OPTIMISING IN VITRO SOMATIC EMBRYOGENESIS IN POLYEMBRYONIC MANGO (*Mangifera indica* L.) VARIETIES

BY

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THESIS

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1995

DECLARATION

I hereby declare that this thesis entitled "Optimising in vitro somatic embryogenesis in polyembryonic mango (*Mangifera indica* L.) varieties" 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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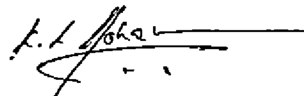
CERTIFICATE

Certified that this thesis, entitled "Optimising in vitro somatic embryogenesis in polyembryonic mango (*Mangifera indica* L.) varieties" is a record of research work done independently by Kum. BINDU. C.P. 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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EXTERNAL EXAMINER

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LIST OF ABBREVIATIONS

ABA	–	Abscisic acid
AC	–	Activated charcoal
BA	–	Benzyl adenine
CH	–	Casein hydrolysate
CW	–	Coconut water
GA_3	–	Gibberellic acid
Glut	–	Glutamine
IBA	–	Indole 3 butyric acid
NAA	–	Napthalene acetic acid
PEG	–	Polyethylene glycol
SUC	–	Sucrose
TSS	–	Total soluble salts
2iP	–	2 isopentenyl adenine
2,4-D	–	2,4- dichlorophenoxy acetic acid

INTRODUCTION

INTRODUCTION

Mango (Mangifera indica L), belonging to the family Anacardiaceae, is one of the six major fruit crops of the world. It is a native of India and is being cultivated for more than four thousand years. Out of the 63 mango growing countries of the world, India is the largest producer. The country has more than one thousand mango varieties.

There are 250 to 300 mango varieties in Kerala, of which 10 to 15 are polyembryonic. Salem, Nileswar Dwarf, Olour, Kurukkan, Mylepelian, Bappakai, Bellary, Goa, Chandrakaran and Goa Kasargod are some of the well known polyembryonic varieties grown in the state (Singh, 1990). Several desirable qualities like large number of fruits per plant, good aroma and juice content, high TSS, regular bearing habit, high productivity per plant and resistance to biotic and abiotic stress conditions are exhibited by the polyembryonic varieties, which can be exploited by the mango breeders. In addition, such varieties are useful rootstocks for several commercial monoembryonic varieties. Decline in the cultivation of polyembryonic varieties has been apparent

in Kerala as a result of the new priorities consequent to the changing socio-economic and family structures, and land use pattern. As such, there is an immediate necessity to preserve the varieties and their cultivation.

In vitro clonal propagation helps to multiply and preserve the rare varieties. It helps to overcome the problem of heterogeneity among the rootstocks as well. As the rootstock influence the growth and quality of the scion, clonal rootstocks would be helpful in ensuring uniform performance of the grafts.

There are reports on the *in vitro* propagation of mango varieties *via* somatic embryogenesis (Litz *et al*., 1982 and 1984; Dewald *et al*., 1989a and 1989b; Litz *et al*., 1991 and 1992). Somatic embryogenesis is a promising route for micropropagation. Somatic embryos are bipolar structures bearing root and shoot apices. Both the meristems, necessary for complete growth, are initiated simultaneously from somatic embryos. Embryogenic cultures can produce large number of embryos need not be separated manually. It has been observed that the polyembryonic varieties respond more readily to *in vitro* treatments than the monoembryonic

varieties. Higher degree of polyembryony has been found to be associated with better _in vitro_ response (Litz, 1984).

The present studies were taken up for optimising the protocols for somatic embryogenesis in a few polyembryonic mango varieties of Kerala like Kalluvarikka, Pulichi, Kilichundan, Vellari, Thalimanga and Varikka. The results may serve as useful guidelines for standardising _in vitro_ propagation techniques for monoembryonic varieties that are comparatively difficult to be standardised.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

In vitro propagation is being considered as a better alternative to the conventional methods, for the large scale clonal propagation of tree crops. *In vitro* propagation is possible *via* enhanced release of axillary buds, somatic organogenesis and somatic embryogenesis (Murashige, 1974). Among the three routes, somatic embryogenesis, with its higher rate of multiplication, is promising for the micropropagation of tree species. Embryogenic cultures produce large number of somatic embryos. Somatic embryos have both shoot and root poles. The embryoids need not be seperated manually.

Mango is a major fruit crop of Kerala occupying an area of 76,675 ha (FIB, 1994). It belongs to the family *Anacardiaceae*. Only very few reports are available on the *in vitro* propagation of mango. Out of them, still few relate to the Indian varieties. There are no reports on the *in vitro* propagation of polyembryonic mango varieties of Kerala. This review highlights the research on the various aspects of *in vitro* propagation *via* somatic embryogenesis in mango and related tree crops.

Somatic embryogenesis is the development of embryos from somatic cells (Mascarenhas, 1989). Somatic embryos closely resemble their zygotic counterparts in structure and biochemistry (Ammirato, 1987). However, a fundamental difference is seen between zygotic and somatic embryos in that the latter tends to mature incompletely without entering a rest phase (Gray, 1987).

Somatic embryogenesis consists of all the stages of embryo development, from the onset of embryogenesis to the production of a fully mature developmentally arrested embryo (Redenbough et al., 1991).

Somatic embryogenesis was first recognised by Reinert (1958) and Steward (1958) in the cultures of carrot tissue. Tisserat et al. (1979) reported somatic embryogenesis in 32 families, 81 genera and 132 species.

Direct embryogenesis proceeds from the pre-embryogenically determined cells while indirect embryogenesis requires the re-determination of differentiated cells, callus proliferation and differentiation of embryogenic determined cells (Kato and Takeuchi, 1966). Potentially, embryogenic

cells are differentiated from cellular aggregates that have differential staining properties, conspicuous size and large number of nucleoli (Konar et al., 1972).

Somatic embryos orginate from pro-embryonic masses of cells that develop from single cells (Raghavan, 1976). They possess a bipolar structure with a vascular system (Haccius, 1978). According to Tisserat et al. (1979), the cells that undergo embryo initiation are pre-determined and their subsequent exposure to exogenous growth substances favours embryogenesis. Sharp et al. (1982) reported that somatic embryogenesis initiated either from pre-embryogenic determined cells or from induced embryogenic determined cells. Williams and Maheswaran (1986) reported that somatic embryos could arise either from single cells or groups of cells.

The most important application of somatic embryogenesis is in the large scale clonal propagation of plants (Janick, 1993). Somatic embryoids are useful for the analysis of molecular and biochemical events (Ammirato, 1987). Production of artificial seeds and direct regeneration from protoplast are favoured by somatic

embryogenesis (Razdan, 1993). Other uses include crop improvement, metabolite production, disease elimination and germplasm preservation.

I. Factors influencing somatic embryogenesis

The key factors which influence somatic embryogenesis are pre-treatments, explants, culture media, culture conditions, genetic stability, density of embryogenic cells and synchronous development of embroyoids (Ammirato 1983). According to Fitchet (1989) the important factors for somatic embryogenesis are the presence of an auxin and a reduced form of nitrogen in the culture medium.

A. Explant

Embryogenesis is largely a function of the explant particularly its type, development, and interaction with the growth medium (Litz and Gray, 1992).

Atree and Fowke (1991) reported that somatic embryos can be induced only from embryonic tissues.

In vitro culture of nucellar explants gives rise to somatic embryos and ehventually to fully developed plants (Kochba *et al*., 1972). Nucellar tissues from both unfertilized and fertilized ovules undergo embryogenesis *in vitro* (Mitra and Chaturvedi, 1972).

Mitra and Chaturvedi (1972) reported that embryos may arise either directly from the nucellus or indirectly from the nucellar callus. Nucellar adventive embryony (polyembryony) occurs in many woody plant species and is identified in species belonging to several plant families (Rangaswamy, 1982).

Stevenson (1956), Rangan *et al*. (1968), Mitra and Chaturvedi (1972), Ben-Hayyim and Neumann (1983) and Navarro *et al*. (1985) used nucellus as explant for inducing somatic embryogenesis in citrus. Eichholtz *et al*. (1979) used nucellar explant for inducing somatic embryogenesis in *Malus domestica*. In *Hevea braziliensis*, Carron and Enjalric (1985) reported somatic embryogenesis from nucellar explants.

In mango, somatic embryogenesis was obtained from nucellar explants in both polyembryonic (Litz *et al*., 1982

and 1984) and monoembryonic (Litz, 1984a) varieties. The stage of development of the ovule was critical and the optimum stage for taking the explant was before the embryo mass has filled the embryo sac (Litz et al., 1992). Although monoembryonic and polyembryonic cultivars appear to respond equally well, somatic embryogenesis was observed to be cultivar dependent (Litz et al., 1991).

Rao (1965) reported somatic embryogenesis in Santalum album from embryo. Gingas and Lineberger (1989) and Vieitez and Barciela (1990) stimulated somatic embryogenesis in Quercus rubra and Camellia japonica, respectively, from zygotic embryo explant on basal medium alone.

Harada (1975) used stem and root as explants for initiating somatic embryogenesis in Actinidia chinensis. Lakshmi Sita et al. (1979) used stem as explant for inducing somatic embryogenesis in Santalum album.

Somatic embryogenesis could be induced from leaf by Sondahl and Sharp (1977) in Coffea arabica and Litz (1986b) in Theobroma cacao.

B. Basal medium

1. Induction

According to Christianson (1985) an event must initially occur that involves a change in the determination or fate of certain cells. This change in the commitment of a cell is referred to as an inductive event.

Somatic embryos have been grown on a range of media from the relatively dilute Whites medium (White, 1963) to the more concentrated formulations of Gamborg *et al*. (1968), Murashige and Skoog (1962) and Schenk and Hildebrandt (1972).

The form in which nitrogen is supplied to the tissue system is a key factor in the determination of embryogenesis. Ammonium ions and casein hydrolysate at low levels strongly stimulate embryogenesis compared to nitrate ions (Halperin and Wetherell, 1965).

Murashige and Skoog medium was used for the induction of somatic embryos in citrus (Ben-Hayyim and Neumann, 1983). In apple (var. Golden Delicious) MS medium was used for the induction of somatic embryoids (Eichholtz *et al*., 1979).

Seventy per cent of the explants were cultured on MS medium or modified MS medium for somatic embryogenesis (Evans et al., 1981). A key element of MS medium is the presence of high levels of nitrogen in the form of ammonium nitrate (Ammirato, 1983). MS medium has a high level of inorganic salt and ammonium (Chen Zhengua et al., 1988).

Litz et al. (1982 and 1984), Litz (1984a), Litz and Schaffer (1987), Dewald et al. (1989a and 1989b) and Mathews and Litz (1990) induced somatic embryogenesis from the nucellus of mango on modified MS medium containing half strength major salts.

Rao et al. (1982) reported the induction of callus from cotyledons of mango on MS medium.

Litz et al. (1991) obtained somatic embryos when nucellus of polyembryonic and monoembryonic mango varieties were cultured on medium containing modified B_5 major salts and MS minor salts and organics.

2. Initiation

When tissues are transferred from an auxin containing medium to an auxin free medium having nitrogenous

compounds like amino acids, embryogenesis was triggered (Reinert, 1959). Butenko et al. (1967) suggested that mineral salts should be present at a high concentration for embryo initiation. Tazawa and Reinert (1969) reported that nitrogen should be present either in an organic form or inorganic form for the initiation of somatic embryoids.

In mango, the initiation medium consisted of modified B_5 major salts, MS minor salts and organics (Litz et al., 1984). Litz (1984a) reported that somatic embryogenesis occurred from nucellar callus of mango on half strength MS medium containing 1-2 mg/l 2,4-D. According to Dewald et al. (1989a) modified B_5 medium was significantly more effective for the production of somatic embryos from nucellar tissue in mango.

3. Maturation

Litz (1984a) subcultured the embryogenic nucellar callus of mango on to modified MS medium containing half strength major salts without any growth regulators to permit maturation of embryos. Dewald et al. (1989b) used maturation medium consisting of modified B5 major salts, MS minor salts and organics.

4. Germination

If somatic embryos are not physiologically mature, they cannot germinate normally and survive. Poor germination is typical in many embryogenic culture systems. Razdan (1993) reported that germination of somatic embryos can occur only when it is mature enough to have functional root and shoot apices capable of meristematic growth.

The germination medium used by Litz (1984a) consisted of modified MS liquid medium without growth regulators and with 20 per cent (v/v) coconut water.

Dewald et al. (1989b) reported that germination was accompanied by slight enlargement and progressive greening of the somatic embryo in mango. Modified B_5 medium consisting of half strength major salt formulation supplemented with coconut water and casein hydrolysate resulted in significantly higher germination rate.

C. Plant growth substances

1. Induction

Generally the presence of an auxin in the medium is essential for embryo initiation. Tissue and calli maintained continuously in an auxin free medium do not form embryos.

Halperin (1970) reported that embryogenesis could be induced only in suspension cultures with an auxin containing medium. The conversion of a somatic cell to an embryo is caused by an increased ratio of nitrogen to auxin (Reinert, 1973).

