Standardisation of *in vitro* techniques for the rapid clonal propagation of mango (*Mangifera indica* L.)

By

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DECLARATION

I hereby declare that this thesis entitled **"Standardisation of *in vitro* techniques for the rapid clonal propagation of mango (*Mangifera indica* L.)"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Vellayani,
27.07.1996.

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CERTIFICATE

Certified that this thesis entitled **"Standardisation of *in vitro* techniques for the rapid clonal propagation of mango (*Mangifera indica* L.)"** is a record of research work done independently by **Smt. G. R. Sulekha** 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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CONTENTS

LIST OF TABLES

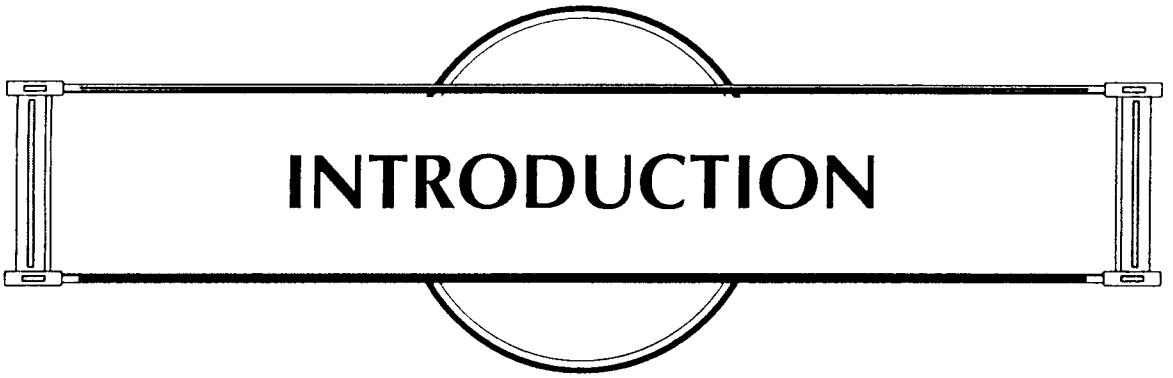
LIST OF FIGURES

LIST OF PLATES

Plate No.	Title
Plate 9.	Somatic embryoids of Neelum in maturation medium (Basal medium containing of B_5 major salts and MS minor salts supplemented with abscisic acid 5.0 mg/l)
Plate 10.	Vellari Manga somatic embryoids in basal medium containing B_5 major salts and MS minor salts supplemented with abscisic acid 4.22 mg/l
Plate 11.	Somatic embryoids of Neelum in liquid maturation media
Plate 12.	A matured somatic embryo of Vellari Manga in germination medium containing B_5 major salts and MS minor salts supplemented with BA 1.0 mg/l
Plate 13.	A germinating somatic embryoid of Vellari Manga in basal medium containing B_5 major salts and MS minor salts supplemented with BA 1.0 mg/l and sodium chloride 0.5 g/l
Plate 14.	Abnormal growth of a germinating somatic embryoid of Vellari Manga when sucrose was reduced in the germination medium
Plate 15.	Developmental stages of somatic embryoids initiated from nucellus of Vellari Manga
Plate 16.	A typical bipolar somatic embryoid of Vellari Manga
Plate 17.	Normal growth of Vellari Manga somatic embryoid in germination medium supplemented with BA 1.0 mg/l, sodium chloride 0.5 g/l and cobalt chloride 10.0 mg/l
Plate 18.	A germinated somatic embryoid of Neelum in medium supplemented with coconut water 200.0 ml/l

LIST OF ABBREVIATIONS

ABA	-	Abscisic acid
AC	-	Activated charcoal
BA	-	6- benzyl aminopurine
CH	-	Casein hydrolysate
CW	-	Coconut water
GA_3	-	Gibberellic acid
IAA	-	Indole -3- acetic acid
IBA	-	Indole -3- butyric acid
IEDCs	-	Induced embryogenic determined cells
NAA	-	Naphthalene acetic acid
PEDCs	-	Pre-embryogenic determined cells
PEG	-	Polyethylene glycol
PGS	-	Plant growth substance
PVP	-	Polyvinyl pyrrolidone
TDZ	-	Thidiazuron
2,4-D	-	2,4 - dichlorophenoxy acetic acid
2 iP	-	2 - isopentenyl adenine



INTRODUCTION

INTRODUCTION

Mango (*Mangifera indica* L.), belonging to the family *Anacardiaceae* is the leading fruit crop of India. At the national level it occupies 42.60 per cent of the area under fruits and contributes to 40.11 per cent of the total fruit production (Singh, 1996). In Kerala, it is the second important fruit crop, occupying an area of 75,000 ha with an annual production of 2,50,000t (FIB, 1996). Mango trees form an integral part of the homesteads of Kerala.

There are over three hundred and fifty mango varieties in South India (Naik, 1963), used for table, juice and / or culinary purposes. The varieties are either monoembryonic or polyembryonic. In polyembryonic varieties, adventive embryos arise from the cells of the nucellus and parts of the ovary wall. These are metriclinal in orgin and are, therefore, highly uniform and give rise to true-to-type plants. Most of the local varieties of mango are polyembryonic. There are ten named polyembryonic varieties in South India (Naik, 1963).

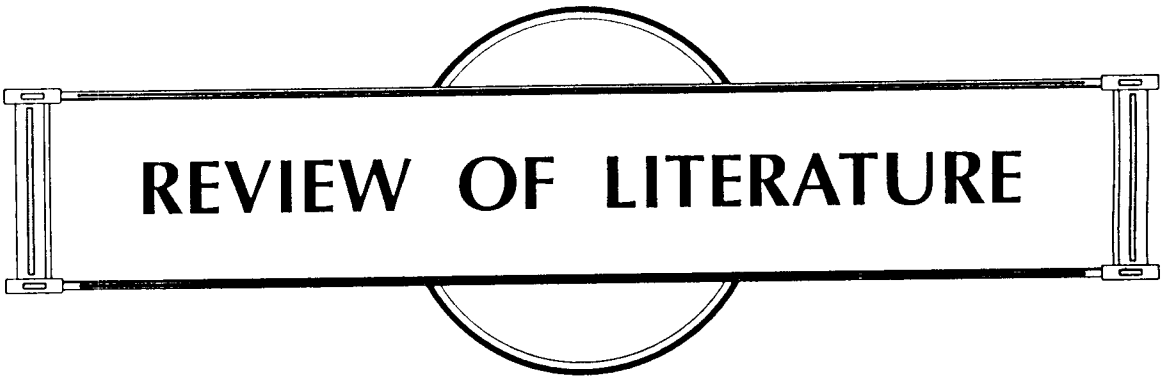
Mango is cross pollinated and is generally propagated by seeds. This leads to variability among the progeny, except in polyembryonic varieties. There is a wide range in the productivity of mango, being 3.5 to 13.5 t/ha/ year (Singh and Pandey, 1993). Conventional vegetative propagation methods

like inarching and stone grafting are quite successful (Singh, 1996). However, the rate of multiplication is not sufficient to meet the demand for superior planting material.

In vitro propagation is useful to ensure high rate of multiplication. It is increasingly being used as an alternative means of asexual propagation of economically important crops. It requires only a limited quantity of plant tissue as the initial explant and is not season dependent. Tissue culture techniques can also aid in the production of disease - free plants and in the cryo-preservation of germplasm. Tissue culture mediated genetic modification serve as complementary to the conventional plant breeding methods.

Evolving a protocol for *in vitro* propagation of mango will help the rapid clonal propagation and early establishment of elite varieties and superior hybrids. Rapid multiplication of scion material and large scale production of clonal root-stocks are also possible. As the root - stock influences the growth and quality of the scion, clonal root stocks would be helpful in ensuring uniform performance of the grafts. Rare and endangered cultivars, especially certain promising local varieties of Kerala, can be multiplied and saved from becoming extinct.

In vitro propagation of mango has been attempted (Litz, 1984a and 1986a; Jaiswal, 1990; Sahijram, 1990; Litz and Gray, 1992; Jana *et al.*, 1994). However, only a few studies relate to Indian varieties. The present study was taken up for developing protocols for the *in vitro* propagation of important mono and polyembryonic varieties of Kerala.



REVIEW OF LITERATURE

Mango is a major fruit crop of Kerala occupying an area of 77,000 ha (FIB, 1996). It belongs to the family *Anacardiaceae*. Only very few reports are available on the *in vitro* propagation of mango. There are no reports on the *in vitro* propagation of monoembryonic and polyembryonic mango varieties of Kerala. This review highlights the research on various aspects of *in vitro* propagation *via* somatic embryogenesis, somatic organogenesis and enhanced release of axillary buds in mango and related tree crops.

A. ROUTES OF *IN VITRO* PROPAGATION

1. SOMATIC EMBRYOGENESIS

Somatic embryogenesis is the development of embryos from somatic cells (Mascarenhas, 1989). Somatic embryos closely resemble their zygotic counter parts in structure and biochemistry (Ammirato, 1987). However, a fundamental difference is 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 (Rendenbough *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.

Sharp *et al.* (1982) reported that somatic embryogenesis initiated either from pre-embryogenic determined cells (PEDC) or from induced embryogenic determined cells (IEDC). Williams and Maheswaran (1986) reported that somatic embryos could arise either from single cells or groups of cells.

Direct embryogenesis proceeds from the pre-embryogenic 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 originate 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), cells that undergo embryo initiation are pre-determined and their subsequent exposure to exogenous growth substances favours embryogenesis.

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.

a. 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 embryoids (Ammirato, 1983).

i. Explant

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

In vitro culture of nucellar explants give rise to somatic embryos and eventually 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 nucellar

tissue 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 Enjarlic (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 filled the embryo sac (Litz *et al.*, 1992). Although monoembryonic and polyembryonic cultivars appeared to respond equally well, somatic embryogenesis was observed to be cultivar dependent (Litz *et al.*, 1991). Attempts to achieve regeneration from other types of explants, including shoot tips, leaves and stem have been unsuccessful (Litz, 1993).

Jaiswal (1990) could induce *in vitro* somatic embryogenesis from nucellus of some Indian mango varieties, namely Langra, Desheri and Bombay Green. Maximum response was in Langra, while the minimum was observed in Bombay Green.

Jana *et al.* (1994) evolved techniques for rapid production of somatic embryos with normal developmental morphology from the nucellar tissue of three monoembryonic varieties namely Alphonso, Mundan and Beneshan.

Through an intermediary callus, *Ranunculus sceleratus* floral primiordia at the sepal initiation stage and flower buds with microscopore

containing anthers have given rise to asexual embryos (Konar and Nataraja, 1965 and 1969). Similarly the callus from flower segments, including petals, sepals, anthers, carpels and involucre bracts of *R. sceleratus* (Nataraja and Konar 1970) and *Pterotheca falconeri* (Mehra and Mehra, 1974) has been reported to generate embryos *in vitro*. Mitra and Chaturvedi (1972) could obtain embryos from callus that originated in unpollinated ovaries of *Citrus aurantifolia* and *C. sinensis*. In *Cucurbita pepo* formation of asexual embryos from callus produced from pericarp was observed (Schroeder, 1965). 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.

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

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

ii. Basal medium

Induction of somatic embryos

According to Christianson (1985), an event must initially occur that involves a change in the determination of 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 White's medium (White, 1963) to the more concentrated formulations of Gamborg *et al.* (1968), Murashige and Skoog (1962) and Schenk and Hildebrandt (1972).

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).

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 *et al.*, 1988).

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).

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 was cultured on medium containing modified B_5 major salts and MS minor salts and organics.

Initiation of somatic embryos

When tissues are transferred from an auxin containing medium to an auxin free medium having nitrogenous compounds like amino acids, embryogenesis will be 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 organic 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 than, for the production of somatic embryos from nucellar tissue in mango.

Maturation of somatic embryos

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 B_5 major salts, MS minor and organics.

Germination of somatic embryos

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 was 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 somatic embryo in mango. Modified B_5 medium consisting half strength major salt formulation supplemented with coconut water and casein hydrolysate resulted in significantly higher germination rate.

iii. Plant growth substances

Induction of somatic embryos

Generally the presence of an auxin in the medium is essential for somatic embryogenesis. 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 2,4-D 1-2 mg/l (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 was not dependent on 2,4-D as a stimulus. However, Dewald *et al.* (1989a) incorporated 4.5 µM 2,4-D to modified MS medium for the induction of somatic embryos in mango.

Somatic embryogenesis could be induced using MS medium supplemented with 2,4-D 5.0 mg/l, GA_3 5.0 mg/l, sucrose 60.0 g/l and activated charcoal 2.5 g/l (Jana *et al.*, 1994).

Initiation of somatic embryos

Litz *et al.* (1982) could initiate somatic embryoids on medium containing coconut water, BA or no growth regulators. Litz *et al.* (1984) reported that efficient somatic embryogenesis occurred when nucellar callus was transferred to liquid medium without 2,4-D.

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

Maturation of somatic embryos

Litz *et al.* (1983), Litz and Gray (1992) and Razdan (1993) reported that maturation of mango somatic embryos occurred 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 embryos, 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 μM).

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

Maturation of somatic embryo was observed in a reduced concentration of MS basal medium supplemented with abscisic acid 1.0 mg/l, coconut water 20 per cent (v/v), casein hydrolysate 100 mg/l, sucrose 40.0 g/l and activated charcoal 2.5 g/l (Jana *et al.*, 1994).

Germination of somatic embryos

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

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

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

Mathews and Litz (1990) studied kanamycin sensitivity of mango somatic embryos.

Jana *et al.* (1994) reported that BA 5.0 mg/l favoured normal germination of the mango somatic embryos. Plantlet development was completed in 75-80 days.

iv. Other supplements

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). Proliferation of somatic embryos occurred when the nucellar tissue was subcultured into 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 embryos 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 source of reduced nitrogen.

Solidifying agent (agar)

Litz *et al.* (1982) reported that efficient somatic embryogenesis occurred 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.

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 inorder to prevent precocious germination of somatic embryoids.

Agents countering polyphenol interference

Charcoal can assist in adsorbing toxic substances which may be present in media as a result of autoclaving, or produced by cultured tissues. Fridborg *et al.* (1978) showed that activated charcoal could adsorb some phenols commonly produced by wounded tissues. In date palm, activated charcoal effectively prevented browning of explants from mature trees (Tisserat, 1982). Hu and Wang (1983) found that treating the explants with polyvinyl pyrrolidone, washing them with sterile water and inclusion of activated charcoal in the medium reduced the oxidation of polyphenols because of the absorption of the oxidation products by these chemicals.

Inclusion of charcoal and PVP in the culture media was not found to be helpful in absorbing phenolic substances in clove cultures. Alternatively, quick transfer of explants on to fresh media at the rate of four to six transfers a day was found useful (Priyadarshan *et al.*, 1989).

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

v. 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 subcultured 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 subcultured 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 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 occurred when somatic embryos were transferred to light.

b. Histological characterization of somatic embryoids

Sagare *et al.* (1995) studied the histology of somatic embryo initiation and development in chick pea (*Cicer arietinum* L.) from different explants. The studies revealed that somatic embryos arose indirectly with an intervening callus phase from immature cotyledons and immature embryo axis, directly from mature embryo axis and by both routes from young leaflets.

In *Cornus florida* (flowering dogwood) the somatic embryos examined histologically suggested that direct somatic embryos were derived from a single cell (Trigiano *et al.*, 1989). Many of the embryos that had developed beyond the globular stage were fused in pairs along the entire length of the hypocotyl - radicle axis. Each embryo had a closed vascular system, a well developed root meristem and a poorly organised shoot meristem.

