.4 | 70.6 | | | 40.2 | 2.3 | 50.2 | 49.8 | | |
| | 2 | 33.4 | 5.5 | 23.2 | 76.8 | | | 41.4 | 3.0 | 60.4 | 39.6 | | |
| ABA | 0.5 | 26.4 | 7.1 | 20.2 | 79.8 | | | 26.2 | 4.2 | 42.2 | 41.8 | 16.0 | |
| | 1 | 23.1 | 4.3 | | 81.7 | 18.3 | | 41.6 | 1.9 | | 37.4 | 62.6 | |
| | 1.5 | 30.9 | 4.4 | | 23.6 | 62.2 | 14.6 | 43.9 | 3.1 | | 49.6 | 50.4 | |
| | 2 | 42.6 | 4.2 | | 20.4 | 19.2 | 60.4 | 42.8 | 4.3 | | 42.9 | 57.1 | |

NAA (1 to 2 mg $l^{-1}$) a few embryoids having a size more than 4 mm were formed. By the addition of 10 per cent CW, the frequency of embryogenesis was found to be similar at all the four levels. But the intensity was increased. Size of the embryoids reduced by addition of CW especially at lower levels.

Better response to 2,4-D was observed at 1.5 and 2.0 mg $l^{-1}$ (41.2, 33.4 and 10.3, 5.5 numbers). However, the size of the embryoids were much less at all the four levels. 2,4-D at very low level of 0.5 mg $l^{-1}$ also was not effective in inducing embryogenesis. The frequency of embryogenesis was increased when 10 per cent CW was added to the medium containing 0.5 mg $l^{-1}$ and 1.0 ug $l^{-1}$ of 2,4-D. With other levels much variation was not observed. Intensity of embryogenesis was however, reduced at these levels. CW also was ineffective in improving the size of the embryoids induced by 2,4-D.

Among the four levels of ABA tested, 2.0 mg $l^{-1}$ was the most effective (42.6% and 4.2 numbers). Intensity of embryogenesis was maximum at 0.5 mg $l^{-1}$ (7.1). A proportionate increase in size of the embryoid was observed with increase in levels of ABA. 60.4 per cent of embryoids formed were of more than 4 mm size at 2 mg $l^{-1}$ of ABA. While at 0.5 mg $l^{-1}$ no such embryoids were formed. At 1.5 mg $l^{-1}$ most embryoids were

of size 2-4 mm. Addition of 10 per cent CW increased the frequency of embryogenesis at 1.0 and 1.5 mg $l^{-1}$. Intensity was not changed much but a reduction in size of embryoid was observed at all the four levels tried.

### 4.2.6 Embryoid induction from explants other than zygotic embryo

Effect of media supplements on callusing of different explants of cocoa, viz., the leaf and stem segments, petals, gynoecium and integument of genotype Amelonado were cultured on media containing MS basal salts, 10 ml $l^{-1}$ of amino acids stock, 4 per cent sucrose and varying levels of ABA, NAA and 2,4-D with and without 10 per cent CW. The results of this experiment are presented in Table 10.

Callus alone was produced with all the explants in all the media tried (Plate 5a & 5c). Callus index (CI) was calculated for each level of growth regulators. CI values were obtained by multiplying percentage of explants initiating callus (P) with the growth score (G). The growth of the callus (G) was assessed based on visual rating (with score 1 to the smallest and score 4 to the largest callus).

Using leaf segments as explants, when ABA alone was used at four levels 0.5-2.0 mg $l^{-1}$, up to 1.5 mg $l^{-1}$ produced similar effects (CI = 100.0-107.4). At 2 mg $l^{-1}$, least

Table 10. Effect of media supplements on callusing of different explants of cocoa

Genotype - Amelonado
Basal medium - MS + Amino acids stock 10 ml $l^{-1}$ + Sucrose 4%

| Growth regulator mg $l^{-1}$ | | Callus index | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Without coconut water | | | | | With coconut water | | | | |
| | | Leaf | Stem | Petal | Gynoecium | Integument | Leaf | Stem | Petal | Gynoecium | Integument |
| ABA | 0.5 | 100.2 | 127.0 | 73.5 | 27.9 | 99.1 | 73.7 | 100.2 | 107.1 | 53.6 | 137.9 |
| | 1.0 | 107.4 | 107.5 | 93.2 | 27.2 | 60.2 | 86.2 | 259.1 | 190.2 | 60.2 | 137.1 |
| | 1.5 | 100.0 | 113.2 | 80.4 | 100.6 | 93.7 | 115.1 | 173.3 | 93.4 | 60.5 | 100.6 |
| | 2.0 | 7.7 | 47.4 | 0.0 | 66.7 | 27.4 | 40.2 | 120.2 | 93.2 | 67.1 | 93.5 |
| 2,4-D | 0.5 | 100.0 | 87.7 | 0.0 | 73.4 | 152.1 | 107.3 | 93.1 | 200.7 | 173.2 | 107.4 |
| | 1.0 | 60.1 | 93.8 | 156.1 | 80.2 | 93.2 | 200.4 | 86.2 | 140.2 | 47.3 | 93.2 |
| | 1.5 | 80.5 | 93.1 | 160.1 | 93.1 | 93.3 | 202.5 | 80.3 | 100.4 | 60.2 | 93.8 |
| | 2.0 | 93.2 | 100.2 | 113.3 | 67.2 | 92.5 | 210.6 | 99.4 | 133.2 | 93.4 | 93.5 |
| NAA | 0.5 | 60.7 | 215.2 | 205.4 | 47.5 | 114.2 | 100.5* | 154.1 | 146.8 | 146.5 | 80.2 |
| | 1.0 | 22.9 | 133.1 | 280.9 | 73.1 | 93.3 | 120.2 | 100.5 | 114.1 | 67.6 | 100.1 |
| | 1.5 | 73.1 | 125.3 | 187.7 | 73.2 | 93.7 | 93.5 | 113.7 | 86.2 | 67.4 | 100.7 |
| | 2.0 | 27.4 | 126.1 | 213.2 | 26.4 | 58.9 | 0.6 | 100.8 | 80.4 | 73.2 | 114.9 |

* 20% rhizogenesis also was noted

response of CI = 7.7 was observed. With 2,4-D, the four levels were showing CI values ranging from 60.1 to 100. NAA at 1.0 and 2.0 mg $l^{-1}$ produced similar effects, CI = 22.9 and 27.4 respectively, but at 0.5 and 1.5 mg $l^{-1}$ higher response of 60.7 and 73.1 were noted. Addition of CW 10 per cent increased callusing at 1.5 and 2.0 mg $l^{-1}$ of ABA, all four levels of 2,4-D and 0.5 to 1.5 mg $l^{-1}$ of NAA. In media with NAA 0.5 mg $l^{-1}$ and 10 per cent CW rhizogenesis was induced in 20 per cent of the cultures.

When stem segments were used as explants, ABA at 0.5-1.5 mg $l^{-1}$ produced callus index of 107.5 to 127.0 and 2.0 mg $l^{-1}$ produced a lesser effect (CI = 47.4). With 2,4-D, CI values ranged between 87.7 and 100.2. At NAA 0.5 mg $l^{-1}$, CI value was highest, that is 215.2. But at other levels it was nearly 130. Addition of 10 per cent CW to the medium increased the response to callusing with higher levels of ABA, decreased the same with NAA and produced no marked effect with 2,4-D.

The response of petals as explants showed that, the treatment with ABA, 2.0, and 2,4-D 0.5 mg $l^{-1}$ produced no callusing at all. NAA at 1.0 mg $l^{-1}$ showed the highest CI value of 280.9. ABA at 0.5-1.5 mg $l^{-1}$ produced CI value of range 73.5-93.2 with 2,4-D (1-2 mg $l^{-1}$) and other levels of NAA it ranged between 113.3 and 213.2. Addition of 10

per cent CW increased the CI values with ABA and 2,4-D 0.5 mg $l^{-1}$. However, a marked reduction in CI values were noticed with CW when NAA was present in the medium.

Culturing of gynoecium on ABA 1.5 and 2,4-D 1.5 mg $l^{-1}$ resulted in higher callus index of 100.6 and 93.1, respectively. The lowest values of 26.4 and 27.2 were shown by NAA 2.0 mg $l^{-1}$ and ABA 0.5, 1.0 mg $l^{-1}$. Addition of CW 10 per cent increased callusing except at ABA 1.5, 2,4-D 1.0 and 1.5 mg $l^{-1}$ and 1.0 and 1.5 mg $l^{-1}$ of NAA.

Considering integument as explant, the highest CI value 152.1 was obtained in 2,4-D 0.5 mg $l^{-1}$ and lowest by ABA 2.0 mg $l^{-1}$, (CI = 27.4). With other levels of growth regulators CI values varied between 58.9 to 114.2. Addition of CW increased callusing except at all levels of 2,4-D and NAA 0.5 mg $l^{-1}$.

Compared to all other explants tried stem segments showed more response to different levels of ABA. But in presence of 2,4-D and NAA, petals were showing more callusing.

The leaf and stem segments as well as petals were cultured on a medium containing MS basal salts, 2,4-D 1.0 mg $l^{-1}$ and sucrose at 4 per cent, alone or in combination with different levels of cytokinins like BA, 2 ip or KIN. Callus index obtained in each treatment are presented in Table 11.

Table 11. Effect of 2,4-D and cytokinin combinations on induction of callus from different explants of cocoa

Basal medium - MS + 2,4-D 1 mg $l^{-1}$ + Sucrose 4%

| Explant | Cytokinins | Callus index at different levels of cytokinins | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 mg $l^{-1}$ | 1 mg $l^{-1}$ | 2 mg $l^{-1}$ | 4 mg $l^{-1}$ | 6 mg $l^{-1}$ | 8 mg $l^{-1}$ |
| Leaf | BA | 113.5 | 75.5 | 122.3 | 32.7 | 56.6 | 20.1 |
| | 2 ip | | 88.2 | 100.4 | 150.1 | 133.7 | 100.0 |
| | KIN | | 100.1 | 100.2 | 100.3 | 100.4 | 75.4 |
| Stem | BA | 80.2 | 100.2 | 77.1 | 88.6 | 89.4 | 53.1 |
| | 2 ip | | 89.5 | 88.2 | 78.5 | 78.4 | 67.2 |
| | KIN | | 75.1 | 75.2 | 86.3 | 63.1 | 78.2 |
| Petals | BA | 110.7 | 100.0 | 133.2 | 110.7 | 110.5 | 110.8 |
| | 2 ip | | 125.2 | 133.2 | 107.5 | 150.3 | 118.5 |
| | KIN | | 100.2 | 138.3 | 110.1 | 118.2 | 133.4 |

When 2,4-D 1 mg $l^{-1}$ alone was used as growth regulator, the callus index was 113.5 in leaf explant. BA at 2 mg $l^{-1}$ only produced a slight increase in CI over control and the least value was obtained at 8 mg $l^{-1}$. 2 ip at 4 and 6 mg $l^{-1}$ caused an increase in CI value over control. Other levels of 2 ip as well as all the levels of KIN showed reduced effect over control.

When leaf explants were cultured in media with 6 mg $l^{-1}$ BA some well organized globular structures were observed in 20 per cent of the cultures (Plate 5b). These were further sub-cultured to different media, but later developed into non-embryogenic callus masses.