Litz *et al*. (1982) obtained somatic embryos from the nucellus of cultured ovule explants of polyembryonic mango cultivars on medium supplemented with either coconut water or benzyl adenine. Nucellar callus was induced on modified MS medium supplemented with 1-2 mg/l 2,4-D (Litz *et al*., 1982; Litz, 1984a). Litz *et al*. (1984) could induce somatic embryogenesis from nucellar explants when cultured on modified MS medium without auxin. Litz and Schaffer (1987) observed that somatic embryogenesis from mango nucelli is not dependant upon 2,4-D as a stimulus. However, Dewald *et al*. (1989a) incorporated 4.5 uM 2,4-D to modified MS medium for the induction of somatic embryos in mango.

2. Initiation

Litz *et al*. (1982) could initiate somatic embryoids initiated on medium containing coconut water, BA or no growth

regulators. Litz et al. (1984) reported that efficient somatic embryogenesis occured when nucellar callus was transferred to liquid medium without 2,4-D.

Atree and Fowke (1991) reported that conifer somatic embryos continued to proliferate on a medium containing both auxin and cytokinin.

3. Maturation

Litz et al. (1983), Litz and Gray (1992) and Razdan (1993) reported that maturation of mango somatic embryos occured on medium without growth regulators.

Cytokinins are important for somatic embryo maturation (Fujimura and Komamine, 1980). It influences the development of cotyledon (Ammirato and Steward, 1971) and shoot apex (Kavathekar and Johri, 1978).

The endogenous concentration of ABA in embryogenic cultures showed a rapid increase during somatic embryo development and reached a peak at maturation (Rajasekharan et al., 1982).

Secondary embryos arise from globular or early heart stage somatic embryos. Ammirato (1983) reported that a pulse of ABA suppressed the formation of secondary embroyos, synchronized the cultures and inhibited aberrant development of embryos and precocious germination.

The importance of the application of ABA for the maturation of somatic embryoids was reported by Ammirato (1987). He observed that somatic embryos grown on ABA containing medium had a higher number of embryos with two cotyledons. Dewald et al. (1989b) reported that ABA in combination with coconut water and low sucrose concentration mediated normal somatic embryo maturation in mango. But, the effect of ABA was masked when it was used with six per cent sucrose.

Abscisic acid influences the quality of somatic embryos (Litz et al., 1992). He observed that mango somatic embryos could tolerate high concentrations of ABA (100 uM).

Razdan (1993) observed that high auxin levels inhibited the development and growth of the shoot meristem of the embryoids.

4. Germination

Mature embryos are cultured on growth regulator free medium for germination and plantlet development (Atree and Fowke, 1991). Razdan (1993) reported that somatic embryos germinate on growth regulator free agar medium.

Exogenously supplied GA_3 caused germination of somatic embryos in Citrus sinensis (Rangaswamy, 1961) and Santalum album (Lakshmi Sita et al., 1979).

Kavathekar and Johri, (1978) reported that cytokinins are sometimes required for the growth of embryos into plantlets.

D. Other supplements

1. Coconut water

Coconut water acts as a source of reduced nitrogen in the medium (Tulecke et al., 1961). Homes (1967) reported that coconut water was not a pre-requisite for inducing embryogenesis. In mango, somatic embryogenesis was induced when coconut water was incorporated in the medium (Litz et

al., 1982 and 1984; Dewald et al., 1989a). Poliferation of somatic embryos occured when the nucellar tissue was subcultured onto medium with twenty per cent coconut water. Mature embryos did not develop into normal plantlets when transferred to medium without coconut water.

According to Dewald et al. (1989b) maturation and germination of somatic embroyos of mango were achieved by sequential transfer of somatic embryos onto medium containing twenty per cent coconut water and reduced sucrose concentration. Litz et al. (1992) routinely added filter sterilized coconut water to the maturation medium in mango.

A substantial amount of reduced form of nitrogen is required for embryo initiation and maturation. Razdan (1993) reported that coconut water or casein hydrolysate could be used as the reduced source of nitrogen.

2. Agar

Litz et al. (1982) reported that efficient somatic embryogenesis occured in liquid medium. Cultures in the initiation medium darkened more quickly in solid medium than

on liquid medium (Dewald et al., 1989a). Larger embryos were formed in liquid maturation media but developmental abnormalities were more. Somatic embryos produced on medium containing gelrite were larger than those produced on agar medium. Developmental anomalies were less when somatic embryos were cultured on medium containing gelrite.

3. Sucrose

Normal somatic embryos were obtained with high sucrose concentration than with low concentration. For maturation of somatic embryos the optimum concentration of sucrose was six per cent (Dewald et al., 1989b).

Litz et al. (1993) reported that sucrose concentration should be maintained at moderately high levels in order to prevent precocious germination of somatic embryoids.

4. Activated charcoal

Activated charcoal hastened the differentiation of somatic embryos in mango (Litz et al., 1984; Litz, 1986a). However, the mortality was very high.

E. Culture conditions

In mango, induction and growth of callus was better in dark than with a normal photoperiod (Rao *et al.*, 1982).

Litz (1984a) and Dewald *et al.* (1989a) maintained the cultures in the induction medium at 16 hour photoperiod and 25°C.

Litz *et al.* (1991) incubated the explants of mango in darkness at 25°C and sub-cultured them daily for the first week.

Mango cultures in the initiation medium were kept in dark (Dewald *et al.*, 1989a). Litz *et al.* (1991) maintained the cultures at 24 - 27°C in darkness and sub-cultured it at two weeks interval.

In mango, the cultures in the maturation media were maintained in darkness in order to prevent precocious germination of somatic embryoids (Dewald *et al.*, 1989b and Litz *et al.*, 1993). The maturation of somatic embryos was found to proceed more in darkness (Razdan, 1993).

Somatic embryos grew vigorously and developed extensive root system when the exposed to 25° C, high light intensity and controlled levels of CO_2 (Kozai and Iwanami, 1988).

According to Litz et al. (1992) germination of mango somatic embryoids was characterised by rapid elongation of the hypocotyl and greening of cotyledons, which occured when somatic embryos were transferred to light.

MATERIALS AND METHODS

MATERIALS AND METHODS

Investigations on optimising techniques for somatic embryogenesis in polyembryonic mango varieties were carried out at the Plant Tissue Culture Laboratory, Department of Horticulture, College of Agriculture, Vellayani from January 1993 to August 1994.

The materials and methods tried for the induction, initiation, maturation and germination of the somatic embryoids are described in this chapter.

A. VARIETIES

Six polyembryonic mango varieties of Kerala namely Pulichi, Kilichundan, Vellari, Kalluvarikka, Thalimanga and Varikka were used for the study.

B. EXPLANTS

Nucellus and embryo mass from the ovules of tender mango fruits (about 30-45 days after fertilization) and segments of tender leaves, were used as explants.

1. Nucellus

Nucellus represents the wall of the megasporangium and is a nourishing tissue for the developing embryo. Generally, the nucellus remains within the confines of the inner integument. Rarely it projects into the micropyle or beyond, forming a nucellar beak. As it is part of the mother plant and has the same ploidy it can be used for clonal propagation.

2. Embryo

The fertilized egg is called zygote. Following a pre-determined mode of development, it gives rise to an embryo, which has the potential to form a complete plant. Irrespective of the mode of development, a mature embryo generally possesses an embryonic root (radicle), an embryonic shoot (plumule) and one or two cotyledons. The occurrence of more than one embryo in a seed is termed polyembryony. Seeds of polyembryonic varieties of mango produce upto five nucellar seedlings along with the zygotic one. Often the zygotic embryo gets degenerated, resulting in the production of nucellar seedlings only. The zygotic seedlings that survive are generally less vigorous. Polyembryony is of

great significance in Horticulture. The adventitious embryos provide uniform seedlings of the maternal type.

C. COLLECTION AND PREPARATION

Tender fruits (thirty to forty five days after fertilization) were collected from healthy trees of polyembryonic mango varieties from different parts of Thiruvananthapuram district. Explants were prepared and inoculated within 24 hours of excision from the tree. The size of the fruits varied with the varieties. The length of fruits ranged from 2.5 to 4.0 cm. Tender leaves from new flushes were collected and inoculated within six hours. The fruits (after removing pedicel) and the leaves (after removing petioles) were washed throughly in tap water with a few drops of the wetting agent 'Labolene', followed by washing with double glass distilled water.

D. SURFACE STERILIZATION

Surface sterilization of the plant materials was carried out inside a laminar air flow chamber. The fruits, after initial cleaning, were transferred to sterile beaker and surface - sterilized using 70 per cent ethyl alcohol for

10 minutes. After draining the alcohol, the fruits were washed with sterile double glass distilled water. They were then treated with freshly prepared sodium hypochlorite (1.0 per cent) for 30 minutes. This was followed by washing four to five times with sterile double glass distilled water. The tender leaves (bronze coloured) were surface-sterilized in a sterile beaker, using sodium hypochlorite (0.8 per cent) for 15 minutes and then washed with sterile double glass distilled water four to five times.

E. INOCULATION AND INCUBATION

All the inoculation operations were carried out under a laminar air flow chamber.

The tools (needles, blades, scalpels, forceps etc.) and glassware required for inoculation were washed thoroughly, rinsed with double glass distilled water, covered with aluminium foil and autoclaved at 121° C and 1.06 Kg/cm^{2} pressure for 40-45 minutes.

The surface - sterilized fruits were dissected using scalpel without injuring the ovule. The ovule was

taken out with foreceps and cut longitudinally into two equal halves with dissection blade fitted on handle. Nucellus and embryo mass were separately scooped out from the ovule. Nucellar tissue and embryo mass with a portion of the ovule also were used for inoculation. The cotton plugs of the test tubes/Erlenmeyer flasks were removed and the mouth was flamed. . The nucellus and embryo mass was then inoculated on the medium and the mouth of the culture vessels was again flamed and closed with cotton plugs.

When disposable sterile plastic petri dishes were used, after the nucellus and embryo mass were inoculated into the medium and after closing the petri dish, the sides were sealed using cling film.

The inoculated culture vessels were kept either in light or darkness.

F. MEDIA

The basal media used for the study were MS (Murashige and Skoog, 1962) modified MS and B_5 (Gamborg _et al_., 1968) media. The chemicals used for the preparation of the culture media were of analytical grade from British Drug

House (BDH, Bombay), Sisco Research Laboratory (SRL, Bombay), Merck (Bombay) and Sigma (USA).

Standard procedures were followed for the preparation of MS and B_5 media (Thorpe, 1980). Stock solutions of major and minor nutrients, amino acids and plant growth substances were prepared by dissolving the required quantity of chemicals in specific volume of double glass distilled water and were stored under refrigerated condition.

All items of glassware used for the preparation of the media were washed with diluted 'Labolene' and rinsed with double glass distilled water. Specific quantities of stock solutions were pipetted out into a 1000 ml beaker. Sucrose, glutamine and myo-inositol were added fresh and dissolved. Coconut water (200 ml.) collected from freshly harvested tender coconut (eight months old), was then added and the volume made upto 950 ml using double glass distilled water. With an electronic pH meter (Philips make, model PP 9046) the pH value of the solution was adjusted between 5.6 and 5.8, using 0.1 N NaOH or 0.1 N HCl. Agar (in the case of soild medium) was then added to the medium and the final volume was made upto 1000 ml.

The solution was then heated by placing the beaker on a heating mantle and stirred throughly for uniform mixing, till agar melted. Activated charcoal, when used in the medium, was added at this stage. The medium was poured to pre-sterilized culture vessels which were rinsed with double glass distilled water. Corning brand test tubes (25 x 150 mm) and Erlenmeyer flask (100 ml) were used as culture vessels. The test tubes and Erlenmeyer flasks were filled with 15.0 ml and 30.0 ml of the medium, respectively. The culture vessels containing the medium were plugged tightly with cotton. They were then autoclaved at $121^{o}C$ and 1.06 Kg/cm^2 pressure for 20 minutes.

For the induction of somatic embryoids, disposable sterile plastic petri dishes (55 mm x 15 mm) were used as culture vessels. In this case, the culture medium was pre-sterilized in suitable Erlenmeyer flasks (by autoclaving at $121^{o}C$ and 1.06 Kg/cm^2 pressure for 20 minutes) and poured while hot into the petri dishes. The petri dishes were opened inside a laminar air flow chamber and sterilized medium (7.0 ml per petri dish) dispensed into each petri dish using sterile glass syringe. The petri dishes were then closed and sealed tightly with cling film.

Agar was not used in the case of liquid media. The sterile petri dishes were opened inside the laminar air flow chamber and sterilized filter paper discs (54 mm radius) were put inside using sterilized forceps. The medium was dispensed (2.0 ml per petri dish) using sterilized glass syringe. The petri dishes were closed and sealed tightly using cling film.