In Celery, Yong-Hwan Kim (1989) conducted histological studies of embryogenic competent callus and indicated that they were isodiametric and easily distinguished from non embryogenic cells by their darkly stained nuclei and nucleoli. Subsequent division of terminal cells formed a multi-celled globular embryo. The suspensor appeared to degenerate and was not observed in mature somatic embryos. A prominent epidermal layer, formed on the globular embryo, was soon followed by vascular strand development at the heart shaped stage. Darkly stained cells extending from the base of the embryo to the cotyledonary regions represent the initiation of vascular strands. Heart shaped embryos developed into torpedo-shaped embryos showing well-developed cotyledons. Secondary embryos occurred with high frequency on the cotyledons and the hypocotyl which inhibited growth of the apical meristem and limited the conversion of primary somatic embryos into normal plantlets.

In Robusta coffee, histological examination revealed that each embryoid had a bipolar structure making it capable of developing into a complete plant (Sumaryono and Tahardi, 1993).

2. SOMATIC ORGANOGENESIS

Generally, a high concentration of auxin and a low concentration of cytokinin in the medium promote abundant cell proliferation with the formation of callus (Skoog and Miller, 1957). On the other hand, low auxin and high cytokinin concentration in the medium result in the induction of shoot morphogenesis. Auxin, alone or in combination with a very low concentration of cytokinin, is important in the induction of root primordia. Somatic organogenesis can be direct or callus mediated (Evans *et al.*, 1981) and is useful in inducing genetic variability or to recover pre-existing natural genetic variability.

Rao *et al.* (1981) observed callus production when shoot tip explants from mature jack trees were cultured on MS medium containing IAA (0.1 ppm) alone or in combination with BA (2.0 ppm). However, the callus failed to differentiate into shoot/root. Callusing could be obtained from inflorescence primordia also. Callus induced from hypocotyl segments differentiated into shoot buds when cultured on MS medium containing IBA or NAA (1.0 ppm) and BA (2.0 ppm).

In mango, Rao *et al.* (1982) reported that the induction of callus from cotyledon tissue and root regeneration on MS medium containing NAA (5.0 mg/l), Kinetin (2.5-5.0 mg/l) and coconut water (150 ml/l). Callus induction occurred two weeks after inoculation. Growth was found to be better in the cultures maintained in the dark. The callus was dark and compact. Distinct

lobes were formed in one month old cultures. Root initials developed during the second week were positively geotropic, somewhat flat and tapering at the top. Root caps were present, but root hairs were absent. Shoot development was not observed.

Morel (1945) was the first to report on the production of callus from stem explants of grapes. Production of callus cultures from explants of stem tissues (Staudt *et al.*, 1972) and immature berries (Hawker *et al.*, 1973) has also been reported.

Callus production from several plant parts of apple has been reported (Mu *et al.*, 1977; Schneider *et al.*, 1978; Fukui *et al.*, 1981). Differentiation of roots and leaves from callus was observed by Mu *et al.* (1977). Chen *et al.* (1979) obtained plantlets regenerated from the callus of explants from M.9 root stocks. Callus was induced from stem segments of MS medium containing 2.2 µM BA, 10.7 µM NAA and 100 mg/l casein hydrolysate. When the callus was transferred to a similar medium without NAA, shoots differentiated. These shoots were then rooted *in vitro* on a medium containing IBA. Differentiation of callus (derived from seedling tissues of Golden Delicious) into leaves, shoots and roots was reported by Mehra and Sachdeva (1979) and Liu *et al.* (1981).

Nair *et al.* (1984) succeeded in inducing adventitious buds from excised leaf explants of custard apple (*Annona squamosa* L.) seedlings on medium containing 0.5 mg/l BA and 0.5 mg/l Kinetin. Various auxins in

combination with the above medium produced callusing of the explants. Maximum number of shoots were obtained using the leaf base with petiole at a temperature of 27°C and a light intensity of 100 lux. Roots were initiated erratically when individual shoots were treated with an auxin and then transferred to an auxin free medium.

Omusa *et al.* (1987) reported the production of callus from leaf explants of pomegranate (*Punica granatum* L.) which in turn regenerated to adventitious shoots after twelve months of culture on MS medium supplemented with 0.5-10 µM BA and 0.05-5 µM NAA.

Callus was induced from petal explants of *Camellia japonica*, cultured on Anderson's Woody plant medium or MS medium, supplemented with Kinetin and 2,4-D (Shults and Haldeman, 1995).

Nehra *et al.* (1990) studied plant regeneration using immature leaf explants taken from *in vitro* shoots and green house grown plants of the strawberry cv. Red Coat. Both types of explants formed callus and multiple shoots at various frequencies in the presence of 5 µM each of BA and 2,4-D in 24 weeks.

3. ENHANCED RELEASE OF AXILLARY BUDS

Morel (1960) was the pioneer in applying shoot tip culture as a clonal multiplication tool. He was successful in cloning the orchid, *Cymbidium* and since then, *in vitro* clonal multiplication gained momentum. Many herbaceous

horticultural species have been successfully multiplied using this technique. The success has been partially due to the weak apical dominance and strong root regenerating capacities of many herbaceous plants (Hu and Wang, 1983).

In 'axillary shoot proliferation' cytokinin is utilized to overcome the apical dominance of shoots and to enhance the branching of lateral buds from leaf axils. This enhanced release of axillary buds with cytokinins was discovered by Wickson and Thimann (1958).

There is no report on the application of shoot tip culture for the micro propagation of mango.

In a tree crop like guava, Amin and Jaiswal (1987) reported that axillary buds were induced *in vitro*. BA (4.5 µM) without any auxin and gibberellin gave the best rate of shoot multiplication.

Hosier *et al.* (1985) achieved shoot proliferation of ligenberry from shoot tips cultured on a low salt medium supplemented with 2iP.

Multiple shoots of Chinese chestnut, from axillary buds of juvenile shoots, were best produced with BA at 0.44 µM. Higher concentration of BA (4.44 µM and 44.4 µM) inhibited buds from sprouting and promoted callus growth (Qi-guang *et al.*, 1986).

Aradhana *et al.* (1989) could develop axillary buds excised from *in vitro* grown seedlings of *Acacia auriculiformis* on Gamborg's (B_5)

basal medium supplemented with coconut milk (5 to 10 per cent) and BA (10^{-6}M).

According to Sugiura *et al.* (1986) dormant bud explants taken from mature trees of Japanese persimmon cv. Hiratanenashi were established successfully on modified MS medium with nitrate reduced to half strength of MS or WPM, both supplemented with BA 2.2 mM.

Cultures were established from shoot apices (1-2 mm) initiated on a medium composed from Knop's macro elements and MS micro elements in the presence of 0.5 mgl^{-1} BA.

Rajmohan (1985) reported MS medium supplemented with GA 1.0 ppm and activated charcoal 1.0 per cent was suitable for the establishment of jack shoot apices.

B. FACTORS INFLUENCING SUCCESS OF *IN VITRO* PROPAGATION

1. EXPLANT

a. Type

Leaf explants and explants formed from the apical part of the embryo were reported to be the most suitable material for *in vitro* propagation in date palm (Falcone and Marcheschi, 1988).

King and Morehart (1988) studied the influence of various explants *viz.*, shoot tip, nodal or internodal sections on morphogenesis in Osage-orange. It was observed that shoots proliferated from both shoot-tip and nodal sections, but not from internodes.

Rao *et al.* (1988) observed that fresh cotyledons followed by leaves were the best source of explants for induction and growth of callus in *Azadiracta indica.*

The stem explants were more responsive than the petiole explants with respect to callus induction, growth and differentiation in mature leguminous trees *viz.*, *Albizzia lebbeck, Cassia fistula* and *C. siamea* (Gharyal and Maheswari, 1980).

b. Surface disinfection

Explants are usually cut to a size larger than that of the final one, surface sterilized and trimmed to the final size before being transferred to the culture vessel (Hussey, 1979). The most commonly used surface sterilant is sodium hypochlorite. For softer tissues, a dilution to lower strength may be needed, but anything below 0.5 per cent may prove ineffective (Sommer and Caldas, 1981). Concentrations ranging from 1.0 to 10 per cent (Kuo and Tasay, 1977) have been used. Generally, a drop of detergent is added to the surface sterilant. Mèrcuric chloride is another commonly used surface sterilant.

2. CULTURAL MEDIUM

a. Basal medium

Wide variety of media have been reported. The choice depends on the plant species and intended use of the culture. The Murashige - Skoog (1962) medium, characterized by high concentration of mineral salts has been widely used for general plant tissue culture and specifically for morphogenesis and plant regeneration (Murashige, 1974).

b. Growth substances

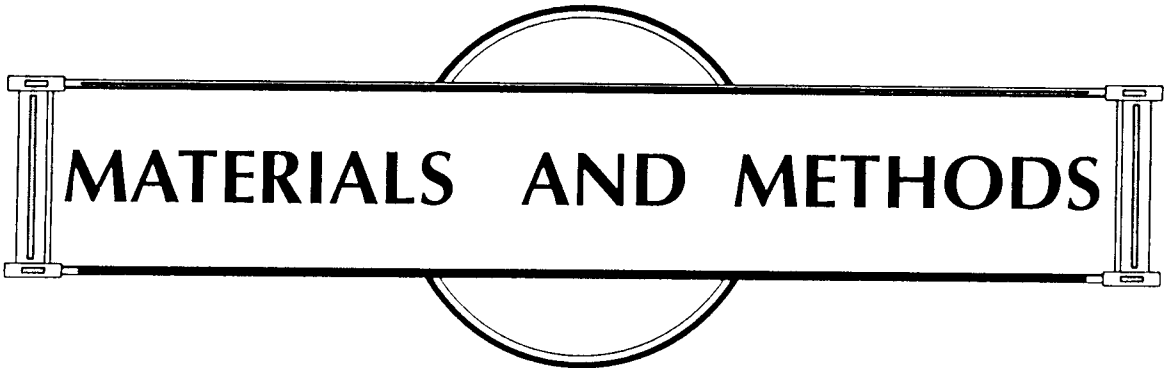
Auxins and cytokinins are inevitable components of plant tissue culture media. BA has been the most effective cytokinin for meristem, shoot tip and bud cultures, followed by Kinetin (Murashige, 1974). Several scientists have reported 2-iso pentenyl adenine as the best cytokinin for multiple shoot induction and callus regeneration (Eltinger and Preece, 1985; Voyatzi and Voyatziz, 1989). Lo *et al.* (1980) reported that high cytokinin content was deleterious to the initiation and elongation of roots of both monocotyledonous and dicotyledonous plants. Young actively growing plant parts have been described as an active site for auxin biosynthesis.

3. POLYPHENOL OXIDATION

Polyphenolic compounds are present in many plants. When tissues of such plants are injured during dissection, phenolic compounds will be oxidised by polyphenol oxidases and the tissues will turn brown or black. The

oxidation products are known to inhibit enzyme activity, kill the explant and darken the tissues and culture media. Such phenomena are serious set backs in the establishment of primary cultures, especially in woody plants (Hu and Wang, 1983). Some of the procedures used to overcome the problem are addition of anti-oxidants to the culture medium, pre- soaking of the explants in an anti-oxidant before inoculation, incubating the primary cultures in reduced light or darkness for an initial period and frequent subculturing of the explants into fresh medium. Ichihashi and Kako (1977) could control the browning of Cattleya shoot tips by the addition of an anti-oxidant into a liquid medium in stationary conditions. Stevenson and Harris (1980) reported reduction in the discoloration of agar medium with PVP-10 (0.01%) in Fuchsia shoot tip cultures. Gupta *et al.* (1981) observed during the initial shoot tip culture of teak that the medium turned black and all the explants died. In order to reduce the blackening, the explants were agitated for 45 minutes in a solution of different anti-browning agents in 0.058 M sucrose. The chemicals tested were H_2O_2 (5%), ascorbic acid (0.28 mM), soluble PVP (0.7%) and polyclar AT (0.7%). Blackening was reduced by all the treatments. Multiple shoots, nevertheless, were formed only from the explants treated with polyclar AT, an insoluble PVP. A less than 4 per cent browning of the garlic meristem dome explants was obtained by Wang and Huang (1974) when the incubation light intensity during the first month was 150 lux. In order to reduce the accumulation of phenolic oxidates, the initial incubation period (1 to 6 weeks) has been carried out in darkness (Adams *et al.*, 1979; Mc Comb and Newton, 1981; Monaco *et al.*, 1977). The tissue and medium discolouration in

thornless blackberry cultures were effectively controlled by Broome and Zimmerman (1978) when the shoot tip explants were transferred to fresh medium one or two days after initial culturing. Litz (1986a) opined that the use of the reducing agents like ascorbic acid and frequent subculturing were necessary to counteract polyphenol interference in mango somatic embryogenesis.



MATERIALS AND METHODS

MATERIALS AND METHODS

Investigations on standardisation of techniques for the rapid *in vitro* clonal propagation of mango (*Mangifera indica* L.) were carried out at the Plant Tissue Culture Laboratory, Department of Horticulture, College of Agriculture, Vellayani, during 1992-96.

Standardisation of techniques for the *in vitro* clonal propagation of mango was done *via.* somatic embryogenesis, somatic organogenesis and enhanced release of axillary buds.

The materials used and the methods tried for the various stages of *in vitro* culture are described in this chapter.

A. VARIETIES

Six monoembryonic mango varieties, namely, Neelum, Bangalora, Prior, Banganpalli, Mundappa, Mulgoa and six polyembryonic varieties, namely, Olour, Kurukkan, Vellayani Varikka, Vellari Manga, Panchara Varikka and Kilichundan were screened for their initial response. Two varieties, one each from the two groups, showing the best response, were selected for further studies.

B. EXPLANTS

Nucellus and embryo mass from the ovules of tender mango fruits (about 30-45 days after fertilization), floral parts (immature flower parts and segments of inflorescence) and segments of tender leaves (15-20 days old) 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 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 asexual seedlings along with the zygotic one. Often the zygotic embryo gets degenerated, resulting in the production of asexual 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.

3. Leaf segments

Segments of tender leaves from new flushes were used for inoculation. Leaf segments, along with midrib (pieces of one and half to two cm dimension) were used for inoculation on the medium.

4. Floral parts

Immature flower buds along with the stalks were cut into pieces of two to three cm and used as explants.

C. COLLECTION AND PREPARATION OF EXPLANTS

Tender fruits (thirty to forty five days after fertilization) were collected from healthy trees of monoembryonic and polyembryonic mango varieties (Plate 1). The size of the fruits varied among the varieties. Girth in the middle of the fruits ranged from 8.0 to 8.3cm in Neelum (monoembryonic), and 7.5 to 7.8cm in Vellari Manga (polyembryonic).