Using stem segments as explant, 2,4-D 1.0 mg $l^{-1}$ alone produced a CI value of 80.2. BA at 1.0 mg $l^{-1}$ along with 2,4-D 1.0 mg $l^{-1}$ produced a slight increase in CI value over control and at 8 mg $l^{-1}$ a decrease in response was noted. Other levels of BA as well as all the five levels of KIN and 2 ip responded similar to control.

Petals showed a callus index of 110.7 in media containing 2,4-D 1.0 mg $l^{-1}$ alone. Addition of cytokinins did not show much variation in callusing response. BA at 2 mg $l^{-1}$, KIN at 2, 6 and 8 mg $l^{-1}$ and 2 ip at all levels except 4 mg $l^{-1}$ produced a slight increase in callus index.

An experiment was conducted to study the effect of higher levels of BA in combination with 2,4-D in direct embryogenesis from leaf and stem explants. The results are presented in Table 12. About 75 per cent of the cultures were lost due to the drying of the explant, as a result of the suspected toxicity of the media. Callusing was observed in some of the cultures and this was more evident in cultures with 15 mg $l^{-1}$ 2,4-D. However, embryogenesis was not observed in any of the cultures, but some hairy out growths on calli was observed in some of them.

## 4.3 Indirect embryogenesis

### 4.3.1 Effect of cytokinins on indirect embryogenesis

In order to find out the effect of cytokinins on indirect embryogenesis an experiment was conducted using cytokinins - BA, 2 ip and KIN at 1, 2, 4, 6 and 8 mg $l^{-1}$ along with MS basal salts and 4 per cent sucrose. Original callus was induced in MS media containing 4 per cent sucrose and 2.0 mg $l^{-1}$ of 2,4-D, using explants like leaf and stem segments and petals. The results are presented in Table 13. No embryogenesis or organogenesis was observed.

With respect to leaf callus, maximum CI (253.5 and 253.1) was observed at BA concentrations 2 and 8 mg $l^{-1}$. With regard to 2 ip maximum response of 244.2 was observed at

Table 12. Effect of higher levels of 2,4-D; BA combinations on leaf and stem segments of cocoa

Basal medium - MS + Sucrose 6% + AC - 1%

| 2,4-D mg $l^{-1}$ | Percentage of cultures showing the response - callusing | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Leaf | | | | | Stem | | | | |
| | BA mg $l^{-1}$ | | | | | BA mg $l^{-1}$ | | | | |
| | 100 | 110 | 120 | 130 | 140 | 100 | 110 | 120 | 130 | 140 |
| 5 | 0.0 | 0.1 | 0.3 | 0.0 | 0.2 | 0.1 | 0.0 | 0.0 | 0.3 | 0.0 |
| 10 | 0.0 | 7.5 | 0.2 | 0.0 | 0.0 | 0.2 | 0.0 | 0.2 | 0.1 | 0.0 |
| 15 | 14.2 | 16.8 | 0.4 | 29.4 | 32.9 | 0.3 | 0.1 | 0.0 | 14.1 | 33.3 |
| 20 | 0.1 | 0.1 | 0.2 | 0.0 | 0.3 | 14.2 | 0.02 | 0.0 | 21.7 | 0.1 |

Table 13. Effect of cytokinins on indirect embryogenesis from callus of different explants of cocoa

Original callus induced in MS + Sucrose 4% + 2,4-D 2 mg $l^{-1}$
Basal medium - MS + Sucrose 4%

| Explant | Cytokinins | Callus index at different levels of cytokinins | | | | |
|---|---|---|---|---|---|---|
| | | 1 mg $l^{-1}$ | 2 mg $l^{-1}$ | 4 mg $l^{-1}$ | 6 mg $l^{-1}$ | 8 mg $l^{-1}$ |
| Leaf | BA | 154.1 | 253.5 | 165.1 | 143.2 | 253.1 |
| | 2 ip | 244.2 | 238.3 | 231.4 | 165.1 | 220.8 |
| | KIN | 100.8 | 225.8 | 225.2 | 225.5 | 250.6 |
| Stem | BA | 190.3 | 110.9 | 33.1 | 33.2 | 33.5 |
| | 2 ip | 99.8 | 110.7 | 33.5 | 88.6 | 66.7 |
| | KIN | 367.4 | 367.2 | 367.4 | 233.1 | 330.8 |
| Petals | BA | 300.2 | 396.3 | 356.5 | 400.4 | 300.2 |
| | 2 ip | 233.1 | 153.2 | 145.2 | 132.5 | 110.5 |
| | KIN | 313.7 | 200.1 | 300.5 | 100.8 | 325.7 |

1.0 mg $l^{-1}$ and gradually reduced up to 6 mg $l^{-1}$ with CI value 165.1. However, 8 mg $l^{-1}$ showed CI value as 220.8. By using KIN highest response was at 8 mg $l^{-1}$ and lowest at 1.0 mg $l^{-1}$.

When callus derived from stem explants were cultured in different cytokinins, maximum response was observed with KIN. CI values ranged from 233.1 to 367.4. With respect to BAP and 2 ip lower concentrations produced CI values of 99.8 to 190.3. But concentrations above 2 mg $l^{-1}$ were found to be inhibitory (CI = 33.1-88.6).

With respect to callus from petals, maximum proliferation was observed with BA (CI = 300.2-400.4). KIN was found to be better than 2 ip. T he effect of 2 ip was maximum at the lowest concentration (1 mg $l^{-1}$).

### 4.3.2 Effect of auxins, ABA and cytokinins on indirect embryogenesis

In order to induce indirect embryogenesis from callus of various explants of cocoa an experiment was conducted using different growth regulator combinations. The initial callus was induced in 2,4-D cytokinin combinations as presented in Table 11. The callus was subcultured to medium containing MS basal salts, 4 per cent sucrose and 3x3 combinations of ABA, NAA or 2,4-D (1, 2, 3 mg $l^{-1}$) and BA or 2 ip (1, 2.5 and 5 mg $l^{-1}$).

Eventhough the aim of the experiment was embryoid induction, no embryogenesis was noticed. Callus was found to be responding to further growth but never turned embryogenic. Callus obtained from the different treatments showed some variation in their structure and colour. Most of the calli were friable and granular while some appeared compact. Colour of the calli showed variation like, white, creamy white, yellowish or brownish. Rhizogenesis to a limited extent was noticed in some of the combinations tried (Plate 5d). This showed some organogenic potential of the calli. The media and explant in which rhizogenesis noticed, are presented in Table 14.

A trial was conducted to induce indirect embryogenesis from petal explants. The petals were first induced to callus in media containing MS basal salts, 4 per cent sucrose, 2,4-D 1.0 and KIN 2.4 mg $l^{-1}$ and MS basal media with sucrose at 3 per cent, 2,4-D 2.0 and KIN 2.0 mg $l^{-1}$. After three weeks of culturing calli were subcultured to embryo induction medium containing BA 1.0 mg $l^{-1}$ and AC 0.2 per cent. Here also no embryogenic response was observed.

### 4.3.3 Effect of suspension culture on indirect embryogenesis

The callus formed from different explants were used for starting suspension cultures. The initial callus was

induced in medium containing 2,4-D 1.0 mg $l^{-1}$ in combination with cytokinins like BA, KIN or 2 ip at 1, 2, 4, 6 and 8 mg $l^{-1}$. The calli produced from leaf, stem and petal explants were subcultured to media containing ABA, NAA or 2,4-D in combination with BA or 2 ip. The calli of all the treatments except in medium containing ABA 3.0 and 2 ip 1.0 mg $l^{-1}$ were found to proliferate without any differentiation. This never turned embryogenic. In above medium some kind of differentiation was shown by stem callus, which originally induced in 2,4-D 1.0 and KIN 8.0 mg $l^{-1}$.

These structures were suspected to be PEM (Proembryogenic masses) and they continued to proliferate when subcultured to in the same medium (MS + ABA 3.0 + 2 ip-1.0 mg $l^{-1}$ + sucrose 4 per cent) or to a lower level of ABA 1.0 mg $l^{-1}$. These globular structures as well as those formed from leaf explants in a medium containing 2,4-D 1.0 and BA 6 mg $l^{-1}$ were further sub cultured to induce embryogenesis. The media were half strength MS salts with 2 per cent sucrose or maltose with or without AC. Even then, only proliferation of the callus masses was observed, never turning embryogenic.

### 4.3.4 Irradiation studies

The callus derived from leaf, stem and floral parts were irradiated with gamma rays at 1 to 10 KR dose and

subcultured immediately to MS medium containing 2 mg $l^{-1}$ of NAA, 10 ml $l^{-1}$ of amino acids stock and 4 per cent sucrose. Here browning of the callus tissue was noticed. The intensity of browning increased with increase in dose of irradiation. After two months of culturing callus proliferation was observed without any embryogenesis.

**4.4 Morphology of embryoids**

The development of cocoa somatic embryos were studied using scanning electron as well as stereo-microscope. Embryoids appeared as single structure or more commonly in small groups or formed clusters over the cotyledon surface. Embryoids in a cluster were found in different stages of development and no synchronisation was observed in their formation. Plate 1b shows a cluster of embryoids at different stages of development and these include the developmental stages from globular to mature ones.

The present investigation on somatic embryogenesis in cocoa with stereo-microscope revealed globular, early heart-shaped, torpedo stage and adult embryoids with well developed cotyledons (Plate 2 a,b,c&d).

A typical adult embryoid showed a long axis and two small cotyledons with prominent veins. Many embryoids were found attached to the parental tissue by a short suspensor

(Plate 3a) but embryoids were also found which lacked them. A very common abnormality observed in somatic embryogenesis was the variable number of cotyledons (Plate 3b). The number of cotyledons varied from nil to many and usually 2 to 4 were found. In some cases an over growth of cotyledonary tissue was observed without producing a normal axis and such embryoids failed to germinate. Fusion of axes of two or more embryoids were also observed in some cultures (Plate 3c).

Embryoids developed from embryo axis exhibited more abnormalities than those developed from cotyledon explants. These were usually produced in clusters of numerous very small embryoids. The cotyledons mostly showed excessive proliferation and most of the cotyledons were fused and expanded. Such embryoids also failed to germinate. In the germination medium only expansion of cotyledons was observed (Plate 3d).

## 4.5 Plantlet regeneration from embryoids

### 4.5.1 Maturation of embryoids

The embryoids were sub-cultured to MS medium supplemented with 1.5 mg $l^{-1}$ ABA for attaining proper maturity. During this the cultures were incubated under dark. When a few of such cultures were transferred to light the fully matured embryoids showed signs of regeneration to

plantlets. Roots and often leaves were produced by some of them. After six weeks incubation in maturation medium, the embryoids were transferred to the germination media.

### 4.5.2 Effect of rinsing and desiccation on embryoid regeneration

Cocoa embryoids are reported to have some inhibitors preventing their germination. Hence they were properly washed and dried before transfer to the germination media. Rinsing the embryoids for 3 minutes with double distilled water followed by a desiccation for 3 minutes was found to enhance the plantlet regeneration. Rinsing for lesser time or desiccation for longer duration resulted in low regeneration. Desiccation for 12-48 hours resulted in browning and death of the embryoid.

### 4.5.3 Germination of embryoids

Embryoids after maturation were properly rinsed and transferred to liquid media for germination. The different treatments tried included change of basal media, variation in salt strength, difference in level of sucrose etc. For germination the cultures were incubated under light. The response of embryoids in different media for germination are presented in Table 15. Embryoids on germination produced elongated axes and green cotyledons.