G. SOMATIC EMBRYOGENESIS

1. Induction of somatic embryoids

a. Treatments

Twenty six treatments were evaluated for their ability to induce somatic embryoids (Table 1). The treatments involved combinations of plant growth substances (2,4-D, GA_3 and BA) activated charcoal, agar, glutamine, sucrose and strength of the basal medium (half and full). Culture media were supplemented with 2,4-D 1.0 - 16.0 mg/l, BA 0.5 - 2.0 mg/l, GA_3 5.0 - 20.0 mg/l, activated charcoal 500.0 mg/l - 5.0 - g/l, agar 4.5 - 7.0 g/l, glutamine 400.0 - 600.0 mg/l and sucrose 30.0 - 60.0 g/l. The treatments were replicated six to twenty four times.

Observations were recorded on the number of cultures initiating embryo mass, callus and multiple embryos.

b. Basal media

Studies were conducted to find out the effect of full strength and half strength of the major salts of MS basal medium with supplements. The treatments were replicated 18 to 24 times.

Observations were made on the number of cultures initiating embryo mass, callus and multiple embryos.

c. Culture conditions

Studies were conducted to find out the effect of light on the initial establishment of the cultures. Darkness was provided by placing the cultures in culture racks covered by black polythene sheets and by covering the culture vessels with aluminium foil. Light (photoperiod 16 hours) was provided by cool white fluorescent tubes, giving a light intensity of 3000 lux.

Observations were recorded on the number of cultures initiating embryo mass, callus and multiple embryos.

Table 1. Treatments tried for the induction of somatic embryoids from nucellus and embryomass

No.	Treatment
T_1	1/2 MS + 2,4-D 2.0mg/l + GA_3 10.0mg/l + Glut 400.0mg/l + Suc 30.0g/l + CW 200.0 ml/l
T_2	1/2 MS + 2,4-D 10.0mg/l + GA_3 10.0mg/l + Glut 600.0mg/l + Suc 30.0g/l + CW 200.0ml/l
T_3	1/2 MS + 2,4-D 2.0mg/l + GA_3 5.0mg/l + Glut 400.0mg/l + Suc 30.0 + Agar 7.0g/l + CW 200.0ml/l + AC 5.0g/l
T_4	1/2 MS + 2,4-D 4.0mg/l + GA_3 5.0mg/l + Glut 400.0mg/l + Suc 30.0g/l + Agar 7.0g/l + CW 200.0ml/l + AC 5.0g/l
T_5	1/2 MS + 2,4-D 8.0mg/l + GA_3 5.0mg/l + Glut 400.0mg/l + Suc 30.0g/l + Agar 7.0g/l + CW 200.0ml/l + AC 5.0g/l
T_6	1/2 MS + 2,4-D 16.0mg/l + GA_3 5.0mg/l + Glut 400.0mg/l + Suc 30.0g/l + Agar 7.0g/l + CW 200.0ml/l + AC 5.0g/l
T_7	1/2 MS + 2,4-D 2.0mg/l + GA_3 10.0mg/l + Glut 400.0mg/l + Suc 30.0g/l + Agar 7.0g/l + CW 200.0ml/l + AC 5.0g/l
T_8	1/2 MS + 2,4-D 4.0mg/l + GA_3 10.0mg/l + Glut 400.0mg/l + Suc 30.0g/l + Agar 7.0g/l + CW 200.0ml/l + AC 5.0g/l
T_9	1/2 MS + 2,4-D 8.0mg/l + GA_3 10.0mg/l + Glut 400.0mg/l + Suc 30.0g/l + Agar 7.0g/l + CW 200.0ml/l + AC 5.0g/l
T_{10}	1/2 MS + 2,4-D 16.0mg/l + GA_3 10.0mg/l + Glut 400.0mg/l + Suc 30.0g/l + Agar 7.0g/l + CW 200.0ml/l + AC 5.0g/l
T_{11}	1/2 MS + 2,4-D 2.0mg/l + GA_3 20.0mg/l + Glut 400.0mg/l + Suc 30.0g/l + Agar 7.0g/l + CW 200.0ml/l + AC 5.0g/l
T_{12}	1/2 MS + 2,4-D 4.0mg/l + GA_3 20.0mg/l + Glut 400.0mg/l + Suc 30.0g/l + Agar 7.0g/l + CW 200.0ml/l + AC 5.0g/l
T_{13}	1/2 MS + 2,4-D 8.0mg/l + GA_3 20.0mg/l + Glut 400.0mg/l + Suc 30.0g/l + Agar 7.0g/l + CW 200.0ml/l + AC 5.0g/l
T_{14}	1/2 MS + 2,4-D 16.0mg/l + GA_3 20.0mg/l + Glut 400.0mg/l + Suc 30.0g/l + Agar 7.0g/l + CW 200.0ml/l + AC 5.0g/l

No.	Treatment
T_{15}	1/2 MS + 2,4-D 5.0mg/l + GA_3 10.0mg/l + BA 2.0mg/l + Glut 600.0mg/l + Suc 60.0g/l + Agar 6.0g/l + AC 5.0g/l + CW 200.0ml/l
T_{16}	1/2 MS + 2,4-D 1.0mg/l + GA_3 10.0mg/l + BA 0.5mg/l + Glut 600.0mg/l + Suc 60.0g/l + Agar 6.0g/l + AC 5.0g/l + CW 200.0ml/l
T_{17}	1/2 MS + 2,4-D 2.0mg/l + GA_3 10.0mg/l + BA 0.5mg/l + Glut 600.0mg/l + Suc 60.0g/l + Agar 6.0g/l + AC 5.0g/l + CW 200.0ml/l
T_{18}	1/2 MS + 2,4-D 3.0mg/l + GA_3 10.0mg/l + BA 0.5mg/l + Glut 600.0mg/l + Suc 60.0g/l + Agar 6.0g/l + AC 5.0g/l + CW 200.0ml/l
T_{19}	1/2 MS + 2,4-D 4.0mg/l + GA_3 10.0mg/l + BA 0.5mg/l + Glut 600.0mg/l + Suc 60.0g/l + Agar 6.0g/l + AC 5.0g/l + CW 200.0ml/l
T_{20}	1/2 MS + 2,4-D 1.0mg/l + GA_3 10.0mg/l + BA 1.0mg/l + Glut 600.0mg/l + Suc 60.0g/l + Agar 6.0g/l + AC 5.0g/l + CW 200.0ml/l
T_{21}	1/2 MS + 2,4-D 2.0mg/l + GA_3 10.0mg/l + BA 1.0mg/l + Glut 600.0mg/l + Suc 60.0g/l + Agar 6.0g/l + AC 5.0g/l + CW 200.0ml/l
T_{22}	1/2 MS + 2,4-D 3.0mg/l + GA_3 10.0mg/l + BA 1.0mg/l + Glut 600.0mg/l + Suc 60.0g/l + Agar 6.0g/l + AC 5.0g/l + CW 200.0ml/l
T_{23}	1/2 MS + 2,4-D 4.0mg/l + GA_3 10.0mg/l + BA 1.0mg/l + Glut 600.0mg/l + Suc 60.0g/l + Agar 6.0g/l + AC 5.0g/l + CW 200.0ml/l
T_{24}	1/2 MS + 2,4-D 4.0mg/l + GA_3 5.0mg/l + BA 0.5mg/l + Glut 400.0mg/l + Suc 60.0g/l + Agar 6.0g/l + AC 1.0g/l + CW 200.0ml/l
T_{25}	1/2 MS + 2,4-D 5.0mg/l + GA_3 5.0mg/l + Glut 400.0mg/l + Suc 60.0g/l + Agar 6.0g/l + AC 2.5g/l + CW 200.0ml/l
T_{26}	MS + 2,4-D 5.0mg/l + GA_3 5.0mg/l + Glut 400.0mg/l + Suc 60.0g/l + Agar 6.0g/l + AC 2.5g/l + CW 200.0ml/l

2. Initiation of somatic embryoids

The cultures from the induction media were transferred to initiation (expression) media.

a. Treatments

Twelve treatments were tried for the initiation of somatic embryoids (Table 2). The treatments involved combinations of 2,4-D (2.00-10.00 mg/l), GA_3 (5.00-10.00 mg/l), BA (0.05-1.00 mg/l) and strength of the basal medium (full and half). The treatments were replicated 12 to 40 times.

Observations on the number of cultures initiating somatic embryoids from nucellus, as well as from embryogenic callus and number of cultures having multiple embryos were recorded three weeks after incubation.

b. Culture conditions

The effect of light and temperature on the initiation of somatic embryoids was studied. Light was provided by keeping the culture vessels under the fluorescent

Table 2. Treatments tried for the initiation of somatic embryoids

No.	Treatment
I_1	1/2 MS + GA 5.0mg/l + Glut 400.0mg/l + Suc 60.0g/l + Agar 6.0g/l + CH 500.0 mg/l + AC 2.5g/l + CW 200.0ml/l
I_2	1/2 MS + 2, 4-D 5.0mg/l + Glut 400.0mg/l + Suc 60.0g/l + Agar 6.0g/l + CH 500.0 mg/l + AC 2.5g/l + CW 200.0ml/l
I_3	1/2 MS + 2, 4-D 10.0mg/l + Glut 400.0mg/l + Suc 60.0g/l + Agar 6.0g/l + CH 500.0mg/l + AC 2.5g/l + CW 200.0ml/l
I_4	1/2 MS + 2,4-D 2.0mg/l + BA 1.0 mg/l + GA 5.0mg/l + Glut 400.0mg/l + Suc 60.0g/l + Agar 6.0g/l + CH 500.0mg/l + AC 2.5g/l + CW 200.0ml/l
I_5	MS + 2, 4-D 10.0mg/l + GA 10.0mg/l + Glut 600.0mg/l + Suc 60.0g/l + AC 2.5g/l + CH 500.0mg/l + Agar 4.5g/l + CW 200.0ml/l
I_6	MS + 2, 4-D 10.0mg/l + GA 10.0mg/l + BA 0.05g/l + Glut 600.0mg/l + Suc 60.0g/l + AC 2.5g/l CH 500.0mg/l + Agar 4.5g/l + CW 200.0ml/l
I_7	1/2 MS + BA 0.5mg/l + Glut 600.0mg/l + Suc 60.0g/l + CH 500.0mg/l + Agar 6.0g/l + AC 2.5g/l + CW 200.0ml/l
I_8	1/2 MS + 2,4-D 10.0mg/l + BA 0.5mg/l + Glut 600.0mg/l + Suc 60.0g/l + CH 500.0mg/l + Agar 6.0g/l + AC 2.5g/l + CW 200.0ml/l
I_9	1/2 MS + GA 5.0mg/l + BA 0.05mg/l + Suc 60.0g/l + CH 500.0mg/l + AC 2.5g/l + Agar 6.0g/l + CW 200.0ml/l + Glut 400.0mg/l
I_{10}	1/2 MS + 2,4-D 5.0mg/l + BA 0.05mg/l + Glut 400.0mg/l + Suc 60.0g/l + CH 500.0mg/l + Agar 6.0g/l + AC 2.5g/l + CW 200.0ml/l
I_{11}	1/2 MS + 2,4-D 10.0mg/l + BA 0.05mg/l + Glut 400.0mg/l + Suc 60.0g/l + CH 500.0mg/l + Agar 6.0g/l + AC 2.5g/l + CW 200.0ml/l
I_{12}	1/2 MS + 2,4-D 2.0mg/l + GA 5.0mg/l + BA 0.05mg/l + Glut 400.0mg/l + Suc 60.0g/l + CH 500.0mg/l + Agar 6.0g/l + AC 2.5g/l + CW 200.0ml/l

tubelights at an intensity of 3000 lux. In order to provide darkness, culture vessels were kept in culture racks/stands covered with black polythene sheet or aluminium foil. The influence of temperature on the initiation of somatic embryoids was studied by keeping the cultures inside the culture room ($26^o \pm 2^oC$) and outside the culture room at room (ambient) temperature.

Observations were recorded on the number of cultures initiating somatic embryoids and the number of embryoids produced per flask.

3. Maturation of somatic embryoids

The initiated somatic embryoids were transferred to the maturation medium.

a. Treatments

i. ABA

For the maturation of somatic embryoids, MS and B_5 basal media were used. The somatic embryoids from the initiation medium were transferred to the maturation medium containing various concentrations of abscisic acid (0.5 - 10.57 mg/l). Somatic embryos from the initiation media

were transferred to 100 ml Erlenmeyer flask containing 40.0 ml of the maturation media. The treatments were replicated eight to twenty three times.

The effects of various treatments were observed for a period of six weeks. Observations were made on the size and colour of the somatic embryoids and on the number of somatic embryoids per flask.

ii. Sucrose

Cultures initiating somatic embryoids were transferred to B_5 medium containing sucrose 15.0, 20.0 and 30.0 g/l. These treatments were compared for their effects on the maturation of somatic embryos.