D. SURFACE STERILIZATION

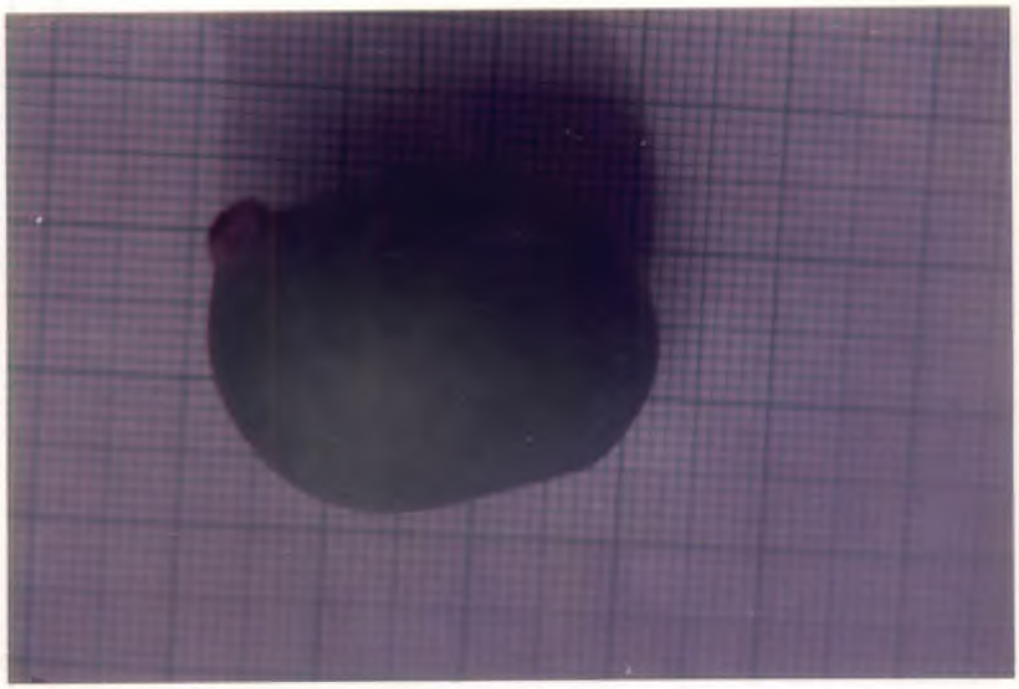
The fruits (after removing pedicel) and the leaves (after removing petioles) and inflorescence were washed thoroughly in tap water with a few

Plate 1

Tender fruit (35 days old) of the mango variety Vellari Manga

Plate 2

Tender mango fruit of Vellari Manga dissected out exposing the nucellus and embryo mass (explants)



drops of the wetting agent 'Labolene' followed by washing with double glass distilled water.

Surface-sterilization of the plant materials was carried out inside a laminar air flow chamber. The fruits, after initial cleaning, were transferred to a sterile beaker and surface-sterilized using 70 per cent ethyl alcohol for ten 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 min. This was followed by washing four to five times with sterile double glass distilled water.

The tender leaves were surface-sterilized in a sterile beaker using sodium hypochlorite (0.8 per cent) for 15 min and then washed with sterile double glass distilled water four to five times.

E. INOCULATION AND INCUBATION

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

The tools (needles, blades, scalpels, forceps etc.) and the glasswares 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 min.

The surface-sterilized fruits were cut open using scalpel, without injuring the ovule. The ovule was taken out with forceps and cut

longitudinally into two equal halves with dissection blade fitted on handle. Nucellus and embryo mass were separately scooped out from the ovules. The cotton plugs of the test tubes / Erlenmeyer flasks were removed and the rim was flamed. The nucellus and embryo mass were then inoculated on the medium. The rim of the culture vessels was again flamed and closed with cotton plugs. When disposable sterile plastic petri dishes were used, the sides were sealed using cling film, after inoculating the explants and closing the dishes.

The cultures were incubated either in light or darkness, as per the treatments.

F. MEDIA

The basal media used for the study were MS (Murashige and Skoog, 1962), modified MS, B5 (Gamborg *et al.*, 1968) and SH (Schenk and Hildebrandt, 1972) media. The chemicals used for the preparation of the culture media were of analytical grade from British Drug House, Bombay; Sisco Research Laboratory, Bombay; Merck, Bombay and Sigma, U.S.A.

Standard procedures were followed for the preparation of MS, B5 and SH media (Thorpe, 1980). Stock solutions of the 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 conditions (4°C).

The glasswares used for the preparation of the media were washed with diluted 'Labolene' and rinsed with double glass distilled water. Specific quantities of the stock solutions were pipetted out into 1000 ml beakers. Sucrose, glutamine and myo-inositol were added fresh and dissolved. Coconut water, collected from freshly harvested tender coconuts (eight-month 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 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 solid medium) was then added to the medium. Activated charcoal, when used in the medium, was also added at this stage. 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 the agar melted. The medium was poured into pre-sterilized culture vessels. Corning brand test tubes (25mm x 150 mm) and Erlenmeyer flasks (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°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. The culture medium was pre-sterilized in suitable Erlenmeyer flasks (by autoclaving at 121°C and 1.06 kg/cm^2 pressure for 20 minutes). The petri dishes were opened inside a

laminar air flow chamber and the 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 tight with cling film. Agar was not used for liquid media.

G. SOMATIC EMBRYOGENESIS

1. Induction of somatic embryoids

a. Treatments

The treatments involved combinations of plant growth substances, activated charcoal, agar, sucrose, glutamine and aminoacids. The treatments were replicated 20 to 31 times.

i. Plant growth substances

Thirty treatments involving 2,4-D, BA and GA_3 in combinations and alone with control were tried for inducing somatic embryogenesis (Table 1).

ii. Sucrose

Cultures were inoculated on a medium containing half strength MS basal medium supplemented with sucrose 30.0, 40.0, 50.0 and 60.0 g/l. These treatments were compared for their effects on the induction of somatic embryogenesis.

Table 1. Plant growth substances tried for inducing somatic embryogenesis

Culture medium - Half strength MS basal medium + Sucrose 60.0g/l + glutamine 400.0mg/l + Coconut water 200.0ml/l + agar 5.0g/l + Activated charcoal 2.5g/l

Treatments	Plant growth substances (mg/l)
T_1	2,4-D 2.0 + GA_3 5.0
T_2	2,4-D 4.0 + GA_3 5.0
T_3	2,4-D 5.0 + GA_3 5.0
T_4	2,4-D 8.0 + GA_3 5.0
T_5	2,4-D 16.0 + GA_3 5.0
T_6	2,4-D 2.0 + GA_3 10.0
T_7	2,4-D 4.0 + GA_3 10.0
T_8	2,4-D 5.0 + GA_3 10.0
T_9	2,4-D 8.0 + GA_3 10.0
T_{10}	2,4-D 16.0 + GA_3 10.0
T_{11}	2,4-D 2.0 + GA_3 20.0
T_{12}	2,4-D 4.0 + GA_3 20.0
T_{13}	2,4-D 5.0 + GA_3 20.0
T_{14}	2,4-D 8.0 + GA_3 20.0
T_{15}	2,4-D 16.0 + GA_3 20.0
T_{16}	BA 0.5 + GA_3 5.0
T_{17}	BA 1.0 + GA_3 5.0
T_{18}	BA 2.0 + GA_3 5.0
T_{19}	BA 0.5 + GA_3 10.0
T_{20}	BA 1.0 + GA_3 10.0
T_{21}	BA 2.0 + GA_3 10.0
T_{22}	BA 0.5 + GA_3 20.0
T_{23}	BA 1.0 + GA_3 20.0
T_{24}	BA 2.0 + GA_3 20.0
T_{25}	2,4-D 5.0 + BA 0.5 + GA_3 5.0
T_{26}	2,4-D 5.0 + BA 1.0 + GA_3 5.0
T_{27}	2,4-D 5.0 + BA 2.0 + GA_3 5.0
T_{28}	2,4-D 5.0 + BA 0 + GA_3 0
T_{29}	2,4-D 0 + BA 0 + GA_3 5.0
T_{30}	2,4-D 0 + BA 2.0 + GA_3 0
T_{31}	Control (No PGS)

iii. Agar (solidifying agent)

Different levels of agar (4.5, 5.0, 6.0 and 7.0 g/l) were tried for the induction of somatic embryogenesis.

iv. Amino acids

Different types of amino acids (L-Glutamine, L-Asparagine and L-Arginine) were tried for the induction of somatic embryogenesis. Different levels of glutamine (400.0, 500.0 and 600.0 mg/l) were also tried.

v. Coconut water

Coconut water at 0, 150.0, 200.0 and 250.0 ml/l in the induction media was tried to study its effect on somatic embryogenesis.

vi. Activated charcoal, Polyvinyl pyrrolidone (PVP), Ascorbic acid and Citric acid

Cultures were inoculated on a medium containing half strength MS basal medium supplemented with activated charcoal (0.5, 1.5, 2.5 and 5.0 g/l and PVP (0.7, 0.8 and 1.0 per cent), ascorbic acid (75.0, 150.0 and 300.0 mg/l) and citric acid (75.0, 150.0 and 300.0 mg/l). These treatments were compared for their effects on the induction of somatic embryogenesis.

Observations were recorded on the number of cultures initiating callus from nucellus as well as from embryo mass. Callus index (CI) was computed

by multiplying per cent cultures initiating callus with growth score (G). Growth of the callus was assessed based on visual rating (with score 1.0 to the smallest and score 4.0 to the largest). The mean score was expressed as growth score, G.

b. Basal media

Studies were conducted to find out the effect of full as well as half strength of major salts of MS and B5 basal media with supplements. The effect of SH medium was also tested. The treatments were replicated 20 to 36 times.

Observations were made on the number of cultures initiating callus, from nucellus and embryo mass. Callus index was also computed.

c. Culture conditions

Studies were conducted to find out the effect of light on the induction of somatic embryogenesis. Darkness was provided by placing the cultures in culture racks covered with black polythene sheets and by covering the culture vessels with aluminium foil. Light (photo period 16 h) was provided by fluorescent tubes, giving an intensity of 3000 lux. The influence of temperature on induction of embryogenic callus was studied by keeping the cultures inside the culture room (26 $\pm$ 2°C) and outside the culture room at ambient temperature (32 $\pm$ 2°C).

Observations were recorded on the number of cultures initiating callus from nucellus.

d. Frequency of subculture

Frequency of subculture was studied by subculturing at an interval of 5, 10 and 15 days in the medium of same composition.

Observations were made on the number of cultures surviving and number of cultures initiating callus.

2. Initiation of somatic embryoids

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

a. Treatments

Treatments involving combinations of plant growth regulators, varying levels of sucrose, casein hydrolysate, coconut water and different types of aminoacids were tried for the initiation of somatic embryoids.

i. Plant growth substances

Twenty treatments involving 2,4-dichlorophenoxy acetic acid (2,4-D), 6-benzyl adenine (BA) and gibberellic acid (GA_3) in combinations and alone were tried to study their effects on the initiation of somatic embryoids (Table 2).

Table 2. Plant growth substances tried for initiatingsomatic embryoids

Culture medium - Half strength MS basal medium + sucrose 60.0 g/l + glutamine 400.0mg/l + CW 200.0ml/l + agar 6.0g/l + AC 2.5g/l + CH 500.0mg/l

Treatments	Plant growth substances (mg/l)
T_1	2,4-D 1.25 + GA_3 5.00 + BA 0.05
T_2	2,4-D 0.50 + GA_3 5.00 + BA 0.05
T_3	2,4-D 1.00 + GA_3 5.00 + BA 0.05
T_4	2,4-D 2.00 + GA_3 5.00 + BA 0.05
T_5	2,4-D 5.00 + GA_3 5.00 + BA 0.05
T_6	2,4-D 1.25 + GA_3 10.00 + BA 0.05
T_7	2,4-D 0.50 + GA_3 10.00 + BA 0.05
T_8	2,4-D 1.00 + GA_3 10.00 + BA 0.05
T_9	2,4-D 2.00 + GA_3 10.00 + BA 0.05
T_{10}	2,4-D 5.00 + GA_3 10.00 + BA 0.05
T_{11}	2,4-D 1.25 + GA_3 5.00 + BA 1.00
T_{12}	2,4-D 0.50 + GA_3 5.00 + BA 1.00
T_{13}	2,4-D 1.00 + GA_3 5.00 + BA 1.00
T_{14}	2,4-D 2.00 + GA_3 5.00 + BA 1.00
T_{15}	2,4-D 5.00 + GA_3 5.00 + BA 1.00
T_{16}	2,4-D 0.50 + GA_3 0 + BA 1.00
T_{17}	2,4-D 0 + GA_3 5.0 + BA 0
T_{18}	2,4-D 5.00 + GA_3 0 + BA 0
T_{19}	2,4-D 5.00 + GA_3 1.00 + BA 0
T_{20}	Control (No PGS)

ii. Sucrose

Different levels of sucrose (40.0, 50.0 and 60.0 g/l) were tried for initiating somatic embryoids from nucellus and embryo mass of Neelum and Vellari Manga.

iii. Casein hydrolysate

Casein hydrolysate (400.0, 500.0 and 600.0 mg/l) were supplemented in the basal medium to study their effects on the initiation of somatic embryoids.

iv. Glutamine

Initial cultures were transferred to half strength MS basal medium with glutamine 400.0, 500.0 and 600.0 mg/l to observe their effects on the initiation of somatic embryoids.

v. Agar

Different levels of agar (4.5, 5.0, 5.5, 6.0 and 6.5 g/l) were tried to study their effects on the initiation of somatic embryoids.

vi. Coconut water

Coconut water at 0, 150.0, 200.0 and 250.0 ml/l were used to observe their effects on the initiation of somatic embryoids.

vii. Activated charcoal

Different quantities of activated charcoal (1.5, 2.5 and 5.0 g/l) were tried in the initiation media.

Observations on the number of cultures initiating somatic embryoids from nucellus as well as from embryo mass and number of embryoids produced per culture were recorded, three weeks after incubation.

b. Basal media

Different basal media such as MS (full strength and half strength major salts), B_5 and SH media were tried. Observations on the number of cultures initiating somatic embryoids and number of embryoids per culture were recorded.

c. 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 tube lights at an intensity of 3000 lux. Inorder 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 ($26 \pm 2^{o}C$) and outside the culture room at ambient temperature ($32 \pm 2^{o}C$).

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

d. Frequency of subculture

The effect of frequency of subculture was studied by subculturing at an interval of 5, 10 and 15 days on to a fresh medium of same composition. A control treatment with no subculturing was also included.

Observations were made on the number of surviving cultures, number of cultures initiating somatic embryoids and number of embryoids per culture.

3. Maturation of somatic embryoids

The initiated somatic embryoids were transferred to the maturation medium.

a. Treatments

i. Abscisic acid

The somatic embryoids from the initiation medium were transferred to the maturation medium containing different concentrations of abscisic acid (0.50, 1.00, 1.50, 2.00, 3.17, 4.22, 5.00, 8.44 and 10.56 mg/l). The treatments were replicated fifteen to twenty four times.

The effects of various treatments were observed for a period of eight weeks. Observations were made on the number of surviving cultures, size (length in cm) and colour of the somatic embryoids.

ii. Sucrose

Cultures initiating somatic embryoids were transferred to a medium containing B_5 major nutrients + MS minor nutrients, supplemented with sucrose 15.0, 20.0, 30.0, 40.0, 50.0 and 60.0 g/l. These treatments were compared for their effects on the maturation of somatic embryoids.

iii. Agar (solidifying agent)

Cultures initiating somatic embryoids were transferred to a medium containing B_5 major nutrients + MS minor nutrients supplemented with agar 4.5, 5.0, 5.5, 6.0 and 7.0 g/l. These treatments were also compared for their effects on the maturation of somatic embryoids.

iv. Casein hydrolysate (CH)

Cultures initiating somatic embryoids were transferred to a medium containing B_5 major nutrients + MS minor nutrients supplemented with casein hydrolysate 100.0 150.0, 200.0, 300.0, 400.0 and 500.0 mg/l. Observations were made on the number of surviving cultures survived and size and colour of embryoids.