Table 15. Effect of different media on the germination of somatic embryos of cocoa

| Treatment (liquid medium) | Percentage of germination size of embryoids | | | |
|---|---|---|---|---|
| | <1 mm | 1-2 mm | 2-4 mm | >4 mm |
| ½MS + sucrose 3 per cent | 0.0 | 0.2 | 0.5 | 12.3 |
| ½MS + sucrose 4 per cent | 0.0 | 0.1 | 7.8 | 26.2 |
| ½MS + sucrose 5 per cent | 0.0 | 1.3 | 27.3 | 83.5 |
| WPM + sucrose 5 per cent | 0.0 | 0.3 | 7.4 | 31.2 |
| MS + sucrose 5 per cent | 0.0 | 0.0 | 0.0 | 0.0 |

Size of the embryoids also influenced the germination. Maximum germination was shown by larger sized embryoids of more than 4 mm, when cultured in half MS medium with 5 per cent sucrose. When these embryoids were cultured on medium with 4 or 3 per cent sucrose a gradual reduction in germination was noted. Embryoids of smaller size (less than 2 mm) did not germinate in all the treatments tried and produced neither shoot nor root. Only greening of the cotyledons was observed. The maximum rate of conversion to plantlet was noted with embryoids of more than 4 mm size followed by embryoids of size 2-4 mm. Embryoids of 2-4 mm size exhibited proper rooting and feeble shoot growth. Further growth was arrested. Embryoids with size more than 4 mm showed proper germination (Plate 4a). Root development was quick and a healthy radicle was produced from most of the embryoids within ten days in germination medium. Excessive prolifertion of roots was observed in many embryoids in the germination media (Plate 4b). Thick stubby and unbranched roots were also observed rarely in germinating embryoids (Plate 4c). Shoot development was very slow and only feeble shoot was produced. Hence shoot and root development did not occur simultaneously. Roots were almost dead by the time of development of the shoot (Plate 4d). Hence plantlet regeneration was found to be difficult.

### 4.5.4 Secondary embryogenesis

Secondary embryogenesis was frequently observed during the course of germination of somatic embryos. Very small embryoids originated from axis or from cotyledons of primary embryoids. These were too small to regenerate. So further development by subculturing was not registered.

### 4.5.5 Shoot induction

As the development of embryoids into shoot was in a slow pace, a trial was conducted for enhancing the regeneration of shoots from the embryoids. Different treatments were tried for inducing shoot growth namely, removal of cotyledons, use of different growth regulators etc. The effects of CW, gibberellin and NAA were tested in combination or alone in basal media (MS and WPM). None of these treatments was effective in inducing proper shoot growth. In most of the combinations tried the embryoids remained green without any further growth. Only in MS and ½MS with NAA 0.2, GA 1.0 mg $l^{-1}$ and CW 15 per cent shoot growth with feeble leaves are noticed. When embryoids were cultured to ½MS medium containing 5 per cent sucrose, very rarely normal shoot growth was obtained. However, none of these plantlets attained proper size for planting out.

### 4.5.6 Micrografting

None of the embryoids grafted to *in vitro* seedlings developed properly. Most of them were contaminated due to the manipulation during the grafting procedure.

Encouraging results were obtained from grafting of cocoa somatic embryos to *ex vitro* seedlings. Eventhough the percentage of success was less, plantlet recovery could be achieved. The embryoid derived plants could also be transferred to the nursery by this method (Plates 6a & b).

# Discussion

# 5. DISCUSSION

Somatic embryogenesis could be exploited for rapid clonal propagation of superior genotypes provided that source of explant for somatic embryogenesis is from sporophytic tissue and the somatic embryos develop into plantlets. In cocoa somatic embryogenesis has been obtained from immature zygotic embryos. Embryoids derived from zygote, however have a limited value for propagation because the zygote is an untested genotype. Although regeneration from embryonic tissues does not vegetatively propagate the maternal clone, it would be of use in multiplying the progeny of selected crosses. An understanding of the process of embryogenesis from zygotic embryos could also provide a basis for attempts to achieve embryogenesis from maternal tissues. Somatic embryogenesis also provides basis for genetic improvement of tree species. Embryogenesis appears to have an epidermal or sub-epidermal origin which allows embryogenic tissues to readily be exposed to Agrobacterium or particle gun mediated gene transfer. The embryogenic process appears to be highly sensitive to external application of chemicals. This can be made use in in vitro screening and selection of tolerant/ resistant plants to Fe, Al or fungal toxicity.

## 5.1 Surface sterilization of explants

In tree micropropagation, the incidence of bacterial and fungal infection in cultures of field explants is a major problem. About 90 per cent of the explants collected from the field were lost because of microbial contamination, regardless of the procedure used for sterilization of the explants (Dublin, 1984). Fungicidal spraying of mother trees, to reduce the culture contamination in cocoa was suggested by Legrand and Mississo (1986). In this experiment the explants, leaf and stem segments were collected from plants grown under glasshouse conditions. These plants were regularly sprayed with contact and systemic fungicides. Mercuric chloride at 0.1 per cent treated for 3 minutes resulted in nearly 90 per cent culture establishment for leaf and stem segments. This is similar to the earlier observation of Antony (1993). The pods were sterilized for one hour in 0.1 per cent mercuric chloride. The cotyledons were separated aseptically and no further sterilization of embryonal tissue was given. Even then nearly 98 per cent culture establishment was obtained. The procedure of further sterilization of embryonic tissues proposed by Adu Ampomah et al. (1988) and Antony (1993) could be avoided. Figueira and Janick (1993) followed the sterilization of pods of cocoa as washing in tap water and flaming in 95 per cent ethanol in a laminar flow, unlike the

mercuric chloride treatment in our studies. For the sterilization of flower buds the procedure was standardised as 0.1 per cent mercuric chloride treatment for 5 minutes. This resulted in 80 per cent of culture establishment.

## 5.2 Basal medium

Embryogenesis has been observed in a range of media starting from the relatively low salt containing Whites medium to the more concentrated MS medium. Evans et al. (1981) noted that for somatic embryogenesis 70 per cent of the explants were cultured on MS medium or modification of MS. Earlier studies on cocoa somatic embryogenesis were conducted in MS medium (Kononowicz et al., 1984; Janick, 1986; Adu-Ampomah et al., 1988). In our studies also MS medium was found to be more effective in inducing embryogenesis. A lower degree of embryogenesis was also noticed in WPM and $B_5$. Antony (1993) reported no embryogenesis in WPM and $B_5$. Only MS medium was found to be effective in inducing embryogenesis in her studies.

## 5.3 Explants and embryogenesis

In cocoa a lot of earlier work has been done by culturing various somatic tissues as source of explant (Archibald, 1954; Ibanez, 1964; Hall and Collin, 1975; Searles et al., 1976; Tsai and Kinsella, 1981; Dufour and Dublin,

1985; Janick, 1986; Adu-Ampomah et al., 1988; Duhem et al., 1989). But success in somatic embryogenesis has been reported only from unripe zygotic embryos. Recent reports indicated a lower degree of embryogenesis from tissues like nucellus, internal integument and flower petals (Chatelet et al., 1992; Lopez-Baez et al., 1993; Figuera and Janick, 1993; Sondhal, et al., 1993). In the present study embryoids were induced only from cotyledons and embryonic axes of immature zygotic embryos. Embryonic, meristematic and reproductive tissues appeared to possess more potential for embryogenic growth (Ammirato, 1983). Immature and mature leaf and stem segments, integument and floral parts failed to respond. Kato and Kateuchi (1963) suggested that direct embryogenesis proceeded from embryogenic determined cells. Leaf and stem segments are differentiated tissues and were lacking embryogenic determined cells and hence would have failed to initiate embryogenesis.

### 5.3.1 Pod maturity and degree of embryogenesis

Pod maturity markedly affected the embryogenic potential of the cotyledon explants in cocoa. This was proposed even in 1979 by Pence and later by Adu-Ampomah et al. (1988) and Antony (1993). In our studies, light pink coloured cotyledons from 100 day old pods produced the maximum frequency of embryogenesis. With increase in maturity of pods, the embryogenic competence of the cotyledons decreased.

Purple coloured cotyledons of 120-130 days maturity failed to regenerate embryoids, but produced only roots. Although embryogenesis was noted from pods of maturity ranging from 90-110 days the optimum maturity of pods for somatic embryogenesis was 100 days.

## 5.4 Physical conditions of culture - Light intensity and temperature

Somatic embryogenesis can occur under light or dark incubation. In black locust, Robinia psuedoacacia L. high light intensity was required for somatic embryogenesis (Arrillaga et al., 1994) while in coffee (Coffea arabica and Coffea canephora) embryoids occurred more normally in complete darkness (Sondahl and Sharp, 1977). Adu-Ampomah et al. (1988) and Duhem et al. (1989) could obtain embryoids on immature cotyledon explant of cocoa when incubated under complete darkness. Similar results were shown by the present studies also. Maximum embryogenic cultures were observed under dark incubation. Embryogenesis also occurred at a lower frequency when incubated under light.

It was seen that temperature has significant impact on in vitro regeneration. It is true with somatic embryogenesis also. For regeneration of embryoids from sugarcane callus it required incubation at 27°C (Fitch and Moore, 1993). In the

present investigation, it was seen that cocoa cultures require a higher temperature of 30 ± 2°C for induction of somatic embryogenesis. Adu-Ampomah *et al*. (1988) also reported a culture room temperature of 31°C for somatic embryogenesis in cocoa. The incubation temperature seemed to be very critical for cocoa somatic embryogenesis since none of the cultures incubated in dark at 26°C turned out to be embryogenic. The optimum physical conditions for cocoa somatic embryogenesis was identified as dark incubation at a temperature of 30 ± 2°C.

## 5.5 Media supplements

Vardi *et al*. (1975) obtained somatic embryos in *Citrus sinensis* without any growth regulators in the media. These are likely to be induced from pre-embryogenic determined cells. Fugimura and Komamine (1980) suggested that explants derived from differentiated tissues require growth regulators in the medium especially auxin or auxin in combination with cytokinin for onset of growth and induction of embryogenesis. Ammirato (1983) proposed the idea that a primary medium with an auxin source and a secondary medium devoid of growth substances but both containing substantial amount of reduced nitrogen was found to be essential to induce embryoids. The growth supplements in the medium for proliferation of the

explant and further differentiation into embryoids was critical.

In the present study using immature cotyledon explants of cocoa, embryogenesis could be obtained by addition of various levels of auxins to the medium. But with other explants, only callusing was noted. Combination of auxins and cytokinins also resulted only in callusing of such tissues.

Among the different auxins tried (NAA and 2,4-D) for inducing embryogenesis from cotyledons, the most superior treatment was with that of NAA 1-2 mg $l^{-1}$. The effect of NAA was not replaceable by other auxins such as 2,4-D. Adu-Ampomah et al. (1988) also reported cocoa somatic embryogenesis in the presence of NAA in the media. Evans et al. (1981) noted that 2,4-D was extremely useful in inducing embryogenesis in 57.1 per cent of cultures. In the present study also 2,4-D was effective in inducing embryogenesis. The number of embryoids per culture was found to be high but they were of smaller size which were sub-optimal for regeneration into plantlet. Kononowicz et al. (1984) also used 2,4-D for indirect embryogenesis in cocoa. There was no mention about plantlet recovery from such embryoids.