The effects of various treatments were observed for a period of six weeks. Observations were made on the size and colour of the somatic embryoids and the number of somatic embryoids per flask.

b. Culture conditions

The effect of light and darkness on the maturation

Table 3. Treatments tried for the maturation of somatic embryoids

No.	Treatment
M_1	1/2 MS + BA 0.5mg/l + IBA 0.1mg/l + GA 5.0mg/l + Glut 400.0mg/l + Suc 40.0g/l + CH 500.0mg/l + Agar 6.0g/l + AC 500.0mg/l + CW 200.0ml/l
M_2	1/2 MS major + 1/2 Minor + Full organics + CH 100.0mg/l + Suc 40.0g/l + Agar 5.5g/l + AC 250.0mg/l + CW 200.0ml/l
M_3	1/2 MS + ABA 0.5mg/l + CH 100.0mg/l + Suc 40.0g/l + Agar 5.00g/l + AC 250.0mg/l + CW 200.0ml/l
M_4	1/2 MS + ABA 1.0mg/l + Suc 40.0mg/l + CH 100.0mg/l + Agar 5.5g/l + CW 200.0ml/l + AC 250.0mg/l
M_5	1/2 MS + ABA 1.5mg/l + CH 100.0mg/l + Suc 40.0g/l + Agar 5.5g/l + CW 200.0ml/l + AC 250.0mg/l
M_6	1/2 MS + ABA 2.0mg/l + CH 100.0mg/l + Suc 40.0g/l + Agar 5.5g/l + CW 200.0ml/l + AC 250.0mg/l
M_7	1/2 MS + ABA 5.0mg/l + CH 100.0mg/l + Suc 40.0g/l + Agar 5.5g/l + CW 200.0ml/l + AC 250.0mg/l
M_8	1/2 MS + ABA 10.0mg/l + CH 100.0mg/l + Suc 40.0g/l + Agar 5.5g/l + CW 200.0ml/l + AC 250.0mg/l
M_9	1/2 MS + CH 100.0mg/l + ABA 4.23 mg/l + Suc 40.0g/l + Agar 6.0g/l + AC 250.0mg/l + CW 200.0ml/l
M_{10}	1/2 MS + CH 100.0mg/l + ABA 4.76mg/l + Suc 40.0g/l + Agar 6.0g/l + AC 250.0mg/l + CW 200.0ml/l

No.	Treatment
M_{11}	1/2 MS + CH 100.0mg/l + ABA 5.29mg/l + Suc 40.0g/l + Agar 6.0g/l + AC 250.0mg/l + CW 200.0ml/l
M_{12}	1/2 MS + CH 100.0mg/l + ABA 10.57mg/l + Suc 40.0g/l + Agar 6.0g/l + AC 250.0mg/l + CW 200.0ml/l
M_{13}	1/2 MS + CH 100.0mg/l 1 + ABA 1.32mg/l + Suc 40.0g/l + Agar 6.0g/l + AC 250.0mg/l + CW 200.0ml/l
M_{14}	1/2 MS + CH 100.0mg/l + ABA 1.0mg/l + PEG 50.0g/l + Suc 40.0g/l + Agar 6.0g/l + AC 250.0mg/l + CW 200.0ml/l
M_{15}	B_5 Major + MS minor + CH 250.0mg/l + ABA 0.79mg/l + Agar 6.0g/l + AC 250.0mg/l + Suc 20.0g/l + CW 200.0ml/l
M_{16}	B_5 Major + MS minor + CH 250.0mg/l + ABA 0.79mg/l + Agar 6.0g/l + AC 250.0mg/l + Suc 15.0g/l + CW 200.0ml/l
M_{17}	B_5 Major + MS minor + CH 250.0mg/l + ABA 0.79mg/l + Agar 6.0g/l + AC 250.0mg/l + Suc 30.0g/l + CW 200.0ml/l

of somatic embryoids was studied. The cultures were incubated in light (3000 lux) and darkness.

Observations were made after four weeks on the size and colour of the embryoids as well as on the number of embryoids produced per flask.

4. Germination of somatic embryoids

The embryoids from the maturation media were transferred to the germination media.

a. Treatments

Thirty treatments were tried for the germination of somatic embryoids (Table 4). MS was used as the basal medium. All the treatments were replicated six to forty times. The treatments included combinations of plant growth substances, sucrose, agar, coconut water, nitrogen source, sodium butyrate and polyethylene glycol (PEG).

The influence of plant growth substances like BA (0.5 - 20.0 mg/l), GA_3 (0.1 - 10.0 gm/l), 2iP (1.0 - 5.0 mg/l) and NAA (0.1 mg/l) on the germination of somatic

embryoids was studied by incorporating them (in various combinations) in the germination media. Nitrogen source of the MS basal medium was modified by using half strength and one fourth strength ammonium nitrate and half strength potassium nitrate. Sucrose was incorporated into the medium at 20.0, 30.0 and 90.0 g/l. The concentration of agar varied from 4.5 to 10.0 g/l in the different treatments. The influence of PEG on the germination of somatic embryoids was studied by incorporating five per cent PEG to the germination medium. Sodium butyrate (10.0 uM) was added to the medium. Coconut water was tried at 200.0 ml. A combination without coconut water was also included.

Observations were recorded on the germination of somatic embryoids, formation of root and shoot, size of embryoids, colour of embryoids and number of embryoids per culture after four weeks.

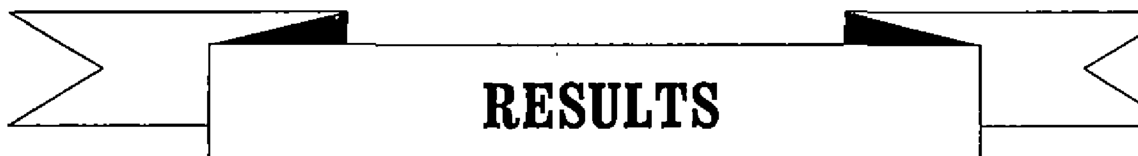
Table 4. Treatments tried for the germination of somatic embryoids

No.	Treatment
G_1	MS + BA 5.0mg/l + Suc 30.0g/l + AC 2.5g/l + Agar 5.5g/l + CW 200.0ml/l
G_2	1/2 MS + BA 10.0mg/l + Suc 20.0g/l + AC 1.0g/l + Agar 5.5g/l + CW 200.0ml/l
G_3	1/2 MS + BA 2.5mg/l + Suc 20.0g/l + AC 1.0g/l + Agar 5.5g/l + CW 200.0ml/l
G_4	1/2 MS + BA 1.25mg/l + Suc 20.0g/l + AC 1.0g/l + Agar 5.5g/l + CW 200.0ml/l
G_5	MS + BA 5.0mg/l + Sue 30.0g/l + AC 2.5g/l + Agar 5.5g/l (No CW)
G_6	MS + BA 5.0mg/l + Suc 30.0g/l + Agar 5.5g/l + CW 200.0ml/l (No AC)
G_7	MS + BA 5.0mg/l + Suc 30.0g/l + CW 200.0ml/l
G_8	MS + BA 10.0mg/l + GA_3 10.0mg/l + Suc 30.0g/l + AC 2.5g/l + Agar 6.0g/l + CW 200.0ml/l
G_9	MS + BA 20.0mg/l + Suc 30.0g/l + Agar 6.0g/l + AC 2.5g/l + CW 200.0ml/l
G_{10}	MS + BA 10.0mg/l + GA_3 5.0mg/l + Suc 30.0g/l + Agar 6.0g/l + AC 2.5g/l + CW 200.0ml/l
G_{11}	MS + GA_3 10.0mg/l + Suc 30.0g/l + Agar 6.0g/l + AC 2.5g/l + CW 200.0ml/l
G_{12}	1/2 MS + BA 10.0mg/l + Suc 30.0g/l + Agar 4.5g/l + AC 2.5g/l + CW 200.0ml/l
G_{13}	MS + Suc 30.0g/l + Agar 6.0g/l + AC 2.5g/l + CW 200.0ml/l
G_{14}	1/2 MS + BA 1.0mg/l + GA_3 5.0mg/l + Suc 30.0g/l + CW 200.0ml/l + Vit C 150ppm + Citric acid 150ppm
G_{15}	1/2 MS + BA 1 + Suc 30.0g/l + Agar 10g/l + AC 2.5g/l + CW 200.0ml/l + Vit C-150ppm + Citric acid 150ppm

No.	Treatment
G_{16}	1/2 MS + BA 1.0mg/l + Suc 90.0g/l + Agar 5.5g/l + AC 2.5g/l + CW 200.0ml/l + Vit C 150ppm + Citric acid 150ppm
G_{17}	Fresh nucellus kept aside embryos in G_8
G_{18}	1/2 MS + BA 1.0mg/l + Suc 30.0g/l + Agar 5.5g/l + AC 2.5g/l + CW 200.0ml/l + Vit C 150ppm + Citric acid 150ppm
G_{19}	1/2 MS + BA 0.5mg/l + GA_3 0.1mg/l + NAA 0.1mg/l + Suc 30g/l + Agar 6.0g/l + AC 2.5g/l + CW 200.0ml/l
G_{20}	1/2 MS + BA 5.0mg/l + 2iP 1.0mg/l + Suc 30.0g/l + Agar 6.0g/l + AC 2.5g/l + CW 200.0ml/l
G_{21}	1/2 MS + BA 1.0mg/l + 2iP 1.0mg/l + Suc 30.0g/l + Agar 6.0g/l + AC 2.5g/l + CW 200.0ml/l
G_{22}	1/2 MS + 2iP 5.0mg/l + Suc 30.0g/l + Agar 6.0g/l + AC 2.5g/l + CW 200.0ml/l
G_{23}	1/2 MS + BA 1.0mg/l + 2iP 5.0mg/l + Suc 30.0g/l + Agar 6.0g/l + AC 2.5g/l + CW 200.0ml/l
G_{24}	1/2 MS + BA 5.0mg/l + 2iP 5.0mg/l + Suc 30.0g/l + Agar 6.0g/l + AC 2.5g/l + CW 200.0ml/l
G_{25}	1/2 MS + BA 5.0mg/l + 2iP 2.0mg/l + Suc 30.0g/l + Agar 6.0g/l + AC 2.5g/l + CW 200.0ml/l
G_{26}	1/2 MS + BA 10.0mg/l + GA_3 10.0mg/l + Sodium butyrate 10 uM + Suc 30.0g/l + Agar 6.0g/l + AC 2.5g/l + CW 200.0ml/l
G_{27}	1/2 MS + BA 1.0mg/l + PEG 50.0g/l + Suc 30.0g/l + Agar 6.0g/l + AC 2.5g/l + CW 200.0ml/l
G_{28}	1/2 MS + (NH_4 NO_3 half) + BA 1.0mg/l + 2iP 1.0mg/l + Suc 30.0g/l + Agar 6.0g/l + AC 2.5g/l + CW 200.0ml/l
G_{29}	1/2 MS + (NH_4 NO_3 1/4th conc) + BA 1.0mg/l + 2iP 1.0mg/l + Suc 30.0g/l + Agar 6.0g/l + AC 2.5g/l + CW 200.0ml/l
G_{30}	1/2 MS + (KNO_3 half) + BA 1.0mg/l + 2iP 1.0mg/l + Suc 30.0g/l + Agar 6.0g/l + AC 2.5g/l + CW 200.0ml/l

Table 5. **Treatments tried for the induction of somatic embryoids from leaf explant**

No.	Treatment
L_1	MS + 2,4-D 2.0mg/l + BA 1.0mg/l + AC 1.0g/l + Agar 6.0g/l + Suc 30.0g/l + CW 200.0ml/l
L_2	MS + 2,4-D 2.0mg/l + BA 1.0mg/l + Suc 30.0g/l
L_3	MS + 2,4-D 0.5mg/l + BA 0.5mg/l + NAA 0.5mg/l + Suc 30.0g/l + CW 200.0ml/l
L_4	MS + 2,4-D 2.0mg/l + BA 1.0mg/l + AC 0.1g/l + Agar 6.0g/l + Suc 30.0g/l
L_5	MS + 2,4-D 0.5mg/l + BA 0.5mg/l + NAA 0.5g/l + Suc 30.0g/l
L_6	1/2 MS + 2,4-D 5.0mg/l + GA_3 5.0mg/l + Glut 400.0mg/l + Suc 60.0g/l + Agar 6.0g/l + AC 2.5g/l + CW 200.0ml/l


RESULTS

RESULTS

The results of the investigations aimed at optimising the *in vitro* production of somatic embryoids in polyembryonic mango varieties are presented in this chapter.

A. Varieties

Nucellus as well as embryo mass from six polyembryonic mango varieties, namely, Thalimanga, Pulichi, Kalluvarikka, Kilichundan, Vellari and Varikka (Table 6) were used for the induction of somatic embryogenesis. The highest per cent (87.50) cultures initiating somatic embryoids from nucellus was observed for Kalluvarikka. The least response (50.00 per cent) was recorded by Vellari. Explants (nucellus as well as embryo mass) of Kilichundan did not respond to the treatments. The response of the other three varieties ranged from 63.15 to 76.92 per cent. Although nucellus from Vellari recorded the least per cent cultures initiating somatic embryoids, embryo mass of the variety was observed to have the highest per cent initiation (91.66). The least response (27.27 per cent) from embryo mass was observed for Varikka. The other three varieties recorded 64.29 to 83.34 per cent initiation.