The effects of various treatments were observed for a period of eight weeks. Observations were made on the number of surviving cultures, size (length in cm) and colour of the somatic embryoids.

ii. Sucrose

Cultures initiating somatic embryoids were transferred to a medium containing B_5 major nutrients + MS minor nutrients, supplemented with sucrose 15.0, 20.0, 30.0, 40.0, 50.0 and 60.0 g/l. These treatments were compared for their effects on the maturation of somatic embryoids.

iii. Agar (solidifying agent)

Cultures initiating somatic embryoids were transferred to a medium containing B_5 major nutrients + MS minor nutrients supplemented with agar 4.5, 5.0, 5.5, 6.0 and 7.0 g/l. These treatments were also compared for their effects on the maturation of somatic embryoids.

iv. Casein hydrolysate (CH)

Cultures initiating somatic embryoids were transferred to a medium containing B_5 major nutrients + MS minor nutrients supplemented with casein hydrolysate 100.0 150.0, 200.0, 300.0, 400.0 and 500.0 mg/l. Observations were made on the number of surviving cultures survived and size and colour of embryoids.

v. Coconut water (CW)

Cultures initiating somatic embryoids were transferred to half strength MS basal medium containing CW 150.0, 200.0 and 250.0 ml/l.

vi. Agents countering polyphenol interference

Different levels of activated charcoal (0.10, 0.15, 0.20 and 0.25 per cent) and PVP (0.7, 0.8 and 1.0 per cent) were also included in the medium to observe their effects on the maturation of somatic embryoids.

vii. Polyethylene glycol (PEG)

Cultures initiating somatic embryoids were transferred to basal medium consisting of B_5 major salts and MS minor salts supplemented with PEG, 5.0, 8.0 and 10.0 per cent and observations recorded on the number of cultures survived.

b. Basal medium

Basal media such as MS, 1/2 MS, B_5, 1/2 B_5, B_5 major + MS minor, MS major + B_5 minor and SH media were tried to standardise the ideal basal medium for the effective maturation of the somatic embryoids.

Observations were made on the number of cultures survived and the size and colour of the embryoids.

c. Culture conditions

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

Observations were made after five weeks on the number of cultures survived and the size and colour of the embryoids.

d. Frequency of subculture

The somatic embryoids were subcultured at five days, ten days, fifteen days and twenty days interval in the same media. A control treatment with no subculturing was also tried.

Observations were made on the number of cultures survived, size and colour of somatic embryoids.

4. Germination of somatic embryoids

a. Basal medium

The embryoids from the maturation media were transferred to the germination media. Basal media such as MS, 1/2 MS, B5, SH, B5 major + MS minor and MS major + B_5 minor were tried. All the treatments were replicated 15 to 25 times.

b. Plant growth substances

The influence of plant growth substances like BA, GA_3, 2ip, IAA, NAA and kinetin were tested by incorporating them alone and in various combinations. Fifty nine treatments (Table 3) were tried for the germination of somatic embryoids.

c. Thidiazuron

Thidiazuron was tried at varying concentrations (1.0, 2.0 and 4.0 mg/l) in liquid media.

d. Sodium butyrate

Sodium butyrate was tried at concentrations (0.55, 1.10 and 1.65mg/l) in the medium to study its effect on the germination of somatic embryoids.

e. Nitrogen content

Nitrogen content of the MS basal medium was modified by using half strength and one fourth strength of ammonium nitrate and half and one fourth strength of potassium nitrate.

Table 3. Plant growth substances tried for the germination of somatic embryoids

Culture medium - Basal medium containing B5 major salts + MS minor + sucrose 40.0g/l + CW 200.0ml/l + agar 5.0g/l + PVP 10.0g/l

Treatments	Plant growth substances (mg/l)	Treatments	Plant growth substances (mg/l)
T_1	BA 0.05 + IAA 0.20	T_{31}	2ip 1.00 + GA_3 5.00
T_2	BA 0.05 + IAA 1.00	T_{32}	2ip 1.00 + GA_3 10.00
T_3	BA 0.05 + IAA 2.00	T_{33}	2ip 2.00 + GA_3 1.00
T_4	BA 0.05 + IAA 5.00	T_{34}	2ip 2.00 + GA_3 5.00
T_5	BA 0.05 + IAA 10.00	T_{35}	2ip 2.00 + GA_3 10.00
T_6	BA 0.05 + IAA 15.00	T_{36}	IAA 0.20 + GA_3 1.00
T_7	BA 0.05 + IAA 20.00	T_{37}	IAA 0.20 + GA_3 5.00
T_8	BA 0.10 + IAA 0.20	T_{38}	IAA 0.20 + GA_3 10.00
T_9	BA 0.10 + IAA 1.00	T_{39}	IAA 1.00 + GA_3 1.00
T_{10}	BA 0.10 + IAA 2.00	T_{40}	IAA 1.00 + GA_3 5.00
T_{11}	BA 0.10 + IAA 5.00	T_{41}	IAA 1.00 + GA_3 10.00
T_{12}	BA 0.10 + IAA 10.00	T_{42}	2ip 2.00 + IAA 0.20
T_{13}	BA 0.10 + IAA 15.00	T_{43}	2ip 2.00 + IAA 1.00
T_{14}	BA 0.10 + IAA 20.00	T_{44}	2ip 2.00 + IAA 2.00
T_{15}	BA 1.00 + IAA 0.20	T_{45}	2ip 2.00 + IAA 5.00
T_{16}	BA 1.00 + IAA 1.00	T_{46}	2ip 2.00 + IAA 10.00
T_{17}	BA 1.00 + IAA 2.00	T_{47}	GA_3 1.00
T_{18}	BA 1.00 + IAA 5.00	T_{48}	GA_3 5.00
T_{19}	BA 1.00 + IAA 10.00	T_{49}	GA_3 10.00
T_{20}	BA 1.00 + IAA 15.00	T_{50}	BA 0.05
T_{21}	BA 1.00 + IAA 20.00	T_{51}	BA 0.10
T_{22}	Kinetin 2.80 + IAA 0.20	T_{52}	BA 0.50
T_{23}	BA 0.10 + 2ip 1.00	T_{53}	BA 1.00
T_{24}	BA 0.10 + 2ip 2.00	T_{54}	BA 2.00
T_{25}	BA 0.10 + 2ip 5.00	T_{55}	BA 4.00
T_{26}	BA 0.10 + GA_3 1.00	T_{56}	BA 6.00
T_{27}	BA 0.10 + GA_3 5.00	T_{57}	BA 8.00
T_{28}	BA 0.10 + GA_3 10.00	T_{58}	BA 10.00
T_{29}	BA 0.10 + GA_3 50.00	T_{59}	BA 50.00
T_{30}	2ip 1.0 + GA_3 1.00		

f. Osmotic regulants

As an osmoticum, sucrose was incorporated into the medium at 20.0, 30.0, 40.0, 50.0 and 60.0 g/l. Agar at 4.5, 5.0, 5.5 and 6.0 g/l was also tried. The influence of PEG on the germination of somatic embryoids was studied by incorporating 5.0 and 10.0 per cent PEG in the germination medium. Different levels of sodium chloride *viz.* (0.05, 0.10, 0.20, 0.40 and 0.80 per cent) were added to the medium, to study their effects on germination.

g. Ethylene inhibitors

Ethylene inhibitors like silver nitrate and cobalt chloride at concentrations of 5.0, 10.0 and 15.0 mg/l were tried in the germination media to study their effects.

h. Coconut water

Coconut water was tried at 0, 150.0 and 200.0 ml/l in the germination medium.

i. Agents countering polyphenol interference

Different levels of charcoal (0.10, 0.15, 0.2 and 0.25 per cent) and PVP (0.7, 0.8 and 1.0 per cent) were also tried for the germination of somatic embryoids.

5. Observations

Observations were recorded on the number of cultures survived and number of cultures with germinating somatic embryoids. Visual observations on the stages of development, abnormalities of the somatic embryoids and size of embryoids were made after four weeks.

6. Statistical analysis

The data generated from the various trails were subjected to statistical analysis, in completely randomised design, wherever necessary, as per Panse and Sukhatme (1978).

7. Morphological studies

The morphology of the developing somatic embryoids of the monoembryonic variety, Neelum and the polyembryonic variety, Vellari Manga were studied. The procedure adopted was :

Fixation → dehydration → Critical point drying → gold coating → examining in the Scanning Electron microscope (SEM).

Samples of somatic embryoids of different stages were collected at random from the cultures of Neelum and Vellari Manga. The samples were treated with a fixative for 24 hours. The fixative consisted of a mixture of glutaraldehyde solution (2.5 per cent) and phosphate buffer (pH 7.2) in 1:9 ratio.

a. Preparation of glutaraldehyde solution

2.5 ml of glutaraldehyde was made up to 100ml with distilled water, to get 2.5 per cent glutaraldehyde solution.

b. Preparation of phosphate buffer (pH 7.2)

Two hundred millilitre phosphate buffer (pH 7.2) was prepared by mixing 190ml of solution A (0.2 M monobasic sodium phosphate), 81 ml of solution B (0.2 M dibasic sodium phosphate) and 100ml distilled water.

Solution A was prepared by dissolving 3.12g monobasic sodium phosphate ($NaH_2 PO_4 2H_2O$) in 100ml distilled water. Solution B was prepared by dissolving 7.16g dibasic sodium phosphate ($Na_2 HPO_4 12 H_2O$) in 100ml distilled water.

After the fixation, the plant tissues were washed in phosphate buffer (pH 7.2) five to six times, to remove the adhering traces of the fixative. The tissues were then dehydrated at room temperature, using the following acetone series.

Strength of acetone (%)	Treatment time
50	25 min
70	20 min
90	60 min
90	12 h
95	25 min
95	25 min
100	30 min
100	30 min
100	30 min

Observations on surface characters of nodular calli and different stages of developing somatic embryoids were made and photographs taken.

8. Histological studies

Histological studies were made to ascertain the status of somatic embryoids formed, both in the monoembryonic variety, Neelum and polyembryonic variety, Vellari Manga. Different developmental stages of somatic embryos starting from the induction of embryogenic calli from nucellus up to germination of embryoids were taken for sampling. Fresh, hand sections of the samples were taken using a sharp blade, stained with dilute safranine and examined.

H. ENHANCED RELEASE OF AXILLARY BUDS

Shoot apices and nodal segments with axillary buds from fresh sprouts of five to ten year old mango trees were used as explants. The explants collected from field were thoroughly washed with running tap water. They were then washed with distilled water and dipped in bavistin (0.10, 0.15 and 0.20 per cent) for 60 min. After washing out the traces of bavistin with sterile water, the explants were dipped in 70 per cent alcohol for 1.0 min. The explants were then treated with 0.7 per cent PVP for 30 minutes in a shaker at 120 rpm.

The explants were then taken to the laminar air flow chamber and surface sterilized with sodium hypochlorite or mercuric chloride solution. A

few drops of 'Teepol' were added to the sterilants. The details of the surface sterilization treatments are given in Table 4.

Table 4. Surface sterilization treatments tried for shoot apices of mango

Sterilant	Concentration (%)	Treatment time (min)
Sodium hypochlorite	1.00 and 2.00	20, 30 and 40
Mercuric chloride	0.05, 0.08 and 0.10	5, 8, 10, 12, 15 and 20.

The explants after surface sterilisation were rinsed (at least five times) with sterile water. The explants were carefully transferred to the test tubes or conical flasks with sterile forceps. The cultures were incubated at 26 $\pm$ 2°C under light (3000 lux) at 16 h photoperiod provided by cool white fluorescent tubes.

Varieties tried - Neelum, Mulgoa, Vellari Manga and Pulichi.

Twenty five trèatments were tried for enhanced release of axillary buds (Table 5).

Table 5. Treatments tried for enhanced release of axillary buds

T_1	MS + BA 5.0mg/l + NAA 0.2 mg/l + Sucrose 30.0g/l + AC 0.1g/l + agar 5.5g/l
T_2	1/2 MS + BA 2.0mg/l + IBA 0.5mg/l + AC 0.05mg/l + Sucrose 30.0g/l + agar 5.5g/l
T_3	1/2 MS + BA 2.0mg/l + IAA 2.0mg/l + Sucrose 30.0g/l + AC 0.05g/l + agar 5.5g/l
T_4	1/2 MS + IAA 2.0mg/l + 2iP 2.0mg/l + Sucrose 30.0g/l + agar 6.0g/l + Vit. C 150.0mg/l + Citric acid 150.0mg/l
T_5	1/2 MS + IAA 2.0mg/l + 2iP 4.0mg/l + Sucrose 30.0g/l + agar 6.0g/l + Vit. C 150.0mg/l + Citric acid 150.0mg/l
T_6	1/2 MS + IAA 2.0mg/l + 2iP 8.0mg/l + Sucrose 30.0g/l + agar 6.0g/l + Vit. C 150.0mg/l+ Citric acid 150.0mg/l
T_7	MS + BA 2.0mg/l + NAA 0.2mg/l + Sucrose 30.0g/l + agar 6.0g/l
T_8	MS + BA 2.0mg/l + IAA 10.0mg/l + Sucrose 20.0g/l + agar 6.0g/l
T_9	1/2 MS + BA 5.0mg/l + Sucrose 20.0g/l + agar 6.0g/l + PVP 7.0g/l
T_{10}	MS + BA 5.0mg/l + Sucrose 20.0g/l + agar 6.0g/l + PVP 7.0g/l + antibiotics 100.0mg/l
T_{11}	1/2 B5 + Sucrose 40.0g/l + BA 20.0mg/l + agar 4.5g/l + AC 1.0g/l
T_{12}	1/2 B5 + BA 20.0mg/l + Sucrose 40.0g/l + agar 4.5g/l + AC 1.0g/l + antibiotics 200.0mg/l
T_{13}	1/2 B5 + 2ip 10.0mg/l + Sucrose 20.0g/l + agar 4.5g/l + antibiotics 200.0mg/l + PVP 10.0g/l
T_{14}	1/2 B5 + 2ip 20.0mg/l + Sucrose 20.0g/l + agar 4.5g/l + antibiotics 200.0mg/l + PVP 10.0g/l
T_{15}	1/2 B5 + IBA 2.0mg/l + Sucrose 40.0g/l + antibiotics 200.0mg/l + AC 1.0g/l + agar 5.5g/l
T_{16}	1/2 B5 + IBA 10.0mg/l + Sucrose 30.0g/l + antibiotics 200.0mg/l + AC 1.0g/l + agar 6.0g/l
T_{17}	B5 major + MS minor + Sucrose 30.0g/l + IAA 2.0mg/l + BA 0.5mg/l + agar 6.0g/l + PVP 10.0g/l
T_{18}	B5 major + MS minor + BA 1.0mg/l + Sucrose 30.0g/l + agar 6.0g/l + PVP 10.0g/l
T_{19}	B5 major + MS minor + BA 1.5mg/l + Sucrose 30.0g/l + agar 6.0g/l + PVP 10.0g/l
T_{20}	B5 major + MS minor + BA 2.0mg/l + Sucrose 30.0g/l + agar 6.0g/l + PVP 10.0g/l
T_{21}	B5 major + MS minor + BA 4.0mg/l + Sucrose 30.0g/l + agar 6.0g/l + PVP 10.0g/l
T_{22}	B5 major + MS minor + BA 0.1mg/l + Sucrose 30.0g/l + agar 5.0g/l + PVP 10.0g/l
T_{23}	B5 major + MS minor + BA 1.0mg/l + Sucrose 30.0g/l + agar 5.0g/l + PVP 10.0g/l
T_{24}	B5 + BA 5.0mg/l + IAA 5.0mg/l + Sucrose 20.0g/l + AC 0.5g/l + agar 5.5g/l + PVP 7.0g/l
T_{25}	SH + BA 5.0mg/l + NAA 0.2mg/l + Sucrose 30.0g/l + AC 1.0g/l + agar 5.0g/l

Observations

The treatments were replicated 18-30 times and observations were made after two weeks. Observations were made on per cent contamination, per cent survival of cultures, number of growing cultures, number of cultures showing direct shoot regeneration, number of multiple shoots etc.