Coconut water favoured embryogenesis in cocoa as evidenced from the present studies. It contains a number of cell division factors and free amino acids (Shantz and Steward, 1952). Addition of 10 per cent CW to MS medium with ABA, 2,4-D or NAA at various levels increased the frequency and intensity of embryogenesis in certain combinations (Table 9). The reduction in the intensity of embryogenesis at higher levels of 2,4-D along with CW shows the inhibitory effects of higher concentrations of auxins in embryogenesis. A modification of the media suggested by Adu-Ampomah et al. (1988) by removing CH from the medium and addition of CW at 15 per cent increased the frequency and intensity of embryogenesis to a greater extend. The beneficial effects of CW in cocoa somatic embryogenesis is evident from this.

The growth inhibitor abscissic acid was found to promote embryogenesis in cocoa. Among the different levels tried ABA 2.0 mg $l^{-1}$ produced embryoids with maximum size and having minimum abnormalities. Ammirato (1974) reported that aberrant morphology of somatic embryos has been remedied in some species by exogenous application of ABA to the culture medium.

The size of the embryoids was found to be negatively correlated with the intensity of embryogenesis. In 2,4-D

supplemented media, the number of embryoids was more but size was reduced and the reverse situation was observed in media where ABA was included. Supplementing ABA medium with 10 per cent CW, however, reduced the size of the embryoids indicating that combinations of these media supplements are not ideal for embryogenesis. CW in general increased the intensity as well as frequency of embryogenesis but, it was found to have an adverse effect on the size of embryoids. But when immature cotyledon explants were cultured on modified Adu-Ampomah medium with 15 per cent CW, the frequency, intensity as well as number of larger sized embryoids were high compared with all other media tested. So it was recognised as standard medium for production of embryoids for further experiments on embryogenesis. This increased effect may be attributed to other components of the medium namely thiamine at 1.0 and NAA at 1.8 mg $l^{-1}$.

Based on the present studies, an ideal medium for somatic embryogenesis from cotyledon explants is proposed. The composition of the medium was MS + NAA 1.8, thiamine 1.0 mg $l^{-1}$ with CW 15 and sucrose 4 per cent.

ABA at 1.5 mg $l^{-1}$ was also used in the media for maturation of embryoids. Pence (1992) suggested that both ABA and sucrose are needed for maturation of zygotic embryos of cocoa *in vitro*. Transfer of cultures to ABA containing medium

increased the number of normal embryoids compared to the cultures maintained in the same medium.

## 5.6 Genotype and embryogenesis

Terzi and Loschiavo (1990) pointed out that, of the several factors that affect the induction of somatic embryogenesis, formost one is the genotype of the starting material. Tissarat et al. (1979) suggested explant and certain of its associated physiological qualities are the most significant determinants of embryo initiation while the in vitro environment acts to enhance the embryogenic process.

A relevant comparative study of different genotypes was not yet conducted on somatic embryogenesis of cocoa. Pence et al. (1979) studied the embryogenesis from immature cotyledons of Amelonado, while Esan (1982) studied embryogenesis in Amazon types. Kononowicz and Janick (1984) reported that the stimulatory effect of GA on embryogenesis was clone specific. Adu-Ampomah et al. (1988) also noted varietal differences in both the rate of embryo production and in size of developed embryoids. Amazon types were superior to Amelonado in their studies. Wen and Kinsella (1992) observed that the rate of embryogenesis was different among zygotic embryo explants from different strains of cultivar Mexico or different pods of Amelonado variety.

The present study indicates that cocoa genotypes expressed qualitative and quantitative differences on the induction of embryogenesis. Screening of different genotypes for ability to regenerate embryoids from immature cotyledon explants revealed that some of the hybrids as well as the genotype GVI 61 were superior to others. The desirable attributes like high frequency of embryogenesis, lower percentage of abnormality and higher number of embryoids of more than 4 mm size were shown by the Series I hybrid 6.5 in the standard medium.

It was seen from this study that the inbreds were showing lesser response to embryogenic pattern of development compared to hybrids and open pollinated varieties. In the standard medium, the frequency and intensity of embryogenesis was minimum in the $S_1$ inbred M 12.21 while in the hybrids and open pollinated varieties higher values were observed. In a medium containing 1.5 mg $l^{-1}$ of ABA also the inbred Amelonado ($S_1$) showed the least frequency as well as intensity of embryogenesis compared to all other genotypes tested. Cocoa being a highly heterozygous cross pollinated crop, much loss in vigour can be expected by selfing. The general decrease in vigour would also have contributed to the lesser response to embryogenesis.

## 5.7 Embryoid induction from leaf and stem segments

In many earlier reports, various somatic tissues of cocoa had been cultured but embryogenesis had been reported only from embryonic tissues. Litz (1986) reported that the callus derived from Amelonado leaf discs turned embryogenic in media containing 2,4-D at high levels of BA and activated charcoal. The same experiment was repeated in our studies and callusing alone was obtained in a few combinations. When these calli were further subcultured to different media, only proliferation was observed without any embryogenesis. Other media supplements namely auxins, cytokinins or coconut water also did not produce any embryogenic response in leaf and stem segments. Hence it can be suggested that leaf and stem segments have little potential for production of embryogenic culture.

## 5.8 Embryogenesis from integument and floral parts

Chatelet et al. (1992) obtained initiation of embryogenic cells and early development of pro-embryos from nucellar tissue and integument. Later these cells accumulated phenolic compounds which may have been responsible for subsequent impaired development. By different media manipulations, only callus growth was obtained from the integument in the present studies.

Somatic embryogenesis and plantlet regeneration from flower parts of cocoa was studied by Lopez-Baez et al. (1993). Sondhal et al. (1993) also reported the development of somatic embryos from petals and nucellar tissues of cocoa. When these media were tried, the petals showed only callusing in our studies. No tissues other than embryonic parts turned out to be embryogenic. The varied responses might be due to the genotypic difference of the explants tried.

## 5.9 Morphology of embryoids

The origin of embryoids as solitary structures or in small groups and different aberrant forms were observed in the present study. This was earlier reported by Santos and Machado (1989) in their Scanning Electron Microscope study of cocoa embryoids. The presence of varied number of cotyledons as well as varied types of suspensors were revealed by their studies. Suspensor-like structures in cocoa embryoids were also reported by Pence et al. (1980) and Kononowicz et al. (1984). In our studies, embryoids lacking suspensors were also found unlike the report of Santos and Machado (1989) that a suspensor-like structure is present in every embryo. Kononowicz et al. (1984) reported that somatic embryos originate in two ways - by budding and nonbudding processes. Embryos which originate by budding process usually have a suspensor as they develop from the cells of the hypocotylary

epidermis while those arising by nonbudding process lack a suspensor as they are embedded in the cotyledonary tissue.

## 5.10 Plantlet regeneration from somatic embryos

### 5.10.1 Embryoid germination

Germination of embryoids can be obtained by transferring them to media devoid of growth regulators or containing growth regulators. Wang and Janick (1984) reported that the process of renewing the liquid medium induced radicle development in 60 per cent of cocoa embryoids. Removal of CH and reducing MS salt concentration promoted radicle development. Embryoids germinated only if they were leached in water. The leachate that inhibited the germination of lettuce seed might likely be p-coumaric acid. For germination removal of cotyledons and addition of exogenous hormones at the proper stage of development are required (Novak et al., 1986). Pence et al. (1979) and Esan (1977) reported that addition of growth hormones to the medium promoted excessive callus formation, especially on solid medium which greatly masked the potential of embryos to form plantlet. Adu-Ampomah et al. (1988) reported continuous production of an endogenous factor especially in the somatic embryo cotyledons, which promoted callus formation. Exogenous application of hormones

amplified this effect. So they suggested the germination of embryoids in hormone free liquid medium after removal of cotyledons, subculturing to a medium with GA and NAA for differentiation into plantlet.

Present study on germination of embryoids confirmed the presence of inhibitor. The germination was maximum in liquid medium containing half strength MS salts and 5 per cent sucrose. Washing, desiccation and renewal of liquid medium were essential to enhance the germination. Washing of the embryoids and renewal of the liquid medium might have reduced the inhibitor present and desiccation induced a stress condition. Embryoids of size more than 4 mm exhibited more conversion to plantlet. This was earlier observed by Adu-Ampomah et al. (1988) and Antony (1993).

Eventhough a number of trials were conducted to obtain normal embryo-derived plantlet to plant out, nothing was found successful. Of all the earlier reports available with cocoa somatic embryogenesis, successful planting out was obtained only by Sondhal et al. (1993). In their studies, the conversion of somatic embryos into seedlings required a pre-culture in liquid medium and transfer to semisolid medium in chambers receiving 20,000 ppm of carbon dioxide.

### 5.10.2 Micrografting

Rooting, hardening and planting out are very crucial steps in micropropagation during which a sizable number of tissue culture derived plantlets are lost. This aspect of micropropagation becomes much more critical in case of the minute plantlets recovered from somatic embryos. By adopting the technique of micrografting, such plantlets could be saved and they could be directly transferred to the field within a short span. Aguilar *et al*. (1992) reported that ten months were needed for complete plantlet regeneration when they attempted micrografting of cocoa embryoids in three week old axenic seedlings. Much of this time could be saved by a slight modification of the technique, in our studies. Micrografting was done on three to four week old *ex vitro* seedlings. Such plants could be hardened easily unlike the *in vitro* plantlets and also could be quickly transferred to the field.

Aguilar *et al*. (1992) observed that the embryoids without cotyledons showed better plantlet recovery by micrografting. In our studies also successful grafts were obtained from germinating embryoids devoid of cotyledons and having at least a single hardened leaflet. Successful grafts could be made ready for field planting within two months,

unlike the ten months period reported by Aguilar and co-workers.

The expenses on micropropagation could also be very much reduced by micrografting on ex vitro seedlings. Rooting was not required, hardening turned out to be very simple and mortality to an established graft cannot be expected. The minute shoot of the embryoid produced leaves as big an those of normal seedlings within a period of two months. The presence of an already established stock converts the minute shoot to a normal plantlet within a short span. This is a remarkable achievement ever obtained in cocoa tissue culture.

The present work describes for the first time a simple method for successful regeneration of plantlet from cocoa somatic embryos. There is no doubt that plant cell totipotency expressed as somatic embryogenesis and the development of entire plants from such embryos is a potential tool for plant propagation and improvement.

# Summary

## SUMMARY

Investigations on plantlet regeneration through somatic embryogenesis in Theobroma cacao L. were carried out at the College of Horticulture, Kerala Agricultural University, Vellanikkara during 1992-94 utilising the facilities in the tissue culture laboratory, Cadbury-KAU Co-operative Cocoa Research Project. The results of the study are summarised below:

1. Treating the leaf and stem segments with mercuric chloride (0.1%) for 3 minutes and flower buds for 5 minutes was found to be the best method of surface sterilization.

2. Among the three basal media tried for somatic embryogenesis namely MS, $B_5$ and WPM, MS medium was found to be the best.

3. Light pink coloured cotyledons from zygotic embryos of 100 day old pods showed maximum degree of embryogenesis. Rhizogenesis was observed in older cotyledons.

4. Maximum frequency and intensity of embryogenesis were observed under dark incubation at a culture room temperature of 30 $\pm$ 2°C.