B. Explants

1. Response

The response of the different explants with respect to the induction of somatic embryoids was studied. Two varieties (Vellari and Kalluvarikka) were used for the purpose. Three types of explants, namely, nucellus, embryo mass and tender leaf segments were used. Embryomass recorded the highest response (91.66 per cent cultures initiating somatic embryos) in Vellari (Table 7). In Kalluvarikka, the best response (87.50 per cent) was observed from the nucellus. But leaf segments of both varieties did not respond to the treatments. However, numerous small creamy globular structures were seen initiated from the leaf explants of Vellari. Such structures failed to develop further on subculture.

2. Orientation

Three types of orientation were tried while inoculating nucellar tissue. In the first type, nucellar tissue was scooped out from the ovule and inoculated on to the medium. In the second type, nucellus along with a portion of the ovule was inoculated with the ovule in

Table 6. Influence of varieties on the induction of somatic embryoids

Culture medium : 1/2 MS + 2,4-D 5.0 mg/l + GA_3 5.0 mg/l + glutamine 400.0 mg/l + sucrose 60.0 g/l + agar 6.0 g/l + AC 2.5 g/l + CW 200.0 ml/l

Variety	Replication		Cultures initiating somatic embryoids /multiple/ zygotic embryos (%)	
	Nucellus	Embryomass	Nucellus	Embryomass
Thalimanga	19	28	63.15	64.29
Pulichi	17	7	70.58	71.42
Kalluvarikka	8	12	87.50	83.34
Kilichundan	23	20	0.00	0.00
Varikka	13	11	76.92	27.27
Vellari	4	12	50.00	91.66

Table 7. Influence of explants on the induction of somatic / multiple / zygotic embryos

Variety	Replication			Cultures initiating somatic embryos/callus/multiple/ zygotic embryos (%)		
	Nucellus	Embryomass	Leaf segment	Nucellus	Embryomass	Leaf segment
Vellari	4	12	24	50.00	91.66	0.00
Kalluvarikka	8	12	18	87.50	83.34	0.00

Table 8. Effect of orientation of explant on the *in vitro* response

Culture medium : 1/2 MS + 2,4-D 5.0 mg/l + GA3 5.0 mg/l + glutamine 400.0 mg/l + sucrose 60.0 g/l + agar 6.0 g/l + AC 2.5 g/l + CW 200.0 ml/l

Treatment	Replication	Cultures iniating somatic embryos (%)
Nucellus placed on the medium	6	16.67
Nucellus + part of ovule inoculated horizontally	6	16.67
Nucellus + part of ovule inoculated vertically	6	66.67

Table 9. Effect of various treatments on the *in vitro* response of leaf explants

Treat-ment *	Repli-cation	Contami-nation (%)	Response (%)
L_1	12	83.30	0.00
L_2	24	66.67	8.33
L_3	12	100.00	0.00
L_4	12	100.00	0.00
L_5	12	100.00	0.00
L_6	18	44.40	0.00

* Composition of treatments given in Table 5.

horizontal position. In the third method, nucellus along with a portion of the ovule in a vertical portion was used. The maximum response (66.67 per cent) was observed in the third type of orientation (Table 8). The response from the first and the second type was 16.67 per cent only.

C. Induction of somatic embryoids

1. Treatments

During the first season (January to May 1993), twenty five treatments involving various combinations of plant growth substances, glutamine, activated charcoal and agar were tried for the induction of somatic embryoids. The most promising treatment was found to be 1/2 MS + glutamine 400.0 mg/l + 2,4 - D 5.0 mg/l + GA_3 5.0 mg/l + sucrose 60.0 g/l + agar 6.0 g/l + AC 2.5 g/l + CW 200.0 ml/l (T_{25}).

Twenty three treatments were tried during the second season (January-April 1994) for two varieties (Pulichi and Kalluvarikka). For both the varieties, the most promising treatment for the induction of somatic embryoids from nucellar tissue (Plates 1 and 2) was found to be T_{25} (Tables

10 and 11). Somatic embryoids from nucellar tissue were initiated in 70.58 per cent of the cultures of Pulichi and 87.50 per cent of the cultures of Kalluvarikka when subjected to T_{25}. In Pulichi, the embryo mass subjected to this treatment exhibited initiation of callus in 14.29 per cent of the cultures and multiple embryos in 57.14 per cent of the cultures. The response from nucellar tissue was 66.67 per cent in the treatments T_4, T_8, T_{17}, T_{18} and T_{24} (Table 10). The treatments T_4, T_8, T_{17} and T_{24} contained 4.0 mg/l 2,4-D while T_{18} had 8.0 mg/l of the plant growth substance. The treatments T_3, T_9 and T_{20} recorded 50.0 per cent response from nucellus. There was no response from nucellar tissue in the treatments T_6, T_7, T_{10}, T_{11} and T_{20}.

The treatment T_{25} was the best for initiating somatic embryoids from the nucellar tissue (87.50 per cent cultures) in Kalluvarikka. This treatment produced multiple / zygotic embryos in 66.67 per cent cultures and callus in 16.67 per cent cultures from embryo mass (Plate 8). The treatment T_4 initiated somatic embryoids from nucellar tissue in 75.00 per cent of the cultures. As influenced by the treatment, 33.30 per cent cultures initiated callus and 16.67 per cent cultures initiated multiple embryos from embryo

mass. The treatment T_8, T_{17}, T_{21} and T_{22} initiated somatic embryoids in 66.67 per cent of the cultures from nucellar tissue. There was no response in the nucellus treated with T_6, T_{10}, T_{11} and T_{14}.

The most effective treatments for initiating callus from embryo mass of Pulichi were T_9 (1/2 MS + 2,4-D 8.0 mg/l + GA_3 10.0 mg/l + glutamine 400.0 mg/l + sucrose 30.0 g/l + agar 7.0 g/l + AC 5.0 g/l + CW 200.0 ml/l), T_{13} (1/2 MS + 2,4-D 8.0 mg/l + GA_3 20.0 mg/l + glutamine 400.0 mg/l + sucrose 30.0 g/l + agar 7.0 g/l + AC 5.0 g/l + CW 200.0 ml/l) and T_{18} (1/2 MS + 2,4-D 3.0 mg/l + GA_3 10.0 mg/l + BA 0.5 mg/l + AC 5.0 g/l + CW 200.0 mg/l + sucrose 60.0 g/l + agar 6.0 g/l + glutamine 600.0 mg/l). All these treatments recorded 50.00 per cent cultures initiating callus. The treatments T_{23} and T_{17} recorded 42.86 per cent and 40.00 per cent response, respectively. In Kalluvarikka, the best treatments were T_{13} and T_{18} which recorded 66.67 per cent cultures initiating callus from embryo mass. Fifty per cent response was recorded for T_{17} (1/2 MS + 2,4-D 2.0 mg/l + GA_3 10.0 mg/l + BA 0.5 mg/l + glutamine 600.0 mg/l + sucrose 60.0 g/l + agar 6.0 g/l + AC 5.0 g/l + CW 200.0 ml/l).

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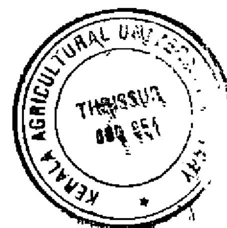
Table 10. Effect of culture media on the induction of somatic embryoids in the mango variety Pulichi

Treatment *	Replication		Cultures initiating embryoids from nucellus (%)	Cultures initiating callus from embryo mass (%)	Cultures initiating multiple/ zygotic embryos from embryo mass (%)
	Embryo-mass	Nucellus			
T_3	2	4	50.00	0.00	50.00
T_4	4	6	66.67	0.00	50.00
T_5	3	3	33.30	0.00	66.60
T_6	4	2	0.00	25.00	25.00
T_7	3	3	0.00	0.00	33.30
T_8	5	3	66.60	20.00	20.00
T_9	4	2	50.00	50.00	0.00
T_{10}	4	3	0.00	25.00	0.00
T_{11}	6	9	0.00	6.67	11.10
T_{12}	7	7	14.29	14.29	28.57
T_{13}	6	6	33.30	50.00	0.00
T_{14}	7	9	33.30	14.29	0.00
T_{16}	2	8	12.50	0.00	50.00
T_{17}	5	6	66.67	40.00	40.00
T_{18}	3	3	66.67	50.00	0.00
T_{19}	3	3	0.00	33.30	0.00
T_{20}	4	8	50.00	25.00	75.00
T_{21}	7	5	60.00	14.29	28.57
T_{22}	6	9	44.40	16.67	16.67
T_{23}	7	9	22.20	42.86	0.00
T_{24}	6	6	66.67	0.00	66.67
T_{25}	7	17	70.58	14.29	57.14
T_{26}	8	10	40.00	12.50	62.50



* Composition of treatment given in Table - 1

Table 11. Effect of culture media on the induction of somatic embryoids in the mango variety Kalluvarikka

Treatment *	Replication		Cultures initiating embryoids from nucellus (%)	Cultures initiating callus from embryo mass (%)	Cultures initiating multiple/ zygotic embryos from embryo mass (%)
	Embryo-mass	Nucellus			
T_3	3	3	33.30	0.00	33.30
T_4	6	4	75.00	33.30	50.00
T_5	3	5	40.00	0.00	66.67
T_6	6	6	0.00	0.00	33.30
T_7	6	6	33.30	0.00	33.30
T_8	4	6	66.67	25.00	25.00
T_9	3	5	20.00	33.30	33.30
T_{10}	6	6	0.00	33.30	16.67
T_{11}	3	3	0.00	33.30	0.00
T_{12}	3	3	33.30	16.67	33.30
T_{13}	6	6	33.30	66.67	16.67
T_{14}	3	6	0.00	33.30	66.67
T_{16}	4	6	50.00	0.00	50.00
T_{17}	4	6	66.67	50.00	25.00
T_{18}	6	6	33.30	66.67	16.67
T_{19}	6	6	33.30	33.30	50.00
T_{20}	4	8	50.00	0.00	50.00
T_{21}	6	8	66.67	16.67	50.00
T_{22}	6	6	66.67	0.00	66.67
T_{23}	6	9	22.20	33.30	66.67
T_{24}	6	8	62.50	0.00	66.67
T_{25}	12	8	87.50	16.67	66.67
T_{26}	9	9	33.30	0.00	44.40

* Composition of treatments given in Table - 1

In Pulichi, the treatment T_{20} (1/2 MS + 2,4-D 1.0 mg/l + GA_3 10.0mg/l + BA 1.0 mg/l + glutamine 600.0 mg/l + sucrose 60.0 g/l + agar 6.0 g/l + AC 0.5 g/l + CW 200.0 ml/l) resulted in the highest per cent cultures (75.00) initiating multiple embryos from embryo mass. Response of less value was recorded by T_5 (66.67 per cent), T_{24} (66.67 per cent) and T_{26} (62.50 per cent). In Kalluvarikka, the treatments T_5, T_{14}, T_{22}, T_{23}, T_{24} and T_{25} produced 66.67 per cent and T_4, T_{16}, T_{19}, T_{20} and T_{21} produced 50.00 per cent cultures initiating multiple embryos.

In Pulichi as well as Kalluvarikka, the nucellar tissue took four to seven weeks for initiating somatic embryoids. Production of callus and multiple embryos from embryo mass took only eight to forteen days.

2. Basal media

Full strength and half strenght of MS basal medium was used for the production of somatic embryoids, callus and multiple embryoids (Table 13). Half strength MS basal medium (1/2 MS + 2,4-D 5.0 mg/l + GA_3 5.0 mg/l + glutamine 400.0 mg/l + sucrose 60.0 g/l + agar 6.0 g/l + AC 2.5 g/l + CW 200.0 mg/l) was found to be the best for the production of

somatic embryoids from nucellar tissue in Pulichi (70.59 per cent) and Kalluvarikka (87.50 per cent). With half strength MS basal medium, 14.29 per cent of the cultures produced callus and 57.14 per cent produced multiple embryos from the embryo mass of Pulichi.

In Kalluvarikka, 16.67 per cent of the cultures initiated callus and 62.50 per cent of the cultures initiated multiple embryos in half strength MS basal medium with supplements.

With full strength MS basal medium, having MS + 2,4 D 5.0 mg/l + GA_3 5.0 mg/l + glutamine 400.0 mg/l + sucrose 60.0 g/l + agar 6.0 g/l + AC 2.5 g/l + CW 200.0 ml/l only 40.00 per cent of the cultures of Pulichi and 33.30 per cent of the cultures of Kalluvarikka initiated somatic embryoids from nucellar tissue. Callus was seen initiated in 12.50 per cent of the cultures and multiple embryos in 62.50 per cent of the cultures of the embryo mass of Pulichi. None of the cultures were observed to initiate callus from the embryo mass of Kalluvarikka. However, 44.40 per cent of the cultures initiated multiple embryos from the embryo mass of Kalluvarikka.