I. SOMATIC ORGANOGENESIS

Immature leaves (15-20 days old) were collected from current season's growth of mature six to ten year old trees. After removing the petiole, leaves were washed thoroughly in distilled water followed by sterile water. They were then dipped in 0.10 per cent Bavistin solution for 30 min and treated with PVP 1.00 per cent for 60 min. Each leaf was cut into two or three pieces. They were subjected to surface sterilization using chemicals as per Table 4. A few drops of the wetting agent 'labolene' were added to the washing solutions as well as sterilizing agents.

The explants, after surface sterilization were rinsed (at least five times) with sterile water. They were trimmed to a size of one to two square cm before transferring to culture media.

Varieties tried - Neelum, Mulgoa, Vellari Manga and Pulichi.

Eleven treatments were tried for inducing somatic organogenesis (Table 6).

Table 6. Treatments tried for somatic organogenesis

T_1	MS + 2,4-D 2.0mg/l + BA 1.0mg/l + sucrose 30.0g/l + agar 6.0g/l + AC 1.0g/l
T_2	MS + 2,4-D 0.5mg/l + kinetin 1.0mg/l + NAA 1.0mg/l + sucrose 30.0g/l + agar 6.0g/l + AC 1.0g/l
T_3	MS + 2,4-D 1.0mg/l + kinetin 1.0mg/l + NAA 1.0mg/l + sucrose 30.0g/l + agar 6.0g/l + AC 1.0g/l
T_4	MS + 2,4-D 2.0mg/l + kinetin 1.0mg/l + NAA 1.0mg/l + agar 5.5g/l + AC 1.0g/l
T_5	MS + 2,4-D 4.0mg/l + kinetin 1.0mg/l + NAA 1.0mg/l + agar 6.0g/l + AC 1.0g/l
T_6	MS + NAA 0.24mg/l + BA 2.0mg/l + sucrose 30.0g/l + agar 6.0g/l + AC 1.0g/l
T_7	1/2 MS + NAA 0.24mg/l + BA 2.0mg/l + sucrose 30.0g/l + agar 6.0g/l + AC 1.0g/l
T_8	B_5 + NAA 0.24mg/l + BA 2.0mg/l + sucrose 30.0g/l + agar 6.0g/l+ AC 1.0g/l
T_9	1/2 B_5 + NAA 0.24mg/l + BA 2.0mg/l + sucrose 30.0g/l + agar 6.0g/l + AC 1.0g/l
T_{10}	B_5 + 2,4-D 2.0mg/l + BA 1.0mg/l + sucrose 30.0g/l + agar 6.0g/l + AC 1.0g/l
T_{11}	Same as T_6 but with initial culturing of the explant in liquid MS basal medium for 24 h and subculturing in solid MS basal medium at a subculturing frequency of 24 h for two days

Observations

Observations were made on number of cultures survived and number of cultures initiating callus.

RESULTS

RESULTS

Investigations were carried out for standardising *in vitro* propagation techniques in mango. Three routes namely somatic embryogenesis, somatic organogenesis and enhanced release of axillary buds were tried. The results of the studies are presented in the following pages.

A. *IN VITRO* PROPAGATION *VIA* SOMATIC EMBRYOGENESIS

1. Varieties

a. Monoembryonic varieties

Initially, the response of six monoembryonic varieties, namely, Neelum, Bangalora, Prior, Banganpalli, Mundappa and Mulgoa was assessed. Nucellus, embryo mass, leaf segments and floral parts were used as explants for the induction of somatic embryogenesis. The highest per cent (66.67) cultures initiating embryogenic callus from nucellus was observed for the variety Neelum (Table 7). The least response (25.00 per cent) was recorded by Mundappa (Fig. 1). The responses of Mulgoa, Bangalora, Prior and Banganpalli were 58.33, 50.00, 33.33 and 41.67 per cent, respectively. Neelum which showed the highest per cent response from nucellus (66.67 per cent) as well as embryo mass (75.00 per cent) was selected for further detailed studies.

Table 7. Effect of monoembryonic mango varieties on the induction of somatic embryogenesis from nucellus and embryo mass

Monoembryonic varieties	Cultures initiating embryogenic callus (%)		Growth score		Callus index	
	Nucellus	Embryo mass	Nucellus	Embryo mass	Nucellus	Embryo mass
Neelum	66.67	75.00	1.49	1.50	99.34	112.50
Bangalora	50.00	41.67	1.33	1.42	66.50	59.17
Prior	33.33	41.67	1.00	1.00	33.33	41.67
Banganpalli	41.67	50.00	1.08	1.00	45.00	50.00
Mundappa	25.00	16.67	1.00	1.00	25.00	16.67
Mulgoa	58.33	83.33	1.42	1.08	82.83	90.00

The data represent the average value of 12 replications

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

The maximum growth score (GS) of 1.49 and callus index (CI) value of 99.34 were also recorded by Neelum. Mulgoa recorded a growth score of 1.42 and a callus index of 82.83 from nucellus and a growth score of 1.08 and callus index of 90.00 from embryo mass. The least response (25.00 per cent) from nucellus and embryo mass (16.67 per cent) was observed for Mundappa.

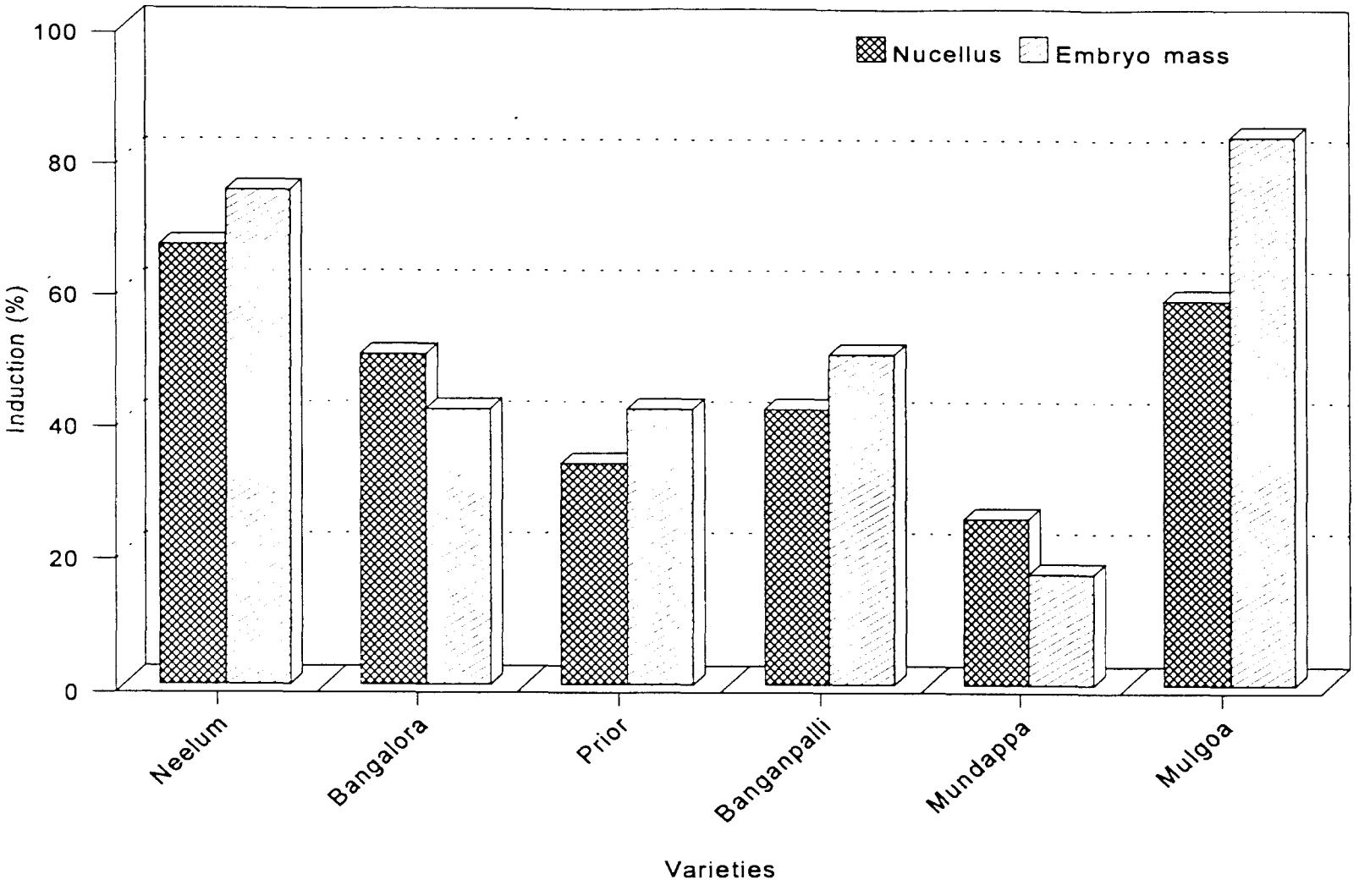


Fig. 1. Effect of monoembryonic mango varieties on the induction of somatic embryogenesis from nucellus and embryo mass

In monoembryonic varieties indirect embryogenesis was observed from both nucellus and embryo mass.

Neelum was selected as the monoembryonic variety for further detailed studies.

b. Polyembryonic varieties

Six polyembryonic varieties such as Olour, Kurukkan, Vellari Manga, Panchara Varikka, Kilichundan and Vellayani Varikka were also compared for their initial response. In polyembryonic varieties, direct somatic embryogenesis from nucellus and indirect somatic embryogenesis from embryo mass were observed.

The highest per cent (83.33) cultures initiating somatic embryoids from nucellus was observed for Vellari Manga (Table 8). Panchara Varikka, another polyembryonic variety recorded 75.00 per cent cultures initiating somatic embryogenesis. Embryo mass of these varieties gave 66.67 per cent cultures initiating embryogenic callus. The least response from nucellus was recorded by Kurukkan (33.33 per cent) and from embryo mass by Kilichundan (33.33 per cent). The nucellar explants of the variety, Vellayani Varikka did not respond to the treatments, whereas its embryo mass recorded 25.00 per cent cultures initiating somatic embryoids (Fig. 2).

Vellari Manga was selected as the polyembryonic variety for further detailed studies.

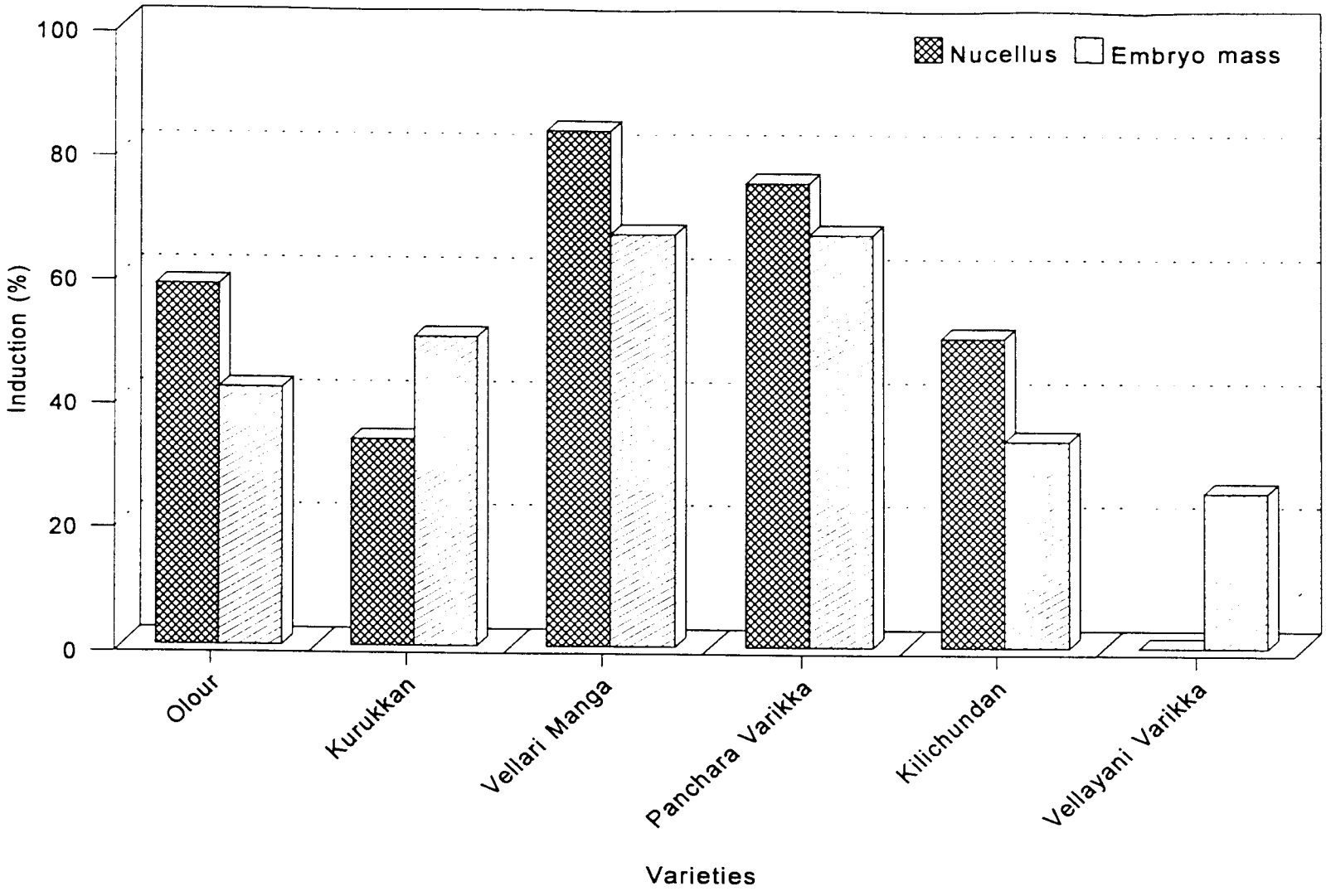


Fig. 2. Effect of polyembryonic of mango varieties on the induction of somatic embryogenesis from nucellus and embryo mass

Table 8. Effect of polyembryonic mango varieties on the induction of somatic embryogenesis from nucellus and embryo mass

Polyembryonic varieties	Cultures initiating somatic embryoids from nucellus (%)	Cultures initiating embryogenic callus from embryo mass (%)
Olour	58.33	41.67
Kurukkan	33.33	50.00
Vellari Manga	83.33	66.67
Panchara Varikka	75.00	66.67
Kilichundan	50.00	33.33
Vellayani Varikka	0	25.00

The data represent the average value of 12 replications

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

2. Explants

In the variety Neelum, embryo mass responded better than nucellar tissue. In Neelum 75.00 per cent cultures initiated embryogenic callus from embryo mass (Table 7). Nucellar tissue initiated callus in 66.67 per cent cultures only. But in Vellari Manga, nucellus was the better explant, recording 83.33 per cent cultures, initiating somatic embryoids (Table 8). The embryo

mass of Vellari Manga initiated embryogenic callus in 66.67 per cent cultures. Leaf segments and immature flower parts did not respond in Neelum and Vellari Manga.

When leaf segments and floral parts were used as explants, the contamination rate was very high. The per cent contamination of the explants ranged from 50.00 to 90.00. In all the varieties tried, both leaf segments and immature flower parts did not respond to the treatments.

3. Phenolics interference

The problem of phenolics interference was very severe which caused browning of media and tissues. In the variety Neelum, incorporation of activated charcoal 2.5 g/l in the induction medium was effective in reducing phenolics problem. Eighty per cent cultures survived in the treatment, out of which 66.67 per cent cultures showed callus initiation in half strength MS basal medium with supplements (Table 9). With 5.0 g/l activated charcoal, though 86.67 per cent cultures survived, only 53.33 per cent responded.

In Vellari Manga, use of activated charcoal 2.5 g/l in the induction medium was the most effective in reducing phenolics problem. The treatment registered the highest percentage of cultures initiating somatic embryoids (80.00), out of which 66.67 per cent cultures survived. The lowest per cent cultures (13.33) initiating somatic embryoids was observed when 1.5 g/l activated charcoal was added in the induction medium.