5. Genotypic variation was observed in response to embryogenesis. Frequency, intensity as well as morphology of embryoids varied with genotype.

6. Among the different genotypes tested, the hybrid H 6.5 of Series I was found to be the ideal explant source because of its higher frequency and intensity of embryogenesis with lesser frequency of abnormal embryos.

7. Among the three growth regulators tried namely ABA, NAA and 2,4-D for somatic embryogenesis, best response was obtained with NAA. ABA promoted the formation of larger embryos but at a lower frequency and intensity. 2,4-D produced smaller embryoids at a higher intensity.

8. The complex organic supplement, coconut water promoted embryogenesis but had an adverse effect on the size of the embryoids. This reduction in size could be overcome by addition of NAA 1.8 and thiamine 1.0 mg $l^{-1}$.

9. Maximum embryogenesis was observed in the media MS + NAA 1.8 + thiamine 1 mg $l^{-1}$ + CW 15 per cent + sucrose 4 per cent.

10. Non-embryonic tissues tried as explants for embryogenesis included leaf and stem segments, gynoecium and integument. All these yielded only non-embryogenic calli in the several media tested for embryogenesis.

11. Presence of NAA in the induction medium favoured rhizogenesis in many cultures derived from non-embryonic tissues.

12. Some organised globular structures were obtained when the leaf explants were cultured in media containing 2,4-D 1.0 and BA 6.0 mg $l^{-1}$.

13. Stem callus in suspension cultures containing 2 ip 1.0 and ABA 3.0 mg $l^{-1}$ also showed some organised globular structures. These structures turned into non-embryogenic calli when cultured in different media for embryogenesis.

14. Embryoids originated from immature cotyledon explants either singly or in clusters. Mostly direct embryogenesis was observed. Each embryoid had an axis and two cotyledons.

15. A large number of aberrant forms were observed among the embryoids. Varied number of cotyledons, fusion of the axes and embryoids of sub-normal size etc. were some of them.

16. A typical embryoid was found to have no suspensor like structure. However, embryoids having suspensor were also not uncommon.

17. Washing the embryoids to remove the inhibitors and desiccating them for some time was found to improve their germination.

18. Liquid medium supplemented with ½MS salts and 5 per cent sucrose was found to be ideal for germination of embryoids.

19. Maximum plantlet recovery was obtained from larger embryoids of size 4 mm or more.

20. Shoot formation in embryoid derived plantlet was favoured by removal of the cotyledons.

21. Plantlet recovery from embryoid was successfully achieved by micrografting germinating embryoids to seedling rootstocks. This is the first report of its kind in cocoa tissue culture.

22. Grafting to *ex vitro* seedlings was found to be easier than *in vitro* seedlings. Presence of atleast one small leaf in the embryoid was essential for the success in micrografting.

23. The technique of micrografting could save much time and resources in tissue culture since rooting could be avoided, hardening became simple and healthy plantlet could be obtained within a short span.

# References

## REFERENCES


Adu-Ampomah, Y., Novak, F.J., Afza, R., Van Durem, M. and Perea-Dallos, M. 1988. Initiation and growth of somatic embryos of cocoa (Theobroma cacao L.). Cafe Cacao The. 32: 187-200

Aguilar, M., Villalobos, V.M. and Varquez, N. 1992. Production of cocoa plants Theobroma cacao via micrografting of somatic embryos. J. In vitro Cell Dev. Bio. 28: 15-19

Ammirato, P.V. 1974. The effects of abscissic acid on the development of somatic embryos from cells of caraway (Carum carvi L.). Bot. Gaz. 135: 328-337

Ammirato, P.V. 1983. The regulation of somatic embryo development in plant cell cultures: suspension culture techniques and hormone requirements. Biotechnology 1: 68-74

Antony, J. 1993. Somatic embryogenesis in cocoa (Theobroma cacao L.) M.Sc. (Ag.) Thesis. Kerala Agricultural University, Thrissur, Kerala

Archibald, J.F. 1954. Culture in vitro of cambial tissue of cocoa. Nature 173: 351-352

Arrillaga, I., Tobolski, J.J. and Merkle, S.A. 1994. Advances in somatic embryogenesis and plant production of black locust. (Robinia psuedoacacia L.). Pl. Cell Rep. 13: 171-175

Bhansali, R.R., Driver, J.A. and Durzan, D.J. 1990. Rapid multiplication of adventitious somatic embryos in peach and nectarine by secondary embryogenesis. Pl. Cell Rep. 9: 280-284

Bonneau, L., Beranger-Novat, N. and Monin, J. 1994. Somatic embryogenesis and plant regeneration in a woody species. The European Spindle Tree (Euonymus europaeus L.). Pl. Cell Rep. 13: 135-138

Chalupa, V. 1990. Plant regeneration by somatic embryogenesis from cultured immature embryos of oak (Quercus robur L.) and linden (Tilia cordata mill.). Pl. Cell Rep. 9: 398-401

Chatelet, P., Machaux-Ferriere, N. and Dublin, P. 1992. Embryogenic potential of the nucellus and the internal integument of immature seeds of cocoa. Theobroma cacao L. Compter Rendus de l' Academic des Sciences. Series 3 Sciences dela vie. 315: 55-62

Deogratius, J.M., Castellani, V., Dosba, F., Juarez, J., Arregui, J.M., Ortega, C., Ortega, V., Llacer, G. and Navarro, L. 1991. Study of growth parameters on apricot shoot tip grafting in vitro (STG) Acta Horticulturae 293: 363-371

Dublin, P. 1984. Extractable Production Section IX, In : Evans, D.D., Sharp, W.R., Ammirato, P.V. and Yamada, Y. (ed). Handbook of Plant Cell Culture. Mac Millan Pub. Co., New York. 541-564

Dufour, M. and Dublin, P. 1985. Quelques donnees sur la multiplication vegetative in vitro des cacaoyers cultiver (Theobroma cacao). Cafe Cacao The. 29: 235-244

Duhem, K., Mercier, N.L. and Boxus, P. 1989. New data on the induction and development of somatic embryos in Theobroma cacao. Cafe Cacao The 33: 220-223

Esan, E.B. 1977. Tissue culture studies on cacao (Theobroma cacao L.). A supplementation of current research. Proceedings Vth International Cacao Research Conference, Ibadan, Nigeria. 1-9 Sept. 1975. Cocoa Research Institute of Nigeria (Ibadan). pp. 116-125

Esan, E.B. 1982b. In vitro studies on morphogenesis and plant regeneration in tissue cultures of cacao and Herrania sp. - a progress report. Proc. 8th Int. Cocoa Res. Conf. Cartagena Columbia 1: 245-252

Evans, D.A., Sharp, W.L. and Flinck, C.F. 1981. Growth and behaviour of cell cultures. Embryogenesis and organogenesis. Thorpe, T.A. (ed). Plant Tissue Culture : Methods and Applications in Agriculture. Academic press, New York, 45-114

Figueira, A. and Janick, J. 1993. Development of nucellar somatic embryos of Theobroma cacao. Acta Horticulturae 336: 231-238

Finer, J.J. and Nagasawa, A. 1988. Development of an embryogenic suspension culture of soybean (Glycine max Merrill.). Pl. Cell Tissue Organ Cult. 15: 125-136

Haberlandt, G. 1902. Sitzupgsber. Mat. Nt. g Kais. Akad. Wiss. Wien. 111: 69-92

Hall, I.R.H. and Collin, H.A. 1975. Initiation and growth of tissue cultures of Theobroma cacao. Ann. Bot. 39: 555-570

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

Hussey, G. 1986. Vegetative propagation of plants by tissue culture. Yeoman, M.M. (Ed.). Plant Cell Culture Technology. Black well scientific publications, London.

Ibanez, M.L. 1964. The cultivation of cocoa embryos in sterile culture. Tropic. Agric. **41:** 325-328

Janick, J. 1986. Somatic embryogenesis in cocoa and jojoba. Proc. Int. Soc. Trop. Hort. 30: 209-213

Kato, H. and Kateuchi, M. 1963. Morphogenesis in vitro starting from single cells of carrot roots. Pl. Cell physiol. **4:** 243-245

Kononowicz, H. and Janick, J. 1984. Response of embryogenic callus of Theobroma cacao L. to gibberellic acid and inhibitors of gibberellic acid synthesis. Z. Pflanzenphysiol. 113: 347-358

Kononowicz, H., Kononowicz, A.K. and Janick, J. 1984. Asexual embryogenesis via callus of Theobroma cacao L. Z. Pflanzenphysiol. 113: 347-358

Levy, L.W. 1981. A large scale application of tissue culture - The mass propagation of pyrethrum clones in Ecuador. Environ. Exp. Bot. 21: 389-395

Legrand, B. and Mississo, F. 1986. Effect of size of the explants and growth regulators on the development of tissues of Theobroma cacao L. Var. Amelonado cultivars in vitro. Cafe Cacao The 30: 239-246

Litz, R.E. 1986. Tissue culture studies with Theobroma cacao Dimick, P.S. (ed.). Proc. Cocoa Biotechnol. Symp. Pennysylvania State Univ. Park. pp.111-120

Lloyd, Z. and McCown, B. 1980. Commercially feasible micropropagation of Mountain Lourel Kalamia latifolia by use of shoot tip culture. Proc. Int. Pl. Prop. Soc. 30: 421-427

Lopez-Baez, O., Bollon, H., Eskes, A. and Peliard, V. 1993. Somatic embryogenesis and plant regeneration from flower parts of cocoa. Comptes Rendus de l' Academic des sciences series sciences 316: 579-584

Michaux-Ferriere, N., Grout, H. and Carron, M.P. 1992. Origin and ontogenesis of somatic embryogenesis in Hevea brasiliences. Am. J. Bot. 79: 174

Michler, C.H. and Bauer, E.O. 1991. High frequency somatic embryogenesis from leaf tissue of Populus sp. Pl. Sci. 77: 111-118.

Monaco, L.C., Sondhal, M.R., Carvalho, H., Crocomo, O.J. and Sharp, W.R. 1977. Applications of tissue culture in the improvement of coffee. J. Reinert and Y.P.S. Bajaj (eds.) Plant Cell Tissue and Organ Culture. Springer Verlag Berlin, 109-129

Murashige, T. 1974. Plant propagation by tissue culture. Ann. Rev. Pl. Physiol. 25: 135-166

Murashige, T. 1977. Clonal propagation of horticultural crops through tissue culture. Barz, W., Reinhard, E. and Zenk, M.H. (ed.). Plant Tissue Culture and Its Biotechnological Applications. Springler-Verlag, New York, 392-408

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15: 473-497

Navardo, L. 1990. Shoot tip grafting in vitro of woody species and its influence on plant age. Rodriguez, R., Tamer, S.R. and Durzan, D.J. (eds.). Plant Aging; Basic and Applied Approaches. Plenum Press, New York, USA.