3. Culture conditions

When the cultures (T_{25}), were kept under light (3000 lux) none of the cultures initiated somatic embryoids from nucellar tissue where as 16.67 per cent and 50.00 per cent of the cultures initiated callus and multiple embryos, from the embryo mass, respectively (Table 12). When the cultures were kept in darkness, 66.67 per cent of the cultures initated somatic embryoids from nucellus, 33.33 per cent initiated callus and 50.00 per cent initiated multiple embryos from the embryo mass. The time taken for differentiation was not found to be influenced by light.

D. Initiation of somatic embryoids

1. Treatments

The explants from the induction media were subjected to twelve treatments for the initiation of somatic embryoids. The treatment I_{10} (1/2 MS + glutamine 400.00 mg/l + 2,4-D 5.00 mg/l + BA 0.05 mg/l + CH 500.00 mg/l + sucrose 60.00 g/l + agar 6.00 g/l + AC 2.50 g/l + CW 200.00 ml/l) was found to be the best, supporting 55.50 per cent cultures (Plates 3 and 4) initiating somatic embryos from

Table 12. Influence of light on the induction of somatic embryoids

Treatment *	Replication		Cultures initiating embryoids from nucellus (%)	Cultures initiating callus from embryo mass (%)	Cultures initiating multiple/ zygotic embryos from embryo mass (%)
	Embryo-mass	Nucellus			
Light (T_{25})	6	6	0.00	16.67	50
Dark (T_{25})	6	6	66.67	33.30	50

Table 13. Effect of basal media on the induction of somatic embryoids

Variety	Treatment *	Replication		Cultures initiating embryoids from nucellus (%)	Cultures initiating callus from embryo mass (%)	Cultures initiating multiple/ zygotic embryos from embryo mass (%)
		Embryo-mass	Nucellus			
Pulichi	T_{25}	7	17	70.59	14.29	57.14
Pulichi	T_{26}	8	10	40.00	12.50	62.50
Kalluvarikka	T_{25}	12	8	87.50	16.67	66.67
Kalluvarikka	T_{26}	9	9	33.30	0.00	44.40

* Composition of treatments given in Table - 1

nucellar tissues (Table 9). In the treatment I_2, 42.50 per cent of the cultures initiated somatic embryos from nucellar tissue (Plates 11 and 12). Somatic embryos were also found initiated from nucellar tissue subjected to the treatments I_7 (41.67 per cent), I_8 (41.67 per cent) and I_4 (40.00 per cent). There was no initiation of somatic embryoids in the treatment I_5 and I_6 in which full strength MS basal medium was used (Table 14).

I_{11} (1/2 MS + 2,4-D 10.00 mg/l + BA 0.05 mg/l + glutamine 400.00 mg/l + CH 500.00 mg/l + sucrose 60.00 g/l + agar 6.00 g/l + AC 2.50 g/l + CW 200.00 ml/l) was observed to be the best treatment for initiating somatic embryoids from embryogenic callus (Plate 5). This treatment recorded 26.67 per cent cultures initiating somatic embryoids. Less response was recorded by I_8 (25.00 per cent) and I_2 (20.00 per cent) in which the concentration of 2,4-D was 10.0 mg/l. The treatments I_1, I_5 and I_6 did not initiate somatic embryoids from embryogenic callus.

Multiple embryos obtained from the induction medium were transferred to the initiation medium with the objective of initiating somatic embryoids. However, only further

growth of the multiple embryos was favoured (Plates 6, 7, 9 and 10). Somatic embryos could not be obtained. All treatments except I_7 favoured the growth of multiple embryos.

2. Culture conditions

The influence of light and temperature on the initiation of somatic embryos was studied. In the presence of light, only 16.67 per cent cultures initiated somatic embryoids, where as 66.67 per cent of the cultures in darkness initiated somatic embryoids (Table 15). The percentage of cultures initiating somatic embryoids remained the same (66.67) when the cultures were kept at 26 $\pm$ 2°C and at room temperature. However the number of embryoids produced per flask was higher (20-30) when the cultures were kept in darkness.

E. Maturation of somatic embryoids

1. Treatments

Sixteen treatments, involving various combinations of ABA, sucrose and basal media, were tried for the

Table 14. Effect of culture media on the initiation of somatic embryoids

Treat-ment *	Repli-cation	Cultures initiating somatic embryoids		Cultures initiating multiple/zygotic embryos(%)
		Nucellus	Embryomass	
I_1	23	30.43	0.00	34.78
I_2	40	42.5	20.00	25.00
I_3	32	18.75	9.38	21.88
I_4	20	40.00	10.00	40.00
I_5	18	0.00	0.00	33.30
I_6	18	0.00	0.00	27.78
I_7	12	41.67	0.00	0.00
I_8	12	41.67	25.00	8.33
I_9	15	13.33	6.67	26.67
I_{10}	18	55.50	16.67	11.11
I_{11}	15	20.00	26.67	20.00
I_{12}	18	33.30	16.67	16.67

* Composition of treatments given in Table - 2

Table 15. Influence of culture conditions on the initiation of somatic embryoids

Treatment	Replication	Cultures initiating embryoids (%)	No. of embryoids per culture
Light	6	16.67	17 - 20
Dark	6	66.67	20 - 30
Low temp. (A/C)	6	66.67	20 - 30
Room temp.	6	66.67	13 - 15

maturation of somatic embryoids. The criteria for assessing the maturation of somatic embryoids was the size of the embryoids.

The maximum size of embryoids was obtained from the treatment M_9 (1/2 MS + CH 100.00 mg/l + ABA 16 uM (10.57 mg/l) + sucrose 40.00 g/l + agar 6.00 g/l + AC 2.50 g/l + CW 200.00 ml/l). In this treatment 100 to 150 cream coloured embryoids per flask were obtained. The size of the embryoids ranged from 0.5 to 1.5 cm. Cream coloured embryoids of the size 0.5 - 1.0 cm were obtained in the treatments M_{15}, M_{16} and M_{17} (Table 16).

The highest number (100-200) of embryoids per flask was recorded in the treatment M_3.

a. Sucrose

Three treatments involving different sucrose level (15.0, 20.0 and 30.0 g/l) were tried for the maturation of somatic embryoids. The basal medium, B_5 was used. There was no increase in the size of embryoids in these three treatments. The colour of the embryoids changed from cream to light brown (Table 17).

Table 16. Effect of culture media on the maturation of somatic embryoids

Treatment *	Replication	Live cultures (%)	No. of embryoids per culture	Size of embryoids (cm)	Colour of embryoids
M_1	23	91.30	2-20	0.5	Cream
M_2	51	82.35	3-12	0.5	Pink
M_3	15	80.00	100-200	< 0.5	Cream
M_4	15	93.33	100-150	< 0.5	Cream
M_5	15	73.33	50-80	0.5-1	Cream
M_6	15	66.67	30-50	0.5	Cream
M_7	12	33.3	6-30	< 0.5	Light brown
M_8	12	16.67	17-25	< 0.5	Light brown
M_9	8	75.00	100-130	0.5-1.5	Cream
M_{10}	10	80.00	100-150	0.5-1.0	Cream
M_{11}	8	62.25	70-100	0.5-1.0	Cream
M_{12}	8	37.50	40-60	< 0.5	Light brown
M_{13}	10	70.00	50-70	0.5	Cream
M_{14}	12	41.67	100-150	< 0.5	Light brown
M_{15}	12	25.00	30-50	< 0.5	Light brown
M_{16}	15	20.00	16-50	0.5	Light brown and transparent

* Composition of treatments given in Table - 3.

Table 17. Effect of sucrose on the maturation of somatic embryoids

Treatment *	Replication	Live cultures (%)	No. of embryoids per culture	Size of embryoids (cm)
M_9	12	41.67	10-30	< 0.5
M_{10}	12	25.00	30-50	< 0.5
M_{11}	12	20.00	16-50	< 0.5

* Composition of treatments given in Table - 3.

Table 18. Effect of light on the maturation of somatic embryoids

Treatment	Replication	Live cultures (%)	No. of embryoids per culture	Size of embryoids (cm)
Light	6	66.67	50-100	< 0.5
Darkness	6	83.33	60-120	< 0.5

2. Culture conditions

There was no difference in the size of the embryoids as influenced by light. When the cultures were kept under light, the percentage of live cultures was 66.67 and when kept under darkness it was 83.33 per cent. In both cases the size of the embryoids remained the same. The average number of embryoids per flask was higher (60 - 120) when kept in darkness (Table 18).

F. Germination of somatic embryoids

Thirty treatments were tried for the germination of somatic embryoids (Table 4). However, normal germination of the somatic embryoids was not observed in any of the treatments (Table 19).

Germination of somatic embryoids was attempted using treatments involving various concentrations of BA. The size of the embryoids was increased in the treatment G_1 (MS + BA 5.0 mg/l + sucrose 30.0 g/l + agar 5.5 g/l + cw 200.0 ml/l + AC 2.5 g/l). The colour of the embryoids turned green in this treatment. But none of the embryoids showed normal germination (Plates 13 and 14). The concentration of BA was

increased to 10.0 mg/l in G_2. In this treatment, 20-80 embryoids were produced per flask, which were pink at first and turned cream later. In the treatments G_3 and G_4, which had reduced concentrations of BA, 20-30 small cream coloured embryoids were produced.

Various combinations of BA and 2iP were tried for the germination of somatic embryoids. Out of the six treatments (G_{20}, G_{21}, G_{22}, G_{23}, G_{24} and G_{25}) only G_{21} having BA 1.0 mg/l and 2iP 1.0 mg/l supported abnormal germination of somatic embryoids. In G_{22} and G_{24} secondary embryoids were formed with high frequency (Plate 15).

None of the cultures surived when GA_3 alone (10.0 mg/l) was supplemented in the germination medium (G_{11}).

The treatments with various combinations of BA and GA_3 (G_8, G_9 and G_{10}) favoured secondary embryogenesis in high frequency.

The treatment G_{19} having BA 0.5 mg/l, GA_3 0.1 mg/l and NAA 0.1 mg/l did not support the germination of somatic embryoids.

In the treatment G_6, lacking AC, somatic embryoids did not survive.

The influence of ammonium ions and nitrate ions on the germination of somatic embryoids was studied by varying the concentration of ammonium nitrate and potassium nitrate in the medium (G_{28}, G_{29} and G_{30}). In these treatments BA 1.0 mg/l and 2iP 1.0 mg/l were added. The results showed that reduction in the concentration of ammonium ions or nitrate ions did not favour the germination of somatic embryoids.

In G_{16}, the concentration of sucrose was increased to 90.0 g/l in order to provide stress condition to enable the germination of somatic embryoids. There was no improvement in the size of embryoids and none of the embryoids germinated.

In order to study the influence of coconut water on the germination of somatic embryoids, two treatments (G_5 and G_{13}) were attempted. When coconut water was not added to the medium the size of the embryoids increased and the colour of the embryoids changed from cream to light green. Abnormal

germination of embryoids was also observed. When only coconut water was added to the medium without any plant growth substances the embryoids did not germinate.

Sodium butyrate was not beneficial to support normal germination of somatic embryoids. In G_{26}, sodium butyrate (10.0 uM) was added along with BA 10.0 mg/l and GA_3 10.0 mg/l. The size of the embryoids increased to 0.5-1.0 cm and there was a colour change from cream to light green. In one particular instance from one embryoid two leafy structures were observed to be developed from the embryoid.

In G_{15}, the concentration of agar was increased to 10.0 g/l in order to provide stress condition for the germination of somatic embyoids. Eventhough there was an increase in the size of the embryoids it soon turned brown and finally black. From the base of these embryoids secondary embryoids started initiating. In G_{12} the concentration of agar was reduced to 4.5 g/l. This treatment did not support the germination of somatic embryoids. Cream coloured small embryoids were being produced continuously.

When five per cent polyethylene glycol was added along with BA 1.0 mg/l (G_{27}) proper germination could not be obtained.

The use of liquid media (G_7 and G_{14}) as well as citric acid and vitamin C (G_{14}) tried for inducing normal germination of somatic embryoids, resulted in mortality of the cultures.

Fresh nucellus was scooped out from tender ovules and kept in close contact with the somatic embryos as a nurse culture technique. Nucellus as well as the cream coloured embryos turned black and finally became dead.