Table 9. Effect of activated charcoal, polyvinyl pyrrolidone (PVP), citric acid and ascorbic acid in eliminating phenolics interference and inducing somatic embryogenesis from nucellus of the mango varieties Neelum and Vellari Manga

Treatment	Surviving cultures (%)		Cultures initiating embryogenic callus in Neelum (%)	Cultures initiating somatic embryoids in Vellari Manga (%)
	Neelum	Vellari Manga		
Activated charcoal (g/l)				
1.5	33.33	26.67	6.67	13.33
2.0	26.67	60.00	13.33	70.33
2.5	80.00	73.33	66.67	80.00
5.0	86.67	66.67	53.33	60.00
PVP (g/l)				
7.0	13.33	6.67	0	0
8.0	40.00	26.67	0	0
10.0	60.00	40.00	0	33.33
Citric acid (mg/l)				
75.0	0	0	0	0
150.0	0	0	0	0
300.0	0	0	0	0
Ascorbic acid (mg/l)				
75.0	0	0	0	0
150.0	0	0	0	0
300.0	0	0	0	0

The data represent the average value of 15 replications

Culture medium for Neelum - Half strength MS basal medium + 2,4-D 5.0mg/l + GA_3 5.0mg/l + CW 200.0ml/l + agar 5.0g/l

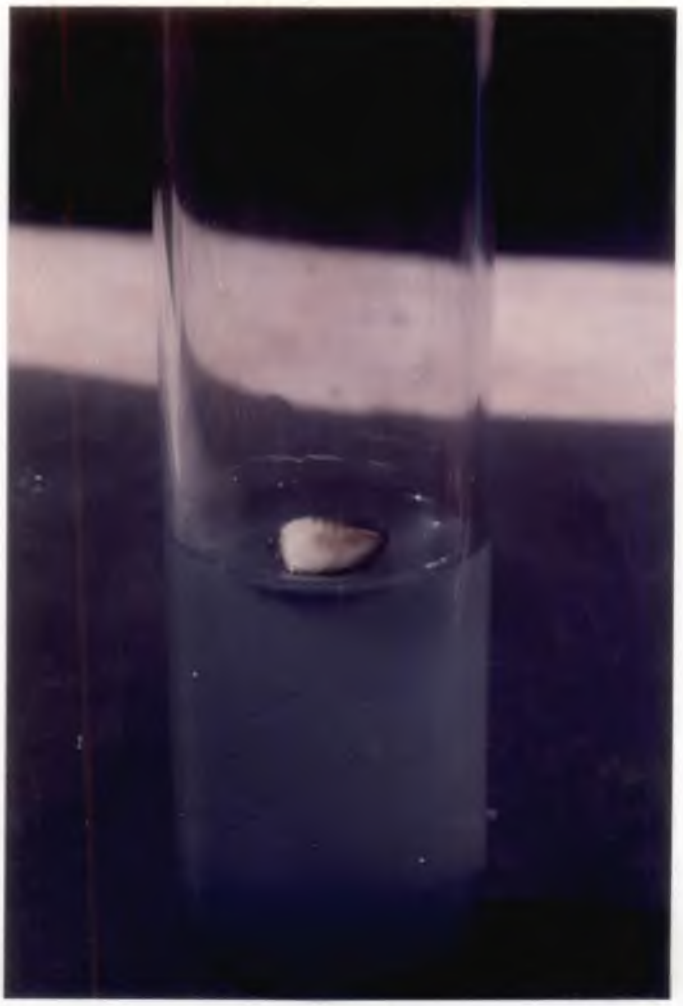
Culture medium for Vellari Manga - Half strength MS basal medium + 2,4-D 2.0mg/l + GA_3 5.0mg/l + CW 200.0ml/l + agar 6.0g/l

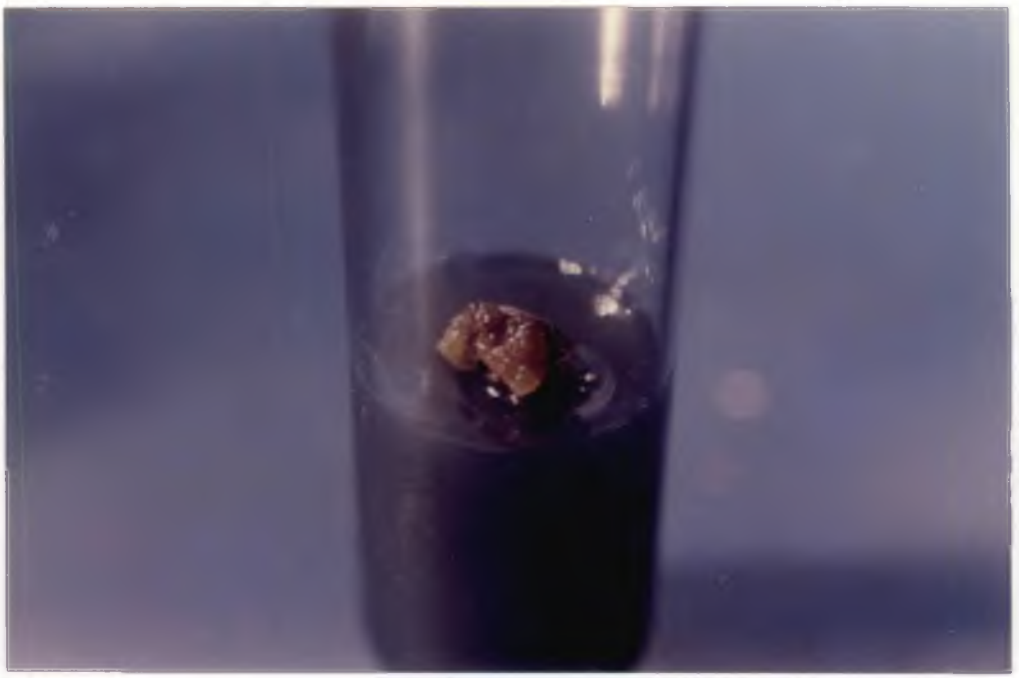
Plate 3

Nucellus of Neelum in induction medium (Half strength MS basal medium supplemented with 2,4-D 5.0 mg/l and GA_3 5.0 mg/l)

Plate 4

Nodular pro-embryos induced from the nucellus of Vellari Manga in half strength MS basal medium supplemented with 2,4-D 2.0 mg/l and GA_3 5.0 mg/l





The treatments with vitamin C or citric acid were not effective in initiating somatic embryoids in Neelum and Vellari Manga. Only the treatments with PVP 8.0 and 10.0 g/l responded. With PVP 8.0 and 10.0 g/l, Vellari Manga registered 20.00 and 33.33 per cent cultures initiating somatic embryoids, respectively.

4. Induction of somatic embryoids

Different basal media were tried for the induction of somatic embryoids in the varieties Neelum and Vellari Manga. Treatments involving various combinations of plant growth substances, glutamine, activated charcoal, sucrose, aminoacids and agar were also tried.

a. Basal media

Basal media such as MS medium (full and half strength), B_5 medium (full and half strength) and SH medium were compared for the induction of somatic embryoids in the variety Neelum. The study revealed that half strength MS basal medium was the best for the production of embryogenic callus from nucellus as well as embryo mass (Plate 3). In this treatment, 66.67 per cent cultures initiated embryogenic callus from nucellus and 20.00 per cent cultures initiated embryogenic callus from embryo mass (Table 10). In full strength MS basal medium, only 26.67 cultures initiated somatic embryoids from nucellus and 13.33 per cent from embryo mass. None of the cultures initiated embryogenic callus from nucellus and embryo mass of Neelum when B_5 and SH media were used. In half strength of MS basal medium with supplements,

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a. Basal media

Basal media such as MS medium (full and half strength), B_5 medium (full and half strength) and SH medium were compared for the induction of somatic embryoids in the variety Neelum. The study revealed that half strength MS basal medium was the best for the production of embryogenic callus from nucellus as well as embryo mass (Plate 3). In this treatment, 66.67 per cent cultures initiated embryogenic callus from nucellus and 20.00 per cent cultures initiated embryogenic callus from embryo mass (Table 10). In full strength MS basal medium, only 26.67 cultures initiated somatic embryoids from nucellus and 13.33 per cent from embryo mass. None of the cultures initiated embryogenic callus from nucellus and embryo mass of Neelum when B_5 and SH media were used. In half strength of MS basal medium with supplements,

the highest growth score of 1.50 and a callus index of 100.00 were registered by nucellus and a growth score of 1.00 and a callus index of 20.00 by embryo mass. Half strength of B_5 basal medium with supplements initiated embryogenic callus from 20.00 per cent cultures of nucellus and 6.67 per cent cultures of embryo mass.

Table 10. Effect of basal media on the induction of somatic embryogenesis from nucellus and embryo mass of the mango variety Neelum

Basal medium	Cultures initiating embryogenic callus (%)		Growth score		Callus index	
	Nucellus	Embryo mass	Nucellus	Embryo mass	Nucellus	Embryo mass
MS	26.67	13.33	1.00	1.00	26.67	13.33
½ MS	66.67	20.00	1.50	1.00	100.01	20.00
B_5	0	0	0	0	0	0
½ B_5	20.00	6.67	1.00	1.00	20.00	6.67
SH	0	0	0	0	0	0

The data represent the average value of 15 replications

Supplements : 2,4-D 5.0mg/l + GA_3 5.0mg/l+ glutamine 400.0mg/l + sucrose 60.0g/l + agar 5.0g/l + AC 2.5g/l + CW 200.0ml/l

The same basal media used for Neelum variety were tried for Vellari Manga. Half strength MS basal medium recorded the highest number of cultures initiating callus from nucellus (80.00 per cent) and from embryo mass (30.00 per cent) (Plate 4). In this treatment a growth score of 1.00 and a callus index of 30.00 were registered by the cultures of embryo mass (Table 11). Full strength of MS basal medium recorded 60.00 per cent cultures initiating embryoids from nucellus and 20.00 per cent cultures initiating embryogenic callus from embryo mass. The corresponding callus index value registered was 20.00 with a growth score of 1.00.. When B_5 and SH media were tried, only 10.00 per cent cultures, initiated somatic embryos from nucellus. None of the cultures initiated callus from embryo mass in these basal media. However, half strength B_5 basal media initiated somatic embryos in 30.00 per cent cultures of nucellus and 10.00 per cent cultures of embryo mass.

b. Plant growth substances

Thirty treatments of plant growth substances incorporated in half strength MS basal medium were tried for inducing somatic embryoids from nucellus as well as embryo mass of Neelum. The treatment T_3 (2,4-D 5.0mg/l and GA_3 5.0mg/l) recorded the highest per cent cultures (62.50) initiating embryogenic callus from nucellus (Table 12). A growth score of 1.40 and callus index value of 87.50 was recorded for this treatment. The same treatment initiated embryogenic callus in 20.83 per cent cultures from embryo mass. A growth score of 1.00 and callus index of 20.83 were observed.

Table 11. Effect of basal media on the induction of somatic embryogenesis from nucellus and embryo mass of the mango variety Vellari Manga

Basal medium	Cultures initiating somatic embryoids from nucellus (%)	Cultures initiating embryogenic callus from embryo mass (%)	Growth score of embryogenic callus from embryo mass	Callus index of embryogenic callus from embryo mass
MS	60.00	20.00	1.00	20.00
½ MS	80.00	30.00	1.00	30.00
B_5	10.00	0	0	0
½ B_5	30.00	10.00	1.00	10.00
SH	10.00	0	0	0

The data represent the average value of 10 replications

Supplements : 2,4-D 2.0mg/l+ GA_3 5.0mg/l + glutamine 400.0mg/l + sucrose 60.0g/l + CW 200.0ml/l + agar 6.0g/l + AC 2.5g/l

The treatments T_5, T_{12}, T_{20}, T_{25}, T_{26} and T_{27} also initiated embryogenic callus (Table 12). Eight treatments (T_4, T_9, T_{11}, T_{13}, T_{21}, T_{29}, T_{30} and T_{31}) did not respond in inducing embryogenic callus from nucellus. Treatments T_{21}, T_{22}, T_{29} and T_{31} were not effective for inducing embryogenic callus from embryo mass.

Table 12. Effect of plant growth substances on the induction of somatic embryogenesis from nucellus and embryo mass of the mango variety Neelum

	Plant growth substances (mg/l)	Cultures initiating embryogenic callus (%)		Growth score		Callus index	
		Nucellus	Embryo mass	Nucellus	Embryo mass	Nucellus	Embryo mass
T_1	2,4-D 2.0+GA_3 5.0	12.50	0	1.00	0	12.50	0
T_2	2,4-D 4.0+GA_3 5.0	20.80	0	1.20	0	25.00	0
T_3	2,4-D 5.0+GA_3 5.0	62.50	20.83	1.40	1.0	57.50	20.83
T_4	2,4-D 8.0+GA_3 5.0	0	0	0	0	0	0
T_5	2,4-D 16.0+GA_3 5.0	45.83	16.67	1.00	1.00	45.83	29.17
T_6	2,4-D 2.0+GA_3 10.0	37.50	8.33	1.00	1.10	37.50	8.33
T_7	2,4-D 4.0+GA_3 10.0	25.00	20.83	1.00	1.00	25.00	20.83
T_8	2,4-D 5.0+GA_3 10.0	16.67	8.33	1.10	1.20	18.34	10.00
T_9	2,4-D 8.0+GA_3 10.0	0	0	0	0	0	0
T_{10}	2,4-D 16.0+GA_3 10.0	29.17	12.50	1.0	1.0	29.17	12.50
T_{11}	2,4-D 2.0+GA_3 20.0	0	0	0	0	0	0
T_{12}	2,4-D 4.0+GA_3 20.0	41.67	0	1.0	0	41.67	0
T_{13}	2,4-D 5.0+GA_3 20.0	0	0	0	0	0	0
T_{14}	2,4-D 8.0+GA_3 20.0	16.67	20.83	2.0	1.0	33.34	20.83
T_{15}	2,4-D 16.0+GA_3 20.0	8.33	0	1.0	0	8.33	0
T_{16}	BA 0.5+GA_3 5.0	8.33	0	1.0	0	8.33	0
T_{17}	BA 1.0+GA_3 5.0	37.50	80.83	1.3	1.0	48.75	80.83
T_{18}	BA 2.0+GA_3 5.0	12.50	8.33	1.1	1.1	13.75	9.16
T_{19}	BA 0.5+GA_3 10.0	4.17	0	1.0	0	4.17	0
T_{20}	BA 1.0+GA_3 10.0	51.17	0	1.20	0	65.01	0
T_{21}	BA 2.0+GA_3 10.0	0	0	0	0	0	0
T_{22}	BA 0.5+GA_3 20.0	8.33	0	1.0	0	8.33	0
T_{23}	BA 1.0+GA_3 20.0	0	20.83	0	1.0	0	20.83
T_{24}	BA 2.0+GA_3 20.0	33.33	0	1.0	0	33.33	0
T_{25}	2,4-D 5.0+GA_3 5.0+BA0.5	41.67	4.17	1.2	1.0	50.01	4.17
T_{26}	2,4-D 5.0+GA_3 5.0+BA1.0	41.67	37.50	1.10	1.0	45.84	37.50
T_{27}	2,4-D 5.0+GA_3 5.0+BA2.0	45.83	16.67	1.0	1.0	45.83	16.67
T_{28}	2,4-D 5.0	20.83	12.50	1.0	1.0	20.83	12.50
T_{29}	GA_3 5.0	0	0	0	0	0	0
T_{30}	BA 2.0	0	8.33	0	1.0	0	8.33
T_{31}	Control (No PGS)	0	0	0	0	0	0

The data represent the average value of 24 replications

Culture medium - Half strength MS basal medium + glutamine 400.0mg/l + sucrose 60.0g + agar 5.0g/l + CW 200.0ml/l + AC 2.5g/l

The polyembryonic variety, Vellari Manga initiated somatic embryoids at lower levels of 2,4-D (2.0 and 4.0 mg/l). Treatments T_1 (with 2,4-D 2.0 mg/l) and T_2 (with 2,4-D 4.0 mg/l) initiated somatic embryoids in 83.33 per cent cultures from nucellar tissues (Table 13). However, 4.17 per cent cultures initiated callus from embryo mass in T_1 medium. In T_2 medium 33.33 per cent cultures initiated embryogenic callus with a callus index value of 33.33.