Novak, F.J., Donini, B. and Owusu, G.K. 1986. Somatic embryogenesis and in vitro plant development of cocoa. Proc. Int. FAO/IAEA Symposium Nuclear Techniques and In Vitro Culture for Plant Improvement. IAEA, Vienna: 443-449

Orchard, J.E., Collin, H.A. and Hardwick, K. 1979. Culture of shoot apices of Theobroma cacao. Physiol. Plant. 47: 207-210

Pence, V.C. 1992. ABA and the maturation of cacao embryos *in vitro*. *Pl. Physiol.* **18**: 1391-1395

Pence, V.C., Hasegawa, P.M. and Janick, J. 1979. Asexual embryogenesis in *Theobroma cacao* L. *J. Am. Soc. Hortic. Sci.* 104: 145-148

Pence, V.C., Hasegawa, P.M. and Janick, J. 1980. Initiation and development of asexual embryos of *Theobroma cacao* L. *in vitro*. *Z. Phlanzenphysiol.* 1-14

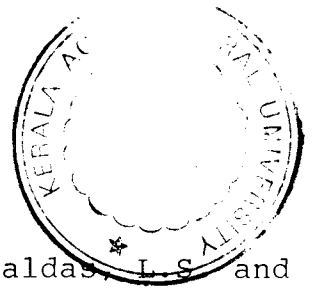
Prior, C. 1977. Growth of *Oncobasidium theobromae*, Talbot and Kaene in dual culture with callus tissue of *Theobroma cacao* L. *J. Gen. Microbiol.* **99**: 219-222

Rao, A.N. and Lee, S.K. 1986. Overview of the *in vitro* propagation of woody plants and plantation crops. Withers L.A. and Alderson, P.G. (ed.) *Plant Tissue Culture and Its Agricultural Applications*. Butterwarths, London: 123-138

Reinert, J. 1958. Morphogenese und ihre kontrolle and Gewebekulturen aus Carotten. *Naturwissenschaft* 45: 344-345

Santos, A.V.P. and Machado, R.D. 1989. A scanning electron microscope study of *Theobroma cacao* somatic embryogenesis. *Ann. Bot.* 64: 293-296

Searles, B.R., Alvim, P. de T. and Sharp, W.R. 1976. Hormonal control of cellular proliferation in cultured callus derived from *Theobroma cacao* L. *Revista Theobroma*. 6: 77-81



Sharp, W.R., Sondhal, M.R., Caldas, L.S. and Maraffa, S.B. 1980. The physiology of in vitro asexual embryogenesis. Hort. Rev. 2: 268-310

Shantz, E.M. and Steward, F.C. 1952. Coconut milk factor: the growth promoting substances in coconut milk. Am. Chem. Soc. 74: 393

Sondhal, M.R., Liu, S., Bellato, C. and Bragin, A. 1993. Cocoa somatic embryogenesis. Acta Horticulturae 336: 245-248

Sondhal, M.R. and Sharp, W.R. 1977. High frequency induction of somatic embryos in cultured leaf explants of Coffea arabica L. Z Pflanzenphysiol. 81: 395-408

Steward, F.C., Maper, O.M. and Moars, K. 1958. Growth and organised development of cultured cells. Organisation in cultured growth from freely suspended cells. Am. J. Bot. 45: 705-708

Tahardi, S. 1984. Tissue cultures of Theobroma cacao. Menara Perkebunan 52: 174-178

Terzi, M. and Loschiavo, F. 1990. Somatic embryogenesis. Bhojwani, S.S. (ed). Plant Tissue Culture : Applications and Limitations Elsevier science publishing company. Inc 54-67

Tisserat, B., Esan, E.B. and Murashige, T. 1979. Somatic embryogenesis in angiosperms. Hortic. Rev. 1: 1-78

Tsai, C.H. and Kinsella J.F. 1981. Initiation and growth of callus and cell suspensions of Theobroma cacao L. Ann. Bot. 48: 549-557

Vardi, A., Spiegel-Roy, P. and Galun, E. 1975. Citrus cell culture; Isolation of protoplasts, plating densities, effect of mutagens and regeneration of embryos. Pl. Sci. Lett. 4: 231-236

Wang, Y.C. and Janick, J. 1984. Inducing precocious germination in asexual embryos of cocoa. Hort. Sci. 19: 839-841

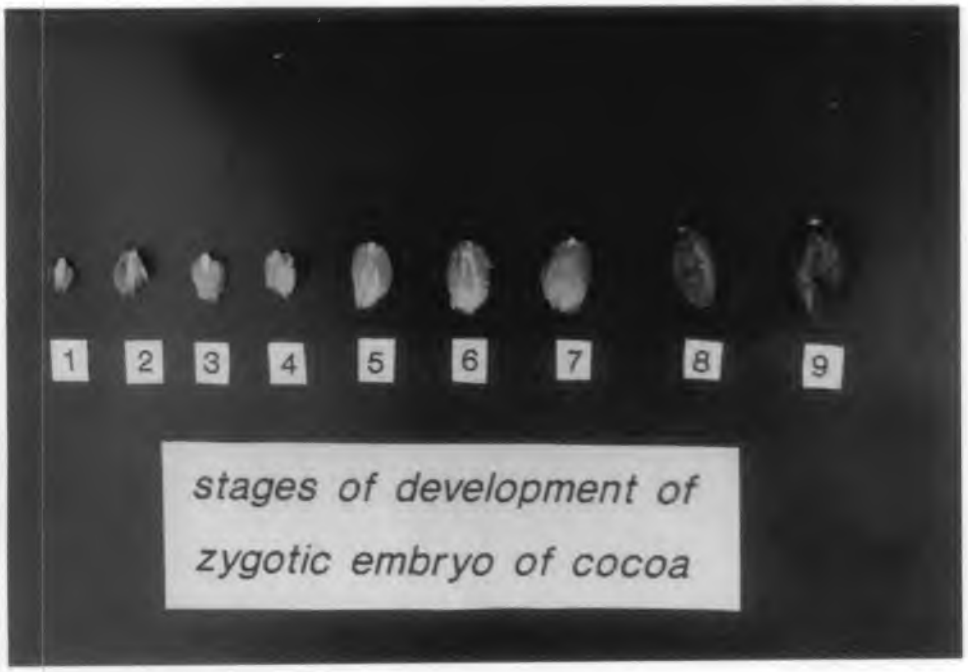
Wen, M.C. and Kinsella, J.E. 1992. Fatty acid composition of suspension cell cultures of Theobroma cacao are altered by culture conditions. J. Food Sci. 57: 1452-1453

# Plates

Plate 1

a. Cocoa zygotic embryos at different stages of development. The ideal maturity of this explant for somatic embryogenesis is light pink coloured stage (No.5)

b. Single cluster of embryoids separated out showing different stages of development. Asynchrony in development was observed as these were in different stages (x 3.25)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stages of development of
zygotic embryo of cocoa

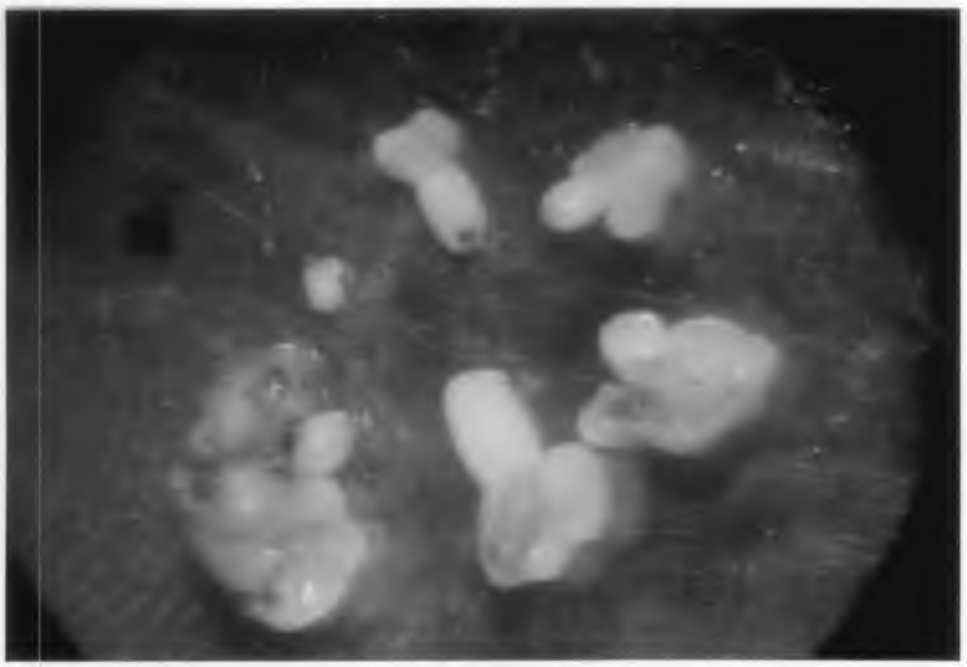


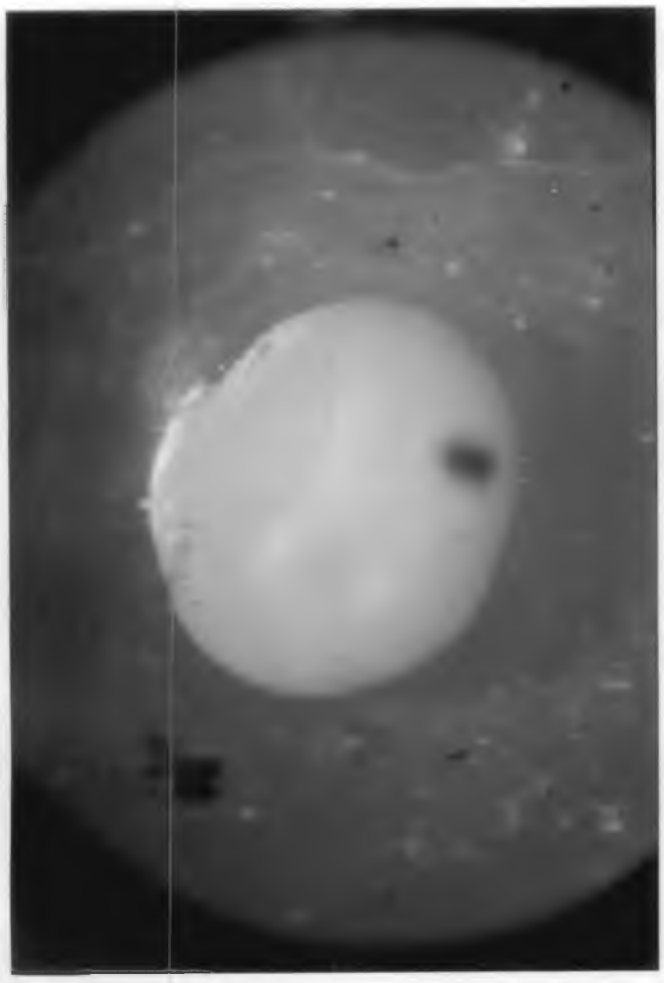
Plate 2. Stages of embryogeny as observed in cocoa somatic embryos