Table 19. Effect of culture media on the germination of somatic embryoids

Treatment *	Replication	Live cultures (%)	Colour of embryoids	Size of embryoids (cm)	Number of embryoids per culture	Other Observations
G_1	10	80.00	Green	1-1.5	3-14	Root & malforined shoots were seen
G_2	10	100.00	Pink	0.5-1	20-80	—
G_3	10	100.00	Cream	< 0.5	20-30	—
G_4	10	90.00	Cream	0.5	20-30	—
G_5	40	72.50	Light green	1-1.5	3-20	Roots were seen in a few culture
G_6	10	—				
G_7	10	—				
G_8	9	77.70	Cream	< 0.5	100	—
G_9	9	77.70	Cream	< 0.5	100	—
G_{10}	9	66.67	Cream	< 0.5	50	—
G_{11}	9	—				
G_{12}	20	80.00	Cream	< 0.5	50	—
G_{13}	12	83.30	Cream	< 0.5	5-20	—
G_{14}	14	—				
G_{15}	8	75.00	Cream	1.5-2.0	5-20	The embryoids finally turned brown
G_{16}	8	75.00	Cream	0.5	50	—
G_{17}	6	—				
G_{18}	6	83.30	Cream	0.5	6-20	—

G_{19}	6	33.3	Cream	0.5	6-20	--
G_{20}	7	71.42	Cream	0.5	6-30	
G_{21}	6	100.00	Cream	0.5	3-12	Roots were formed in some embryoids. In some leafy structures were seen.
G_{22}	6	50.00	Cream	< 0.5		Numerous embryoids were clustered together which could not be seperated, appearing more or less like callus.
G_{23}	6	66.67	Cream	0.5	4-15	--
G_{24}	6	16.67	Cream	< 0.5		Numerous embryoids were clustered together which could not be seperated, appearing more or less like callus.
G_{25}	6	66.67	Transparent	< 0.5	40-60	--
G_{26}	8	75.00	Light green & cream	0.5-1.0	3-15	2 leafy structures were observed from one embryoid.
G_{27}	8	50.00	Cream	0.5	10-30	
G_{28}	6	66.67	Cream	1.0	17-30	
G_{29}	8	62.50	Cream	0.5-1.0	25-30	
G_{30}	10	70.00	Cream	0.5	30-50	

* Composition of treatment given in Table - 4.

Table 20. Effect of form of nitrogen on the germination of somatic embryoids

Treatment *	Replication	Live cultures (%)	No. of embryoids per culture	Size of embryoids (cm)	Colour of embryoids	Other observations
G_{21}	6	100.00	3-12	0.5	Cream	Roots and leafy structures were formed in a few embryoids.
G_{28}	6	66.67	17-30	1.0	Cream	
G_{29}	8	62.50	25-30	0.5-1.0	Cream	
G_{30}	10	70.00	30-50	0.5	Cream	

* Composition of treatments given in Table - 4.

Table 21. Effect of coconut water on the germination of somatic embryoids

Treatment *	Replication	Live cultures (%)	No. of embryoids per culture	Size of embryoids (cm)	Colour of embryoids	Other observations
G_5	40	72.5	3-20	1-1.5	Cream & light green	Roots were formed in a few embryoids
G_1	10	80	3-14	1-1.5	Green	

* Composition of treatments given in Table - 4.

Table 22. Effect of sucrose on the germination of somatic embryoids

Treatment *	Replication	Live cultures (%)	No. of embryoids per culture	Size of embryoids (cm)	Colour of embryoids
G_{16}	8	75	50	0.5	Cream
G_{18}	6	83.3	6-20	0.5	Cream

* Composition of treatments given in Table - 4.

Plates 1 and 2

Nucellus in induction medium for somatic embryogenesis.

Plates 3 and 4

Somatic embryoids initiated from mucellus

Plate 5

Somatic embryoids initiated from embryogenic callus derived from embroyomass

Plate 6

Multiple embryos formed from embryo explant

Plate 7

Multiple embryos formed from embryo explant

Plate 8

Developing zygotic embryo

Plates 9 and 10

Developing zygotic embryo

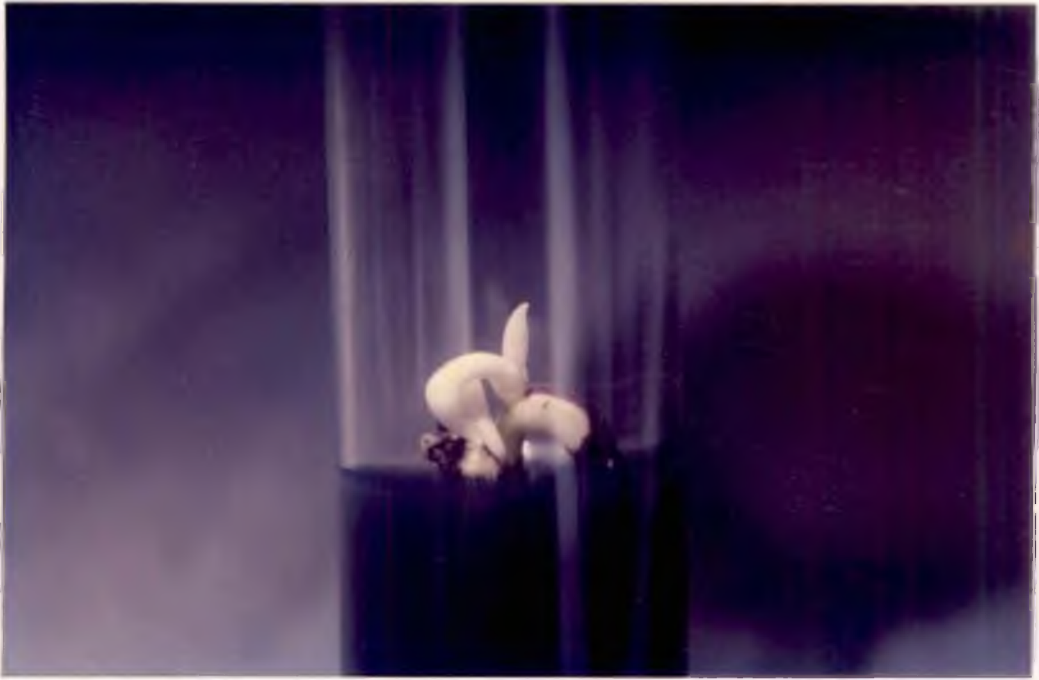


Plate 11

Developmental stages of somatic embryoids initiated from nucellus

Plate 12

Somatic embryoid in torpedo stage

Plate 13

Somatic embryoid showing development of root and shoot

Plate 14

Abnormal germination of somatic embryoid

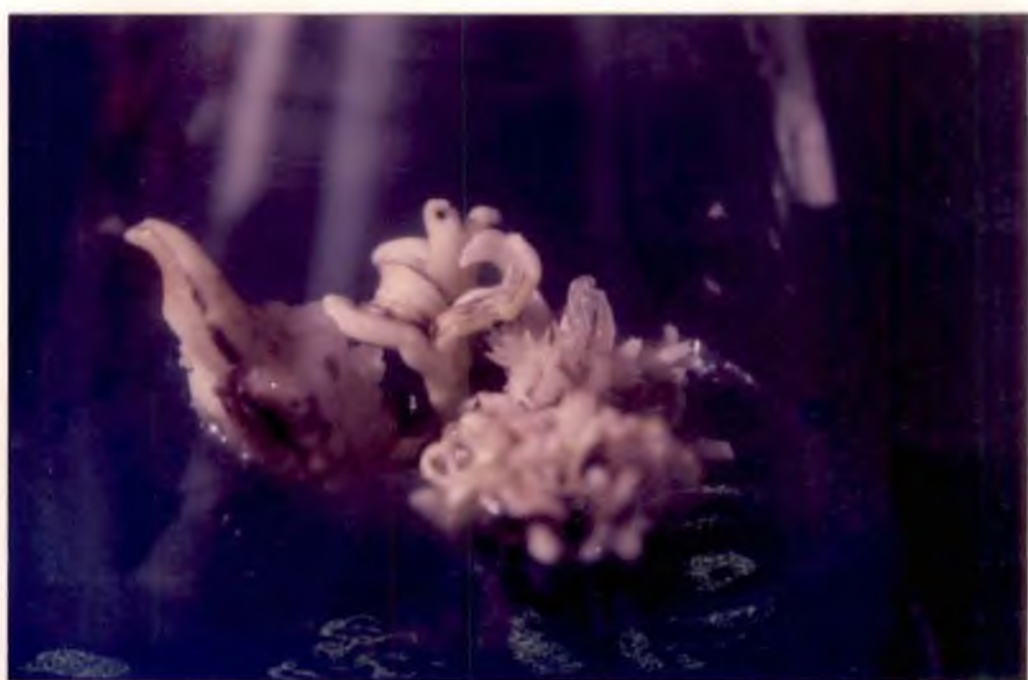


Plate 15

Secondary somatic embryoids

DISCUSSION

DISCUSSION

There are 250 to 300 mango varieties in Kerala. About 10 to 15 of them are polyembryonic. Generally, the polyembryonic types have several desirable characters from the breeding point of view, such as regular bearing habit, large number of fruits per plant, good aroma and juice content, high TSS and resistance to biotic and abiotic conditions. They are widely used for domestic consumption. Polyembryonic varieties are used as rootstocks in mango grafting. There are chances of these varieties becoming extinct due to the changing socio-economic factors and the new priorities in cultivation.

Large scale clonal propagation of polyembryonic rootstocks will be useful to bring about uniformity in the performance of mango grafts. This will help in preserving the genetic wealth of mango as well. There are reports on the *in vitro* propagation of mango, especially of polyembryonic varieties *via* somatic embryogenesis. It has been observed that the polyembryonic varieties respond more readily to *in vitro* propagation than the monoembryonic varieties. The present studies were initiated for optimising

somatic embryogenesis in a few polyembryonic varieties of Kerala (Pulichi, Kalluvarikka, Vellari, Varikka, Thalimanga and Kilichundan). The outcome of the investigations are discussed in the following pages.

Six polyembryonic mango varieties of Kerala (Kalluvarikka, Pulichi, Kilichundan, Thalimanga, Vellari and Varikka) were used for the induction of somatic embryogenesis. Among these, Kalluvarikka and Vellari showed better degree of response. The variety Kilichundan did not respond to any of the induction treatments. Mathews and Litz (1990) reported that certain cultivars like 'Red Itamaraca' did not respond to the treatments for inducing somatic embryogenesis. Litz et al. (1984) reported that somatic embryogenesis occurred in five out of the nine polyembryonic cultivars used in their study. The response appeared to be related to the degree of polyembryony in the ovules at the time of culture.

In the present investigations, nucellus, embryo mass and segments of tender leaves were used as explants for the induction of somatic embryogenesis. Only nucellus and embryo

mass produced somatic embryoids. Nucellus recorded 70.58 per cent response in Pulichi and 87.50 per cent, in Kalluvarikka. Embryo mass recorded 71.42 per cent response in Pulichi and 83.34 per cent, in Kalluvarikka. In perennial fruit crops, somatic embryogenesis was reported from the nucellar tissue of Citrus spp. (Stevenson, 1956) for the first time. Somatic embryogenesis was subsequently obtained from nucellus in Pyrus communis (Janick, 1982), Mangifera indica (Litz et al., 1982, 1984 and 1992; Litz, 1982 and Dewald et al., 1989a and 1989b), Syzygium spp. (Litz, 1984b) and Eriobotrya japonica (Litz, 1985). Pence et al. (1979) and Pleigo - Alfaro and Murashige (1988) observed somatic embryogenesis with Theobroma cacao and Persea americana, respectively, from embryo explant. Muralidharan et al. (1994) reported that in mango, somatic embryogenesis occurred only from zygotic embryo and not from the nucellus.

Difference in response due to the orientation of explant was reported by many workers. In the present instance also, orientation of the explant in the medium influenced the initial response. Maximum response (66.67 per cent) was observed when the nucellus along with a portion of the ovule was inoculated in a vertical position.

The status of a cell at the time of taking the explant is the most important factor for somatic embryogenesis (Tisserat *et al*., 1979). When nucellus was used as the explant, somatic embryoids were seen produced directly without an intermediary callus phase. Mathews and Litz (1990) reported that somatic embryogenesis can occur directly from the explant without an intermediate callus phase as observed in the nucellus of monoembryonic *Citrus* sp. (Rangan *et al*., 1968), *Malus domestica* (Eichholtz *et al*., 1979) and polyembryonic mango (Litz *et al*., 1982). According to Sharp *et al*., (1980), this direct permissive pattern of somatic embryogenesis is due to the presence of pre-embryogenically determined cells (PEDC) in the explant. They also described indirect somatic embryogenesis as observed in the induced embryogenically determined cells (IEDC). Following the induction of morphogenetically competent cells, development occurs in a permissive, indirect manner according to the pre-determined fate of these cells. It was observed that when embryo mass was used as the explant, somatic embryoids were produced only after undergoing an intermediary callus phase.

The strength of the basal medium influenced the production of somatic embryoids in mango. Half strength MS basal medium was found better for the production of somatic embryoids from the nucellar tissue in Pulichi and Kalluvarikka compared to full strength MS basal medium. The same strength was identified as the best by Litz _et al_. (1982 and 1984) and Litz (1984a).

In IEDC, somatic embryogenesis is usually induced by an auxin. When somatic embryogenesis occur directly from the explant without an intermediate callus phase, plant growth substances are not necessary in the medium, but it is often stimulated by auxins as well as coconut water (Mathews and Litz, 1992). In the present studies, 2,4-D 5.0 mg/l and GA3 5.0 mg/l along with 20.0 per cent coconut water was found the best for the induction of somatic embryoids from nucellar tissue.