When no plant growth substances were used in the induction medium, none of the cultures initiated embryogenic callus in both Neelum and Vellari Manga.

c. Solidifying agent (agar)

Effect of the solidifying agent, agar on the induction of embryogenic callus using nucellar plants in Neelum and Vellari Manga was studied. Significant difference was observed among the levels of agar with respect to the per cent cultures initiating embryogenic callus. Agar 5.0 g/l produced the highest per cent cultures (55.56) initiating embryogenic callus in Neelum which was on par with agar 6.0g/l (Table 14). However, in Vellari Manga, the effects of different levels of agar were not significant in inducing somatic embryoids. The highest and lowest levels of agar tried reduced the per cent cultures inducing somatic embryoids in both the varieties.

Table 13. Effect of plant growth substances on the induction of somatic embryogenesis from nucellus and embryo mass of the mango variety Vellari Manga

	Plant growth substances (mg/l)	Cultures initiating somatic embryoids from nucellus (%)	Cultures initiating embryogenic callus from embryo mass (%)	Growth score of embryogenic callus from embryo mass	Callus index of embryogenic callus from embryo mass
T_1	2,4-D 2.0+GA_3 5.0	83.33	4.17	1.00	4.17
T_2	2,4-D 4.0+GA_3 5.0	83.33	33.33	1.00	33.33
T_3	2,4-D 5.0+GA_3 5.0	75.00	8.33	1.10	9.16
T_4	2,4-D 8.0+GA_3 5.0	37.50	25.00	1.00	25.00
T_5	2,4-D 16.0+GA_3 5.0	0	8.33	1.00	8.33
T_6	2,4-D 2.0+GA_3 10.0	41.67	0	0	0
T_7	2,4-D 4.0+GA_3 10.0	12.50	16.67	1.00	16.67
T_8	2,4-D 5.0+GA_3 10.0	45.83	0	0	0
T_9	2,4-D 8.0+GA_3 10.0	8.33	4.17	1.00	4.17
T_{10}	2,4-D 16.0+GA_3 10.0	12.50	0	0	0
T_{11}	2,4-D 2.0+GA_3 20.0	70.83	16.67	1.10	18.34
T_{12}	2,4-D 4.0+GA_3 20.0	41.67	0	0	0
T_{13}	2,4-D 5.0+GA_3 20.0	12.50	12.50	1.00	12.50
T_{14}	2,4-D 8.0+GA_3 20.0	0	0	0	0
T_{15}	2,4-D 16.0+GA_3 20.0	0	0	0	0
T_{16}	BA 0.5+GA_3 5.0	12.50	8.33	1.00	8.33
T_{17}	BA 1.0+GA_3 5.0	25.00	0	0	0
T_{18}	BA 2+GA_3 5.0	4.17	0	0	0
T_{19}	BA 0.5+GA_3 10.0	33.33	0	0	0
T_{20}	BA 1.0+GA_3 10.0	75.00	12.50	1.10	13.75
T_{21}	BA 2.0+GA_3 10.0	37.50	0	0	0
T_{22}	BA 0.5+GA_3 20.0	20.83	0	0	0
T_{23}	BA 1.0+GA_3 20.0	0	0	0	0
T_{24}	BA 2.0+GA_3 20.0	8.33	0	0	0
T_{25}	2,4-D 5.0+GA_3 5.0+BA0.5	62.50	0	0	0
T_{26}	2,4-D 5.0+GA_3 5.0+BA1.0	75.00	0	0	0
T_{27}	2,4-D 5.0+GA_3 5.0+BA2.0	41.67	0	0	0
T_{28}	2,4-D 5.0	0	0	0	0
T_{29}	GA_3 5.0	0	0	0	0
T_{30}	BA 2.0	0	4.17	1.00	4.17
T_{31}	Control (No PGS)	0	0	0	0

The data represent the average value of 24 replications

Culture medium - Half strength MS basal medium + glutamine 400.0mg/l + sucrose 60.0g/l +CW 200.0ml/l + agar 6.0g/l + AC 2.5g/l

Table 14. Effect of agar on the induction of somatic embryogenesis from nucellus of the mango varieties Neelum and Vellari Manga

Agar (g/l)	Cultures initiating embryogenic callus in Neelum (%)	Cultures initiating Somatic embryoids in Vellari Manga (%)
4.5	27.78 (31.64)	27.78 (31.64)
5.0	55.56 (48.75)	39.89 (38.80)
6.0	43.44 (41.27)	42.86 (40.65)
7.0	22.22 (27.76)	25.66 (30.06)
CD (5%)	12.30	NS

The data represent the average value of 18 replications

Values in parenthesis are the transformed values

NS - Not significant

Culture medium for Neelum - Half strength MS basal medium + 2,4-D5.0mg/l + $GA_3$5.0mg/l + glutamine 400.0mg/l + sucrose 60.0g/l + AC 2.5g/l + CW 200.0mg/l

Culture medium for Vellari - Half strength MS basal medium + Manga 2,4-D2.0mg/l + GA_3 5.0mg/l + glutamine 400.0mg/l + sucrose 60.0g/l + AC 2.5g/l + CW 200.0mg/l

d. Activated Charcoal

Among the various levels of activated charcoal tried, 2.5g/l registered significantly higher per cent initiation of embryogenic callus in Neelum (78.24) and somatic embryoids in Vellari Manga (66.13) from nucellar explants (Table 15). There was also significant difference among treatments in the initiation of embryogenic callus from embryo mass in Neelum. Activated charcoal 2.5g/l induced the highest per cent cultures producing embryogenic callus (62.33). However, there was no significant difference among the levels of activated charcoal in the initiation of embryogenic callus from the embryo mass of Vellari Manga. When activated charcoal was not used in the medium, none of the cultures initiated either somatic embryoids or embryogenic callus in both the varieties.

e. Sucrose

Sucrose 60.0g/l registered significantly higher per cent cultures initiating embyogenic callus from the nucellar explants of Neelum (66.54). The response of sucrose 50.0g/l was on par with sucrose 60.0g/l. The percentage induction decreased with decreasing levels of sucrose (Table.16). Sucrose 30.0g/l recorded the least response (25.08 per cent). Sucrose 60.0g/l also recorded significantly the highest per cent cultures initiating somatic embryoids from nucellar tissue of Vellari Manga (66.54). For the induction of embryogenic callus from embryo mass of Neelum, the highest per cent response was observed for sucrose 50.0g/l. In this treatment 83.33 per cent cultures initiated embryogenic callus. In Vellari Manga the most ideal concentration of sucrose was 40.0g/l, for inducing embryogenic callus from embryo mass (66.67 per cent).

Table 15. Effect of activated charcoal on the induction of somatic embryogenesis from nucellus and embryo mass of the mango varieties Neelum and Vellari Manga

Charcoal (g/l)	Cultures initiating embryogenic callus / somatic embryoids from nucellus (%)		Cultures initiating embryogenic callus from embryo mass (%)	
	Neelum	Vellari Manga	Neelum	Vellari Manga
0.5	11.66 (19.61)	19.52 (25.57)	24.47 (31.23)	38.28 (37.78)
1.5	26.18 (30.32)	23.16 (28.57)	36.24 (36.67)	54.21 (47.59)
2.5	78.24 (62.32)	66.13 (54.75)	58.57 (50.38)	62.33 (52.68)
5.0	63.25 (52.96)	45.45 (42.37)	50.17 (45.02)	53.70 (47.76)
CD	9.54*	11.89*	12.78**	NS

The data represent the average value of 21 replications

Values in parenthesis are the transformed values

* Significant at 5% level

** Significant at 1% level

NS Not significant

Culture medium for Neelum - Half strength MS basal medium + 2,4-D5.0 mg/l + $GA_3$5.0mg/l + Sucrose 60.0mg/l + CW 200.0ml/l + agar 5.0g/l + glutamine 400.0mg/l

Culture medium for Vellari Manga - Half strength MS basal medium + 2,4-D2.0mg/l + GA_3 5.0mg/l + glutamine 400.0mg/l + sucrose 60.0g/l + CW 200.0mg/l + agar 6.0g/l

Table 16. Effect of sucrose on the induction of somatic embryogenesis from nucellus and embryo mass of the mango varieties Neelum and Vellari Manga

Sucrose (g/l)	Cultures initiating[a] embryogenic callus / somatic embryoids from nucellus (%)		Cultures initiating[b] embryogenic callus from embryo mass (%)	
	Neelum	Vellari Manga	Neelum Manga	Vellari
30.0	25.08 (27.71)	36.13 (36.35)	16.67	33.33
40.0	36.14 (36.87)	38.50 (38.25)	33.33	66.67
50.0	54.52 (47.65)	58.71 (50.42)	83.33	50.00
60.0	66.54 (55.01)	66.54 (55.01)	66.67	50.00
CD (5%)	10.53	16.12		

a - The data represent the average value of 36 replications

b - The data represent the average value of 6 replications

Values in parenthesis are the transformed values

Culture medium for - Neelum: Half strength MS basal medium + 2,4-D5.0mg/l + $GA_3$5.0mg/l + glutamine 400.0mg/l + CW 200.0ml/l + agar 5.0g/l + AC 2.5g/l

Culture medium for - Vellari Manga: Half strength MS basal medium + 2,4-D2.0mg/l + GA_3 5.0mg/l + glutamine 400.0mg/l +CW 200.0ml/l + agar 6.0g/l + AC 2.5g/l

f. Amino acids

Different types of amino acids such as glutamine, arginine and asparagine 400.0mg/l were tried for inducing somatic embryoids in both Neelum and Vellari Manga (Table 17). Arginine and asparagine were not useful for the induction of embryogenic callus, whereas glutamine gave the highest per cent cultures initiating somatic embryoids from nucellus of Neelum (60.00) and Vellari Manga (80.00) and from embryo mass of Neelum (20.00) and Vellari Manga (40.00). Three levels of glutamine 400.0, 500.0 and 600.0mg/l were tried for inducing embryogenic callus. There was no significant difference among the response of the different levels tried when nucellus of Neelum and embryo mass of Vellari Manga were used as explants (Table 18). In Vellari Manga glutamine 600.0mg/l produced significantly the highest per cent cultures initiating somatic embryoids (69.39) from nucellar explants. In Neelum, embryo mass produced significantly the highest per cent cultures initiating embryogenic callus, when glutamine was used in the medium at 400.0mg/l (58.26).

g. Coconut water

The effect of coconut water in the induction of embryogenic callus in Neelum and somatic embryoids in Vellari Manga was studied. It was observed that 200.0ml/l of coconut water favoured initiation of embryogenic callus in 45.08 per cent cultures of nucellar explants of Neelum. This treatment was on par with coconut water 150.0ml/l supporting 38.00 per cent cultures initiating embryogenic callus (Table 19).

Table 17. Effect of aminoacids on the induction of somatic embryogenesis from nucellus and embryo mass of the mango varietiesNeelum and Vellari Manga

Amino aicd	Cultures initiating embryogenic callus / somatic embryoids from nucellus (%)		Cultures initiating embryogenic callus / from embryo mass (%)	
	Neelum	Vellari Manga	Neelum	Vellari Manga
Glutamine (400.0mg/l)	60.00	80.00	20.00	40.00
Arginine (400.0mg/l)	20.00	40.00	20.00	0
Asparagine (400.0mg/l)	40.00	40.00	0	0

The data represent the average value of 5 replications

Culture medium for Neelum - Half strength MS basal medium + 2,4-D 5.0mg/l + $GA_3$5.0mg/l + CW 200.0ml/l + agar 5.0g/l + AC 2.5g/l + Sucrose 60.0g/l

Culture medium for Vellari Manga - Half strength MS basal medium + 2,4-D2.0mg/l + GA_3 5.0mg/l + CW 200.0ml/l + agar 6.0g/l + AC 2.5g/l + Sucrose 60.0g/l

Table 18. Effect of glutamine on the induction of somatic embryogenesis from nucellus and embryo mass of the mango varieties Neelum and Vellari Manga

Glutamine (mg/l)	Cultures initiating embryogenic callus / somatic embryoids from nucellus (%)		Cultures initiating embryogenic callus from embryo mass (%)	
	Neelum	Vellari Manga	Neelum	Vellari Manga
400.0	48.26 (43.96)	54.69 47.95)	58.26 (49.98)	40.87 (39.48)
500.0	30.50 (33.16)	31.90 (34.25)	31.43 (33.79)	33.58 (34.65)
600.0	32.25 (34.07)	69.39 (56.86)	41.31 (39.91)	43.14 (41.00)
CD	NS	12.76	9.93	NS

The data represent the average value of 18 replications

Values in parenthesis are the transformed values

NS - Not significant

Culture medium for Neelum - Half strength MS basal medium + 2,4-D5.0mg/l + $GA_3$5.0mg/l + CW 200.0ml/l + agar 5.0g/l + AC 2.5g/l + Sucrose 60.0g/l

Culture medium for Vellari Manga - Half strength MS basal medium + 2,4-D2.0mg/l + GA_3 5.0mg/l + CW 200.0g/l + agar 6.0g/l + AC 2.5g/l + Sucrose 60.0g/l

Table 19. Effect of coconut water on the induction of somatic embryogenesis from nucellus and embryo mass of the mango varieties Neelum and Vellari Manga

Coconut water (ml/l)	Cultures initiating embryogenic callus / somatic embryoids from nucellus (%)		Cultures initiating embryogenic callus from embryo mass (%)	
	Neelum	Vellari Manga	Neelum	Vellari Manga
150.0	38.00 (38.01)	38.36 (38.19)	44.83 (42.47)	28.73 (32.25)
200.0	45.08 (42.10)	37.64 (37.79)	48.08 (43.91)	30.84 (33.54)
250.0	26.16 (30.64)	23.65 (28.82)	30.02 (32.87)	27.40 (31.38)
Control	5.28 (10.60)	5.28 (10.60)	0	0
CD (5%)	8.35	9.21	NS	NS

The data represent the average value of 18 replications

Values in parenthesis are the angular transformed values

NS - Not significant

Culture medium for Neelum - Half strength MS basal medium + 2,4-D5.0mg/l + $GA_3$5.0mg/l + agar 5.0g/l + AC 2.5g/l + Sucrose 60.0g/l + gutamine 400.0mg/l

Culture medium for Vellari Manga - Half strength MS basal medium + 2,4-D2.0mg/l + GA_3 5.0mg/l + agar 6.0g/l + AC 2.5g/l + Sucrose 60.0g/l + gutamine 400.0mg/l

In Vellari Manga, 150.0ml/l and 200.0ml/l coconut water produced the highest per cent cultures initiating somatic embryoids from nucellus (38.36 and 37.64 respectively). The treatments without coconut water recorded only 5.28 per cent cultures initiating embryogenic callus from nucellus of Neelum as well as Vellari Manga. Coconut water 150.0ml/l, 200.0ml/l and 250.0ml/l though favoured the initiation of embryogenic callus from embryo mass of Neelum and Vellari Manga, their effects were not significant. None of the cultures initiated embryogenic callus when the embryo mass of both the varieties were cultured in media without coconut water.

h. Culture conditions

Dark, rather than light condition, combined with low temperature was ideal for inducing somatic embryoids both in Neelum and Vellari Manga. When the cultures were kept under dark at low temperature (26 $\pm$ 2°C), 57.14 per cent cultures initiated embryogenic callus from nucellar explants of Neelum and 85.71 per cent cultures initiated somatic embryoids from nucellus of Vellari Manga (Table 20). However, when the cultures were kept under dark at room temperature (32 $\pm$2°C), only 14.29 per cent cultures initiated somatic embryoids in Vellari Manga. None of the cultures of Neelum responded under the same conditions. Under light (3000 lux and 16h photo period) at both low and room temperature, nucellus of Neelum and Vellari Manga did not initiate somatic embryoids or embryogenic callus.