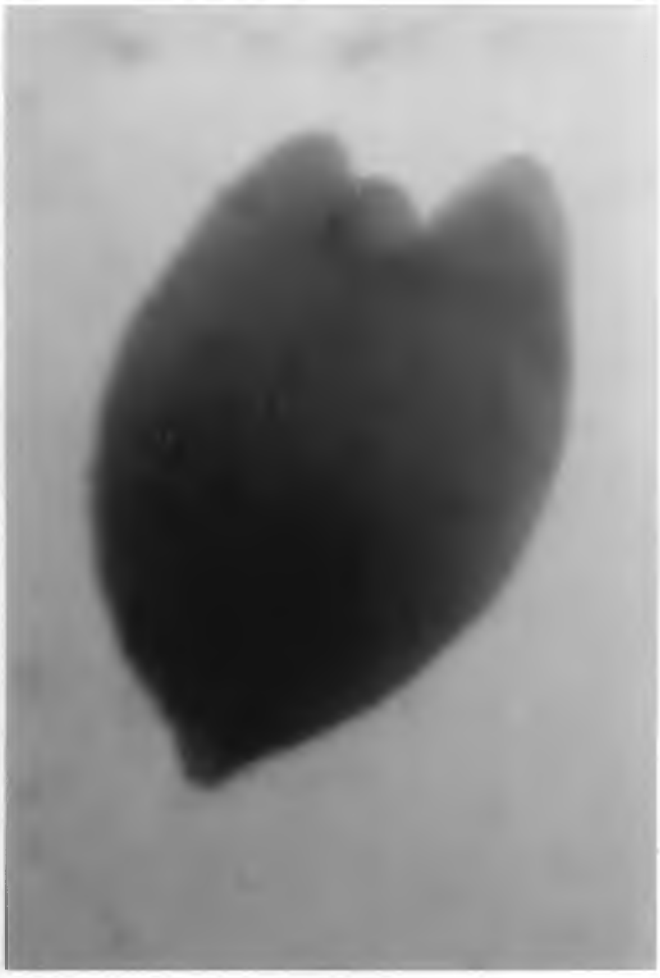
a. Globular stage (x 11.25)

b. Heart stage indicated by a characteristic three lobed stage where cotyledonary initials are separated from the root pole (x 20)

c. Torpedo stage - an elongated form of the heart shaped embryo (x 11.25)

d. Mature embryoid - Typical embryoid with an axis and two cotyledons. This germinate into discernable small seedling with primary root and shoot (x 5.5)





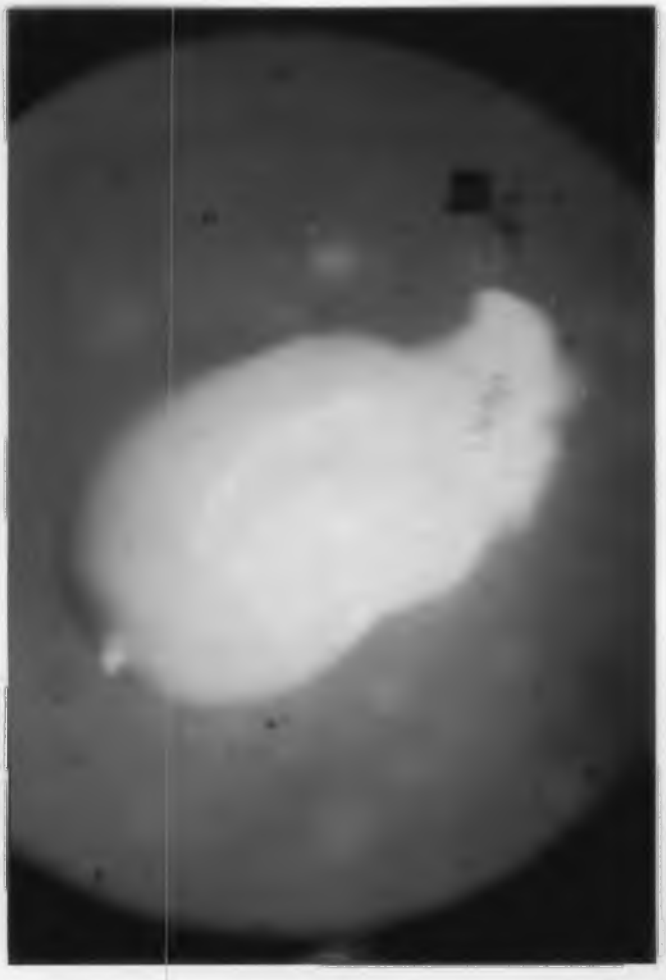


Plate 3

a. An embryoid with a suspensor. Presence of the suspensor is often a subject of controversy (x 5.375)

b. Presence of more than two cotyledons is an abnormality commonly observed in cocoa somatic embryos (x 3.5)

c. Fusion of axes often observed in somatic embryos of cocoa (x 6)

d. Excessive proliferation of cotyledons observed in some abnormal cocoa embryoids. Embryonic axis is highly reduced with such somatic embryos (x 3.75)

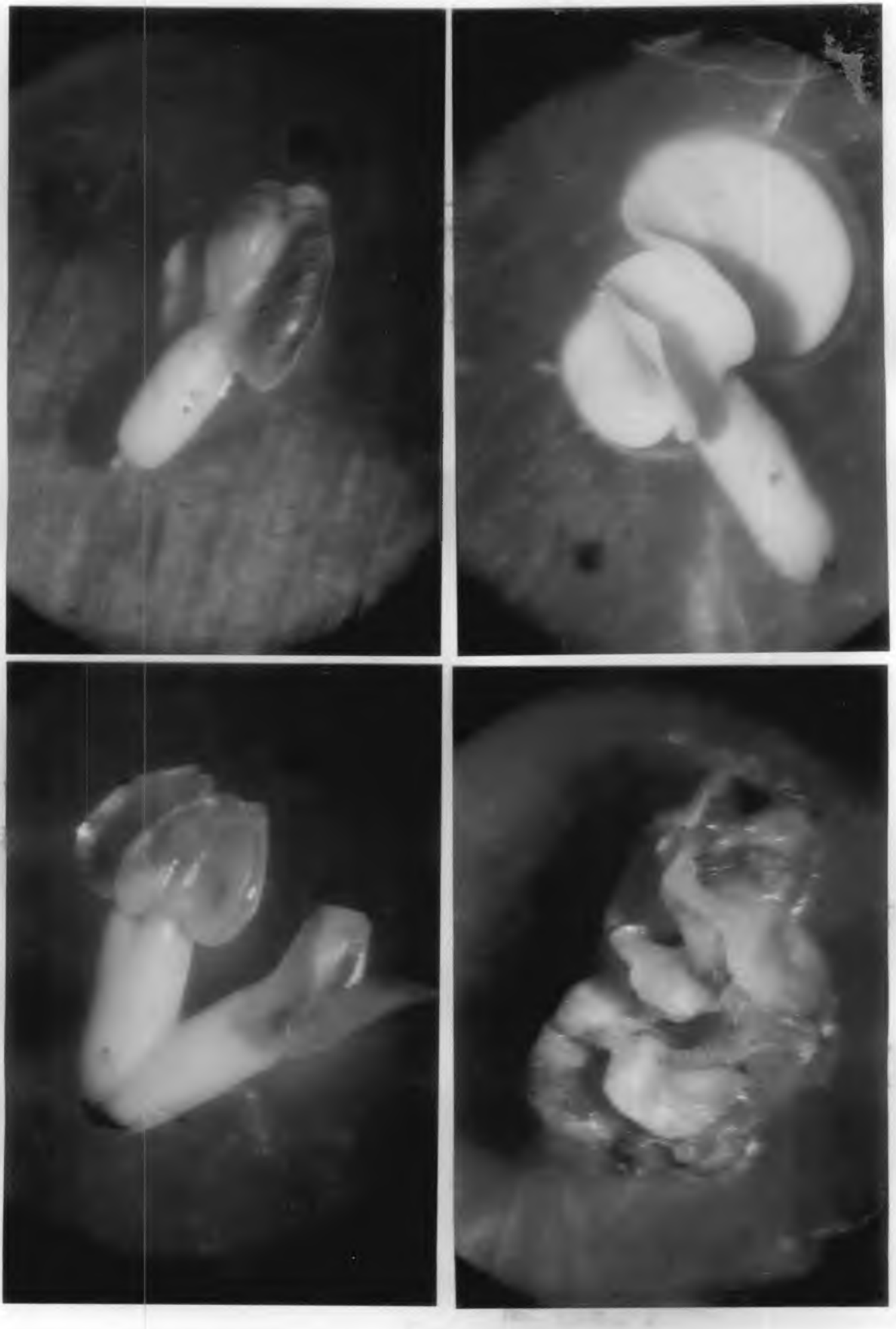


Plate 4

a. Somatic embryoid germinating to plantlet in liquid medium containing half MS salts and 5 per cent sucrose.

b. Excessive proliferation of roots observed in some embryoids at the time of germination.

c. Thick stubby and unbranched roots observed in germinating embryoids (x 2.5)

d. A cocoa embryoid germinated to plantlet. Presence of at least one such expanded leaf ensures success in micrografting (x 2.25)

Plate 5

a. Callus induced from floral parts of cocoa when cultured in media for induction of somatic embryos (x 3)

b. Calloid structures observed in cultures of leaf explant in media containing 2,4-D 1.0 and BA 6.0 $mgl^{-1}$. These got converted into callus on further subculturing (x 6.25)

c. Callus induced from internodal segments of cocoa when cultured in media for somatic embryogenesis.

d. Rhizogenesis observed in cultures for somatic embryogenesis in auxin containing media.

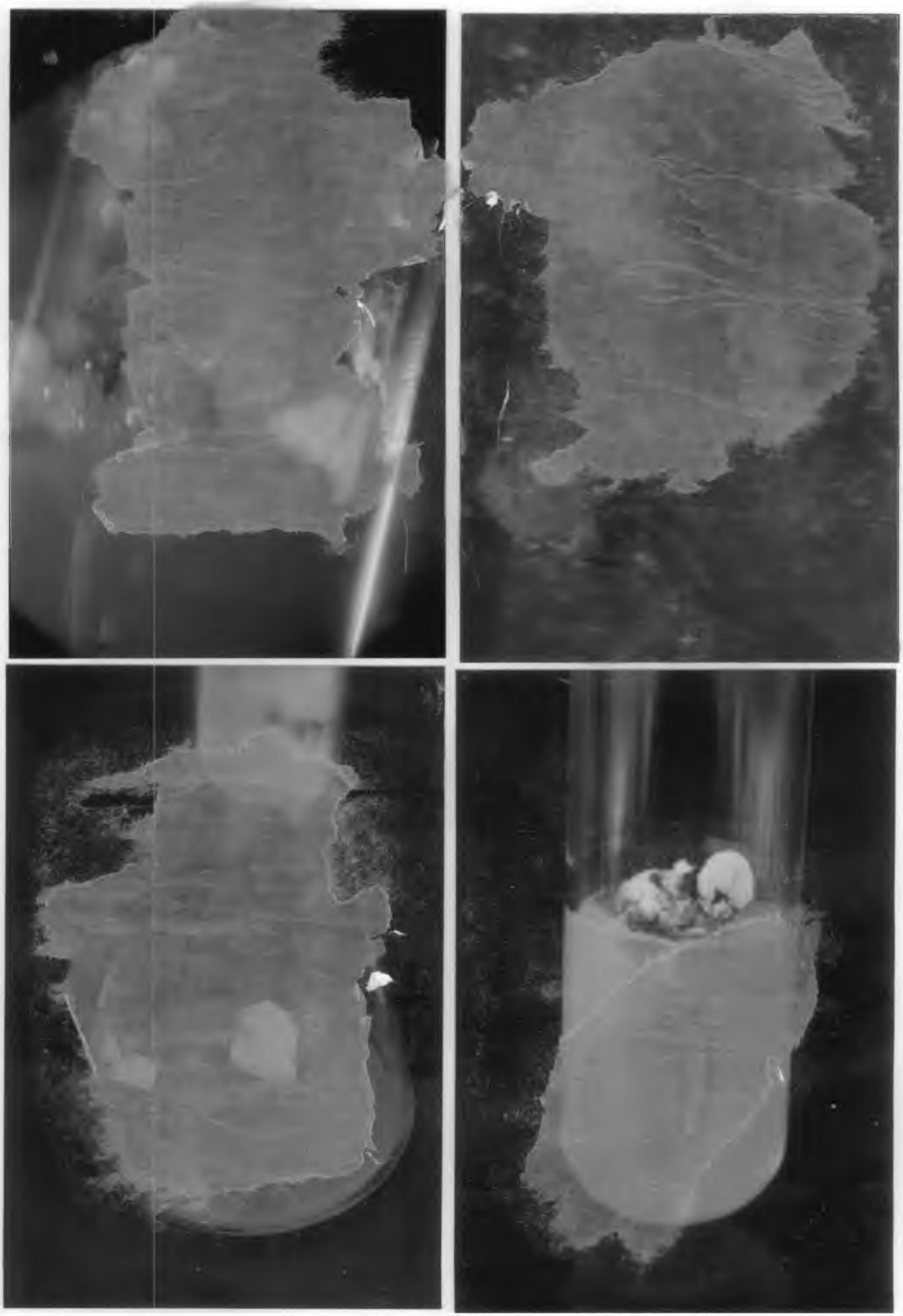


Plate 6

a. Cocoa somatic embryoid being regenerated to plantlet by micrografting to three weeks old *ex vitro* seedling.

b. Two months old embryoid derived plantlet regenerated through micrografting. Growth was abnormal in this plantlet at later stages of development due to the production of cotyledonary leaves as in the embryoid.