Culture conditions influence the response of the explants. When the cultures in the induction medium were kept under darkness 66.67 per cent of the cultures initiated embryo mass from the nucellus whereas none of the cultures responded when kept under light. Litz _et al_. (1992)

maintained the nucellar cultures in the induction medium in darkness at 25°C.

The concentration of inorganic salts in the basal medium influenced the initiation of somatic embryoids. Half strength MS basal medium was identified as the best for the initiation of somatic embryoids. In the present studies, when full strength MS basal medium was used, none of the cultures initiated somatic embryoids. According to Litz et al. (1982) modified MS basal medium consisting of half strength major salts was more effective than full strength MS basal medium for the initiation of somatic embryoids in mango. However, Dewald et al. (1989a) reported that somatic embryo production in mango was the lowest on MS and modified MS basal media. According to them modified B_5 basal medium was optimum for somatic embryogenesis and for the maintenance of embryogenic masses.

For the initiation of somatic embryoids from embryo mass, produced from nucellar tissue, 2,4-D 5.0 mg/l and BA 0.05 mg/l and from embryogenic callus, 2,4-D 10.0 mg/l and BA 0.05 mg/l were ideal.

Light influenced the initiation of somatic embryoids. When kept in darkness, 66.67 per cent of the cultures initiated somatic embryoids whereas when kept under light, only 16.67 per cent initiated somatic embryoids.

According to Litz et al. (1992) control of somatic embryo maturation was the most critical and difficult process in mango regeneration. The growth inhibitor ABA influenced the quality of somatic embryos. In the present studies, various concentrations of ABA (0.50 mg/l to 10.00 mg/l) were tried in the different treatments for the maturation of somatic embryos. The maximum size of somatic embryoids was observed when 4.23 mg/l (16.0 uM) ABA was incorporated in the medium. Litz et al. (1992) reported that 3.0 uM ABA had a significant effect on the appearance of mango somatic embryos. Abscisic acid is a growth retardant which inhibits somatic embryo development beyond the early stages and is useful in suppressing secondary embryoid formation (Ammirato, 1983).

According to Dewald et al. (1989b) sucrose 60.0 g/l was essential as a carbon source for the maturation of somatic embryos. In the present studies, attempts were made

to find out the effect of reduced concentrations of sucrose in the maturation medium. All the embryoids turned brown and finally black when the concentration of sucrose was reduced below 40.0 g/l. This indicates that moderately high levels of sucrose is necessary during the early maturation period. Litz et al. (1992) reported that in order to prevent precocious somatic embryo development, sucrose had to be maintained at moderately high levels during the early maturation period.

The effect of light on the maturation of somatic embryoids was studied. It was observed that the size of the embryoids was not influenced by light. However, Dewald et al. (1989b) found that incubating the maturing cultures in darkness was beneficial.

Poor germination is typical in many embryogenic culture systems. In culture systems in which well developed embryos are formed, poor germination cannot be attributed to abnormal somatic embryo development (Gray, 1990). Mango is a recalcitrant plant with large somatic embryos. Near normal somatic embryo development and germination have been achieved by maintaining the early globular and heart stage somatic

embryos in the darkness on medium with 60.0 g/l sucrose supplemented with 200 ml/l coconut water (Litz, 1988; Dewald et al., 1989b). In the present instance, germination of somatic embryoids was attempted using thirty treatments. In certain treatments, abnormal germination was observed. Growth regulating factors and osmolarity were recognised as important factors for controlling development in somatic embryogenesis (Dewald et al., 1989b). Treatments involving growth regulators like BA, GA, 2iP and NAA and their combinations in various concentrations were attempted for the germination of somatic embryoids. For regulating the osmotic potential, treatments involving polyethylene glycol and increased concentrations of agar and sucrose were tried. These treatments were not very useful. Sodium butyrate was incorporated in the medium in order to support germination of somatic embryoids. Sodium butyrate is known to influence histone deacetylation and the expression of genes that are switched off in the developmental sequence (Perry and Chalkley, 1981). This chemical, however, was not beneficial in the present instance. Somatic embryogenesis is dependent on the optimal concentration and form of nitrogen (Sharp et al., 1980). When the effect of reduced concentrations of ammonium and nitrate ions was studied, it was observed that

reduced concentrations of ammonium and nitrate ions did not influence the germination of somatic embryoids. Ammirato (1983) reported the benefit of using reduced nitrogen in addition to nitrate nitrogen for somatic embryogenesis. Steward and Shantz (1959) suggested that reduced nitrogen could be supplied in the form of complex addenda such as coconut water. In the present studies also treatments with and without coconut water was tried. In both treatments only near normal germination could be achieved. Fresh nucellus from tender mangoes was scooped out and kept in close contact with the somatic embryos in the germination medium with the hope that the fresh nucellus would supply vitamins, minerals and plant growth substances necessary for the germination of somatic embryos. However, the treatment was not very useful.

The culture media and culture conditions for the first two stages of somatic embryogensis, namely induction and initiation, could be standardised with respect to two varieties of mango (Pulichi and Kalluvarikka) in the present investigations. however, proper maturation and normal germination of the embryoids could be successfully induced. Mango somatic embryoids seem to be peculiar in several respects. Size of the embryoids appears to be deciding in

maturation and germination process. A strong genotypic influence is apparent in embryogenesis. The conditions favouring successful maturation and germination of embryoids in the varieties Alphonso, Mundan and Baneshan were not useful for Pulichi and Kalluvarikka optimum duration of embryoid maturation also seems important. The role of ethylene in inhibiting the process of maturation and germination needs special attention. Ethylene inhibitors like silver nitrate, cobalt chloride and amino ethoxy vinyl glycine might be useful for success. Triazole compounds could also be resorted for inducing normal germination of embryoids. A better understanding of the inherent or induced inhibitors during the course of embryogenesis may also be helpful. Considering the success in inducing and initiating somatic embryoids from nucellar tissue in the present investigations and successful reports in certain other varieties, it may not be very difficult to evolve protocols for successful somatic embryogenesis in the varieties Pulichi and Kalluvarikka.

SUMMARY

SUMMARY

Attempts were made to optimise the techniques for *in vitro* somatic embryogenesis in polyembryonic mango varieties. Six varieties namely Kalluvarikka, Pulichi, Vellari, Varikka, Thalimanga and Kilichundan were used. Responses of the varieties as well as explants were studied. Standardisation of basal media, culture medium components and culture conditions during various stages of somatic embryogenesis, namely, induction, initiation, maturation and germination was attempted. The studies were conducted from January 1993 to August 1994 at the Plant Tissue Culture Laboratory, Department of Horticulture, College of Agriculture, Vellayani.

The salient findings of the studies are summarised below :

1. Variations were observed in the response of different polyembryonic mango varieties in the induction of somatic embryoids. The highest per cent cultures initiating somatic embryoids from nucellus was observed for Kalluvarikka (87.50). The least response

was recorded by Vellari (50.00 per cent). Kilichundan did not respond to any of the treatments. Vellari, recorded the maximum (91.66 per cent) cultures initiating somatic embryoids from embryomass and the least response (27.27 per cent) was recorded in Varikka.

2. Three types of explants viz. nucellus, embryo mass and segments of tender leaves were tried. Embryo mass recorded the maximum response (91.66 per cent) in Vellari, while nucellar tissue recorded the maximum response (87.50 per cent) in Kalluvarikka. Leaf segments did not respond to treatments.

3. The maximum response from nucellar tissue (66.67 per cent) was observed when the nucellus along with a portion of the ovule was used for inoculation in a vertical position.

4. Somatic embryoids were produced from the nucellar tissue directly. There was no intermediary callus phase.

5. Three types of response were observed from the embryomass. In the first type callus was produced initially and somatic embryoids were produced when the callus was transferred to the initiation medium. In the second type, multiple embryos were produced which further developed into plantlets. In the third type, the embryo developed directly into a plantlet.

6. Half strength MS basal medium was better for the production of somatic embryoids from nucellar tissue compared to full strength.

7. 2,4-D 5.0 mg/l + GA_3 5.0 mg/l was found to be the best combination of plant growth substances for the induction of somatic embryoids from nucellus.

8. Eight to fourteen days were required for the production of callus and multiple embryos from embryo mass, whereas nucellus took four to seven weeks for the initiation of somatic embryoids.

9. Induction of somatic embryoids was better when the cultures were kept under darkness (66.67 per cent) compared to those under light (16.67 per cent).

10. Half strength MS basal medium with supplements was found ideal for the initiation of somatic embryoids compared to full strength MS basal medium with supplements.

11. Development of somatic embryoids from embryo mass was highest (55.50 per cent) in the medium containing 2,4 - D 5.0 mg/l + BA 0.05 mg/l.

12. For initiating somatic embryoids from embryogenic callus 2,4-D 10.0 mg/l + BA 0.05 mg/l was the best.

13. Initiation of somatic embryoids was observed in 66.67 per cent cultures kept under darkness as against 16.67 per cent under light.

14. The percentage of cultures initiating somatic embryoids remained the same (66.67) when the cultures were kept at room temperature and in culture room temperature (26 ± 2°C).

15. The maturation of somatic embryoids was assessed based on the size of embryoids. Abscisic acid at 16.0 uM in

the maturation media recorded the maximum size (0.5 - 1.5 cm) of embryoids.

16. Light did not influence the size of the embryoids.

17. The embryoids did not survive in liquid medium as well as in the treatments having no activated charcoal.

18. Among the various combinations of 2iP and BA tried for the germination of somatic embryoids, 2iP 1.0 mg/l + BA 1.0 mg/l was found to support near normal development of somatic embryoids. Leafy structures and roots having near normal morphology were produced.

19. None of the cultures survived when GA alone (10.0 mg/l) was supplemented in the germination medium.

20. Reduction in the concentration of ammonium ions or nitrate ions did not favour the germination of somatic embryoids.

21. Stress condition was provided to the embryoids by increasing the concentration of agar and sucrose in

the germination medium. However, these treatments did not support the germination of somatic embryoids.

22. When 10.0 uM sodium butyrate was added along with BA and GA, the size of embryoids increased.

23. Polyethylene glycol (50.0 g/l) along with BA (1.0 mg/l) incorporated in the medium was not very useful for the normal germination of embryoids.

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OPTIMISING *IN VITRO* SOMATIC EMBRYOGENESIS IN POLYEMBRYONIC MANGO (*Mangifera indica* L.) VARIETIES

BY

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ABSTRACT

Studies were conducted to optimise the *in vitro* propagation techniques *via* somatic embryogenesis in polyembryonic mango varieties (Vellari, Kalluvarikka, Thalimanga, Kilichundan, Pulichi and Varikka) of Kerala, during 1993-1994 at the Department of Horticulture, College of Agriculture, Vellayani.

Culture media and conditions could be standardised for the first two stages of somatic embryogenesis, namely induction and initiation. However, attempts for inducing normal maturation and germination of the embryoids were not so successful.

Five out of the six varieties of mango (except Kilichundan) responded to the induction treatments for somatic embryogenesis. Kalluvarikka recorded the highest per cent cultures (87.50) initiating somatic embryoids from the nucellar tissue. Pulichi was observed to initiate the highest per cent cultures (91.66) initiating somatic embryoids from embryo mass cultured.

Somatic embryoids were induced and initiated from nucellus as well as embryo mass. From the nucellus, the embryoids were produced directly, without any intervening callus. The embryo mass gave rise to embryogenic callus, multiple embryos or zygotic embryos.

The somatic embryoids from nucellar tissue were best induced when cultured in darkness on half strength Murashige and Skooge basal medium supplemented with 2,4-D 5.0 mg/l, GA_3 5.0 mg/l, glutamine 400.0 mg/l, sucrose 60.0 g/l, coconut water 200.0 ml/l, agar 6.0 g/l and activated charcoal 2.5 g/l.

Somatic embryoids from nucellar tissue were found to be initiated in 55.50 per cent cultures on half strength Murashige and Skoog basal medium supplemented with 2,4-D 5.0 mg/l, BA 0.05 mg/l, glutamine 400.0 mg/l, casein hydrolysate 500.0 mg/l, sucrose 60.0 g/l, coconut water 200.0 ml/l, agar 6.0 g/l and activated charcoal 2.5 g/l. Darkness was essential for the initiation. Ambient temperature and in the culture room temperature (26°C) were equally effective for the initiation.

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Abscisic acid was tried, among other treatments for inducing proper maturation of the somatic embryoids initiated from nucellar tissue. The maximum size of the embryoids was observed on half strength Murashige and Skoog basal medium supplemented with ABA 16.0 uM, casein hydrolysate 100.0 mg/l, sucrose 40.0 g/l, coconut water 200.0 ml/l, agar 6.0 g/l and activated charcoal 2.5 g/l. Size of the embryoids was not influenced by light.

Attempts for inducing normal germination of the somatic embryoids from the maturation medium were made using treatments involving plant growth substances (BA, 2iP, GA_3 and NAA), factors known to impart osmotic stress (Polyethelene glycol and high concentrations of sucrose and agar), sodium butyrate, known to influence histone deacetylation, and activated charcoal, capable of absorbing inhibitors. However, the treatments were not very useful in inducing normal germination of the embryoids.