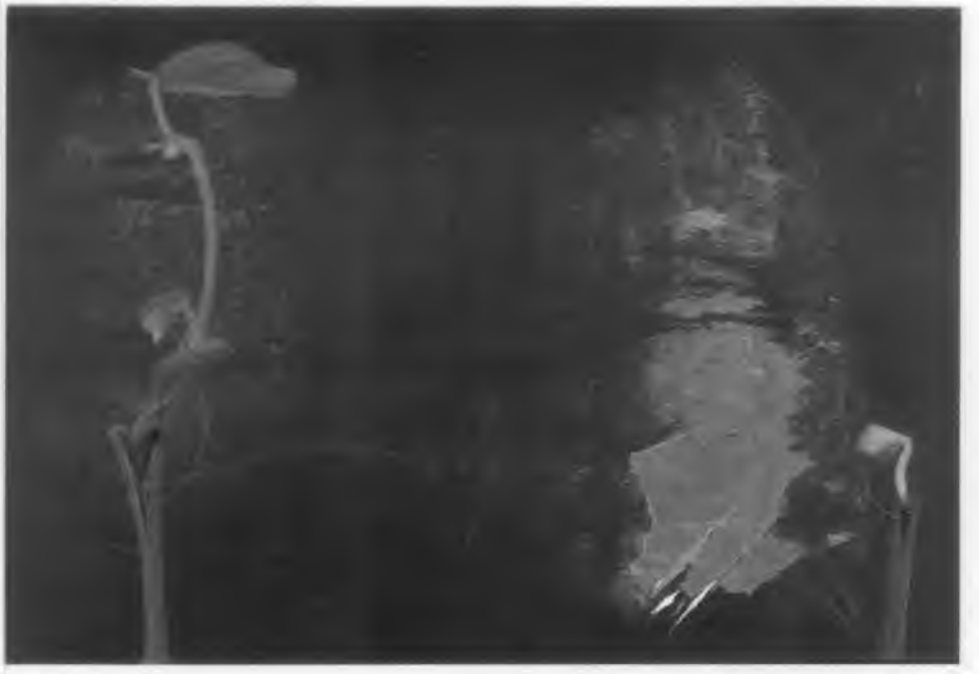
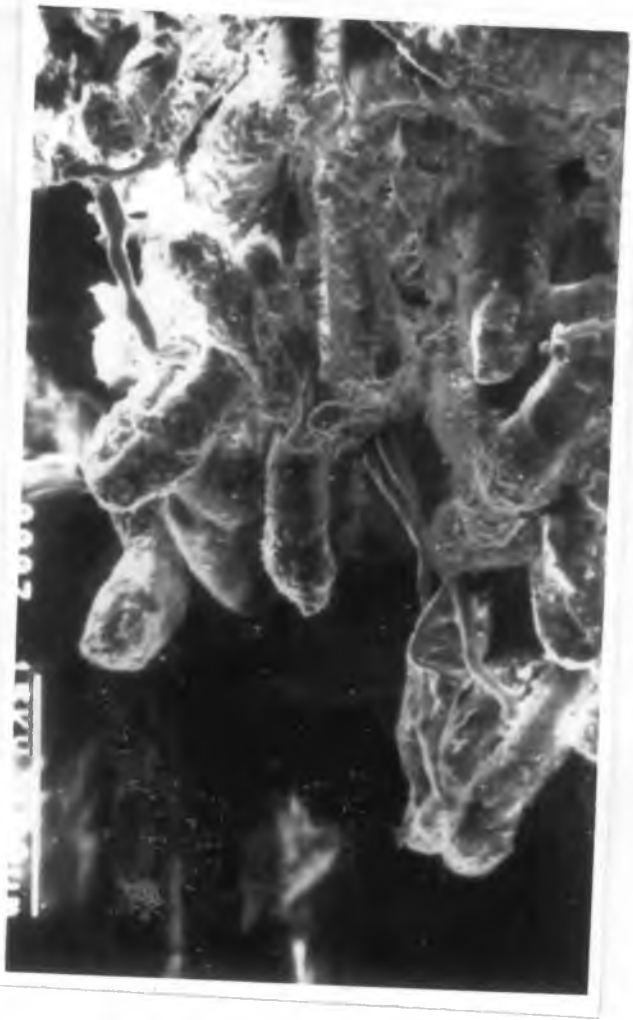
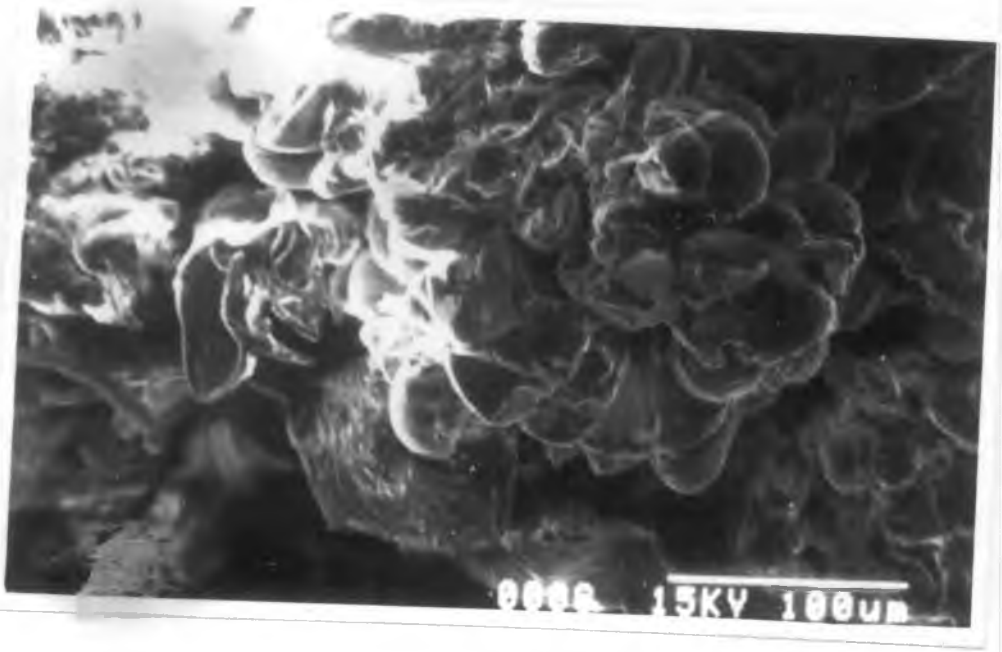


Plate 7

a. Globular somatic embryos of cocoa originating in clusters on the cotyledon surface as observed under Scanning Electron Microscope.

b. Clusters of embryoids in the torpedo stage of development originating on the surface of immature zygotic embryos as observed under Scanning Electron Microscope.

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# PLANTLET REGENERATION THROUGH SOMATIC EMBRYOGENESIS IN COCOA (*Theobroma cacao* L.)

By

**JIJI JOSEPH**

## ABSTRACT OF A THESIS

Submitted in partial fulfilment of the requirement for the degree of

**Master of Science in Agriculture**

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Kerala Agricultural University

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COLLEGE OF HORTICULTURE

Vellanikkara - Thrissur

**1994**

## ABSTRACT

Investigations on 'Plantlet regeneration through somatic embryogenesis in cocoa' were undertaken in the Department of Agricultural Botany, College of Horticulture, Kerala Agricultural University, Vellanikkara during 1992-94. Studies were made to identify the most suitable medium, the most responsive genotype and most favourable conditions for embryogenesis in cocoa. Conditions for germination of embryoids to plantlet were also standardised.

Among the different media tested for embryogenesis namely, MS, WPM and $B_5$, MS medium was found to be the most ideal. Embryoids could be induced only from the tender cotyledon and embryonal axis of immature embryos of 100 day old pods. Other vegetative tissues like leaf, stem, petal, gynoecium, integument etc. yielded only non-embryogenic calli in media for somatic embryogenesis.

An important finding in the present study was the standardisation of an ideal medium which favoured maximum embryogenesis from embryonic tissues. This medium was MS + NAA 1.8 + thiamine 1 mg $l^{-1}$ + CW 15 per cent + sucrose 4 per cent. This is a modification of medium proposed for cocoa somatic embryogenesis by Adu-Ampomah *et al*. (1988).

As already reported by other workers, the maximum embryogenesis occurred under dark incubation. The ideal incubation temperature was 30 $\pm$ 2°C.

The embryoids originated singly or in clusters from the cotyledon explants. Most of the embryoids lacked a suspensor but some of them did have a suspensor. A typical embryoid had an embryonic axis and two cotyledons. However, aberrant forms were not uncommon with excessive proliferation of cotyledons as well as with disproportionate axes and cotyledons.

The study helped to identify some genotypes which showed maximum degree of embryogenesis. The Series I hybrid H 6.5 was found to be the ideal source of explant giving high frequency and intensity of embryogenesis as well as with larger sized embryoids having lesser percentage of abnormalities. The selfed progeny ($S_1$) of this out-breeding crop exhibited minimum degree of embryogenesis. This indicates that the degree of embryogenesis may be associated with the vigour of the explant.

Germination of embryoid to plantlet was a difficult process. Liquid media with ½MS salts and 5 per cent sucrose was found to favour germination. A pretreatment was required to remove the inhibitors by washing and desiccation. Only

embryoids of larger size (>4 mm) germinated properly to plantlet. The recovered plantlets were too small for field establishment.

The most significant achievement in the present study was the plantlet regeneration from somatic embryoid and its planting out in the nursery. This was achieved by micrografting the embryoid derived plantlet to a three week old seedling rootstock. The presence of cotyledons was found to be inhibitory and at least a small leaflet in the embryoid derived plantlet was essential for success in micrografting.