Table 20. Effect of culture conditions on the induction of somatic embryogenesis from nucellus of the mango varieties Neelum and Vellari Manga

Culture conditions	Cultures initiating embryogenic callus (%) Neelum	Cultures initiating somatic embryoids (%) Vellari Manga
Light (3000 lux; 16h photoperiod) + Low temperature (26 ± 2°C)	0	0
Light (3000 lux; 16h photoperiod) + Room temperature	0	0
Darkness + Low temperature (26 ± 2°C)	57.14	85.71
Darkness + Room temperature	0	14.29

The data represent the average value of 7 replications

Culture medium for - Neelum: Half strength MS basal medium + 2,4-D 5.0mg/l + GA_3 5.0mg/l + CW 200.0ml/l + agar 5.0g/l + AC 2.5g/l + Sucrose 60.0g/l + glutamine 400.0mg/l

Culture medium for - Vellari Manga: Half strength MS basal medium + 2,4-D 2.0mg/l + GA_3 5.0mg/l + agar 6.0g/l + AC 2.5g/l + cw 200.0 ml/l + Sucrose 60.0g/l + glutamine 400.0mg/l

i. Frequency of subculturing

When subcultured at five days interval, nucellar tissue of Neelum recorded 30.0 per cent cultures initiating embryogenic callus and Vellari Manga, 40.0 per cent cultures initiating somatic embryoids (Table 21). But when the cultures were kept without subculturing, the nucellar explants of Neelum and Vellari responded less by producing 10.0 and 20.0 per cent cultures initiating embryogenic callus and somatic embryoids respectively (Fig. 3).

Minimum time (6-7 weeks in Neelum and 4-5 weeks in Vellari Manga) were taken by the explant in initiating embryogenic callus / somatic embryoids when sub cultured at five days interval. But when the cultures were kept without subculturing maximum time (8-9 weeks in Neelum and 6-7 weeks in Vellari Manga) were taken for inducing somatic embryogenesis.

5. Initiation of somatic embryoids

a. Plant growth substances

The embryogenic calli from the induction media were transferred to initiation media. Twenty treatments of plant growth substances were tried for initiation of somatic embryoids.

In the variety, Neelum, the treatment, T_{14} (2,4-D-2.0 mg/l + GA_3 5.0 mg/l and BA 1.0 mg/l) supplemented in the basal medium registered the highest

per cent cultures (72.72) initiating somatic embryoids from embryogenic callus of nucellus (Table 22; Plate 5). This was followed by T_{10} (63.64 per cent) and T_5 (54.55 per cent).

Table 21. Effect of frequency of subculture on the induction of somatic embryogenesis from nucellus of the mango varieties Neelum and Vellari Manga

Subculturing interval (days)	Cultures initiating embryogenic callus (%)	Cultures initiating somatic embryoids (%)
	Neelum	Vellari Manga
5	30.0	40.0
10	20.0	40.0
15	20.0	20.0
0	10.0	20.0

The data represent the average value of 10 replications

Culture medium for - Neelum: Half strength MS basal medium + 2,4-D 5.0mg/l + GA_3 5.0mg/l + CW 200.0ml/l + agar 5.0g/l + AC 2.5g/l + Sucrose 60.0g/l + glutamine 400.0mg/l

Culture medium for - Vellari Manga: Half strength MS basal medium + 2,4-D 2.0mg/l + GA_3 5.0mg/l + agar 6.0g/l + AC 2.5g/l + Sucrose 60.0g/l + glutamine 400.0mg/l

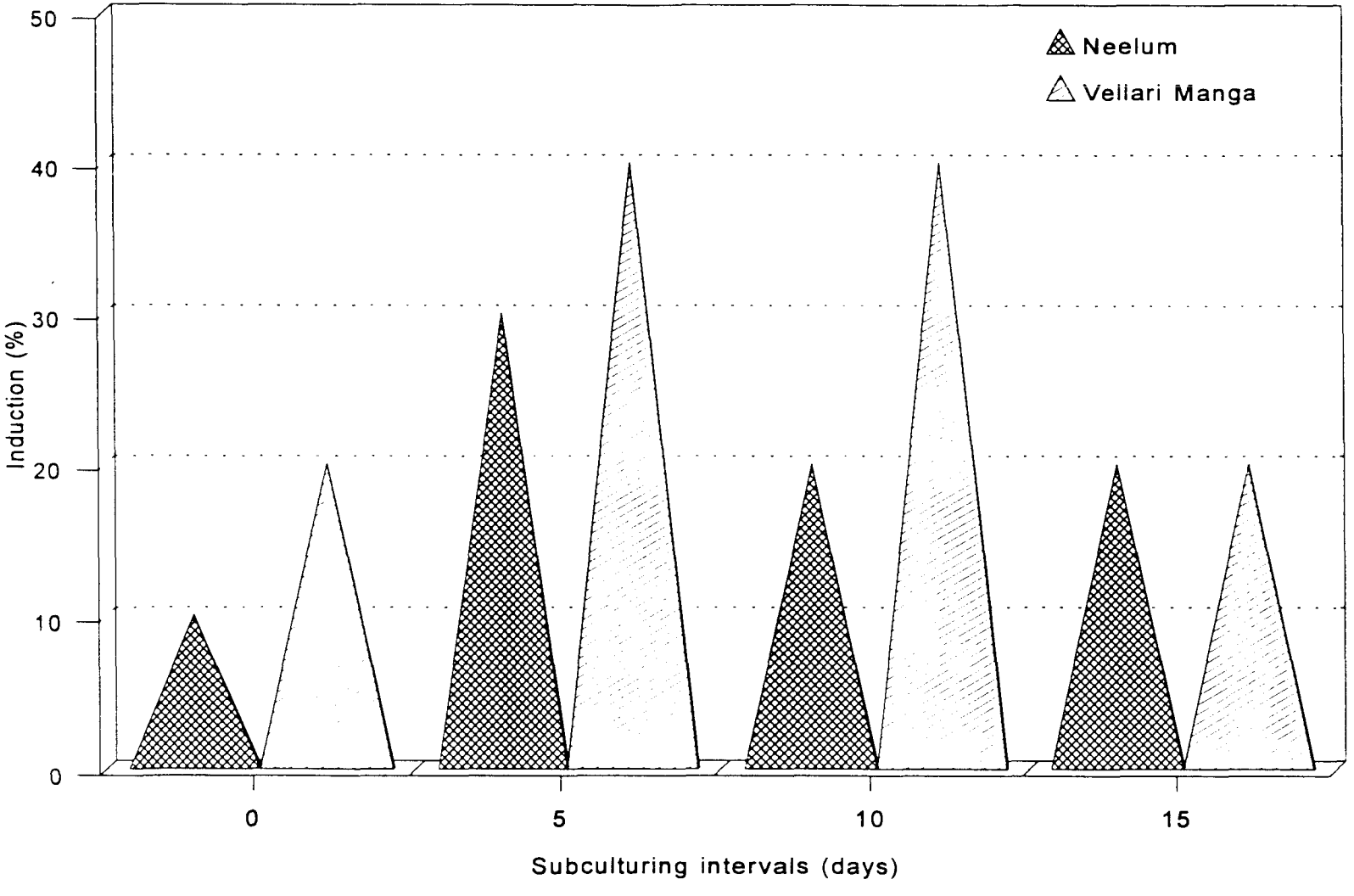


Fig. 3. Effect of frequency of subculture on the induction of somatic embryogenesis from nucellus of the mango varieties Neelum and Vellari Manga

Table 22. Effect of plant growth substances on the initiation of somatic embryoids from nucellus and embryo mass of the mango variety Neelum

Plant growth substance (mg/l)		Cultures initiating[a] somatic embryoids (%)		Number of[b] embryoids/culture	
		Nucellus	Embryo mass	Nucellus	Embryo mass
T_1	2,4-D 1.25 + GA_3 5.00 + BA 0.05	27.27	9.09	11.50	4.25
T_2	2,4-D 0.50 + GA_3 5.00 + BA 0.05	18.18	0	12.25	0
T_3	2,4-D 1.00 + GA_3 5.00 + BA 0.05	27.27	9.09	31.00	4.50
T_4	2,4-D 2.00 + GA_3 5.00 + BA 0.05	45.45	18.18	23.50	10.75
T_5	2,4-D 5.00 + GA_3 5.00 + BA 0.05	54.55	0	46.25	0
T_6	2,4-D 1.25 + GA_3 10.00 + BA 0.05	0	9.09	36.25	7.00
T_7	2,4-D 1.00 + GA_3 10.00 + BA 0.05	18.18	27.27	19.00	10.50
T_8	2,4-D 1.00 + GA_3 10.00 + BA 0.05	0	0	0	0
T_9	2,4-D 5.00 + GA_3 10.00 + BA 0.05	9.09	9.09	45.75	10.0
T_{10}	2,4-D 5.00 + GA_3 10.00 + BA 0.05	63.64	0	19.75	0
T_{11}	2,4-D 1.25 + GA_3 5.00 + BA 1.0	36.36	36.36	11.75	9.25
T_{12}	2,4-D 0.50 + GA_3 5.00 + BA 1.0	45.45	0	16.75	0
T_{13}	2,4-D 1.00 + GA_3 + 5.00 + BA 1.0	54.55	0	20.50	0
T_{14}	2,4-D 2.00 + GA_3 5.00 + BA 1.0	72.72	27.27	36.50	12.50
T_{15}	2,4-D 5.00 + GA_3 5.00 + BA 1.00	0	18.18	0	7.0
T_{16}	2,4-D 0.50 + GA_3 0 + BA 1.00	45.45	45.45	18.25	9.75
T_{17}	2,4-D 0 + GA_3 5.0 + BA 0	27.27	9.09	21.50	5.50
T_{18}	2,4-D 5.00 + GA_3 0 + BA 0	18.18	9.09	29.75	7.75
T_{19}	2,4-D 5.0 + GA_3 10.0 + BA 0	9.09	18.18	18.75	4.75
T_{20}	Control (No PGS)	0	0	0	0

a. The data represent the average of 11 replications

b. The data represent the average of 4 replications

Culture medium - Half strength MS basal medium + sucrose 60.0g/l + glutamine 400.0mg/l + CH 500.0 mg/l + agar 5.0 g/l + CW 200.0 mg/l + AC 2.5g/l

The highest number of embryoids produced per culture was 46.25 when the embryogenic callus from nucellar explants was subcultured on T_5 medium.

The response of the embryogenic callus from embryo mass was the best in T_{16}. The treatment initiated somatic embryoids in 45.45 per cent cultures. However, the mean number of embryoids per culture was the highest in T_4 (12.50), followed by T_9 (10.00). When the plant growth substances were not included in the medium, none of the cultures initiated somatic embryoids from both nucellus and embryo mass.

In the variety, Vellari Manga the treatment T_{12} (2,4-D 0.5 mg/l + GA_3 5.0 mg/l + BA 1.0 mg/l) initiated somatic embryoids in 86.67 per cent cultures (Table 23; Plate 6). This was followed by T_2 (73.30), T_6 (60.00) and T_{16} (60.00). The treatment, T_3, T_8, T_{14} and T_{15} did not respond. Initiation of somatic embryos in 13.33 per cent cultures was observed in basal media without plant growth substances.

The highest number of embryoids per culture was obtained when the embryogenic callus of nucellus was cultured in T_{12} (90.50), followed by T_{16} (81.50) and T_9 (75.25).

The embryogenic callus derived from embryo mass of Vellari Manga, when transferred to the treatments T_4 (2,4-D 2.0 mg/l, GA_3 5.0 mg/l and BA 0.05 mg/l) and T_{12} (2,4-D 0.5 mg/l + GA_3 5.0 mg/l and BA 1.0 mg/l) the highest percentage initiation of somatic embryos (40.00 per cent) resulted. T_5 (2,4-D 5.0 mg/l + GA_3 5.0 mg/l + BA 0.05 mg/l) recorded 33.33 percentage initiation of somatic embryoids. None of the cultures in T_1, T_9, T_{11}, T_{16} and T_{20} responded.

Plate 5

Different stages of somatic embryoids initiated from the nucellus of Neelum in half strength MS basal medium supplemented with 2,4-D 2.0 mg/l, GA_3 5.0 mg/l and BA 1.0 mg/l

Plate 6

Different stages of somatic embryoids initiated from the nucellus of Vellari Manga in half strength MS basal medium supplemented with 2,4-D 0.5 mg/l, GA_3 5.0 mg/l and BA 1.0 mg/l

Table 23. Effect of plant growth substances on the initiation of somatic embryoids from nucellus and embryo mass of the mango variety Vellari Manga

Plant growth substance (mg/l)		Cultures initiating[a] somatic embryoids (%)		Number of[b] embryoids/culture	
		Nucellus	Embryo mass	Nucellus	Embryo mass
T_1	2,4-D 1.25 + GA_3 5.00 + BA 0.05	20.00	0	16.50	0
T_2	2,4-D 0.50 + GA_3 5.00 + BA 0.05	73.33	20.00	33.75	5.50
T_3	2,4-D 1.00 + GA_3 5.00 + BA 0.05	0	13.33	0	8.50
T_4	2,4-D 2.00 + GA_3 5.00 + BA 0.05	40.00	40.00	40.00	20.25
T_5	2,4-D 5.00 + GA_3 5.00 + BA 0.05	33.33	33.33	50.00	10.25
T_6	2,4-D 1.25 + GA_3 10.00 + BA 0.05	60.00	20.00	62.50	11.50
T_7	2,4-D 0.05 + GA_3 10.00 + BA 0.05	46.67	13.33	18.75	6.25
T_8	2,4-D 1.00 + GA_3 10.00 + BA 0.05	0	6.67	0	13.75
T_9	2,4-D 2.00 + GA_3 10.00 + BA 0.05	20.0	0	75.25	0
T_{10}	2,4-D 5.00 + GA_3 10.00 + BA 0.05	20.0	13.33	8.25	6.75
T_{11}	2,4-D 1.25 + GA_3 5.00 + BA 1.00	13.33	0	36.50	0
T_{12}	2,4-D 0.50 + GA_3 5.00 + BA 1.00	86.67	40.00	90.50	15.25
T_{13}	2,4-D 1.00 + GA_3 5.00 + BA 1.00	46.67	13.33	40.25	14.00
T_{14}	2,4-D 2.00 + GA_3 5.00 + BA 1.00	0	6.67	0	8.0
T_{15}	2,4-D 5.00 + GA_3 5.00 + BA 1.00	0	20.0	0	10.25
T_{16}	2,4-D 0.50 + GA_3 0 + BA 1.00	60.00	0	81.50	0
T_{17}	2,4-D 0 + GA_3 5.00 + BA 0	46.67	20.00	49.75	7.50
T_{18}	2,4-D 5.00 + GA_3 10.00 + BA 0	0	26.67	0	10.25
T_{19}	2,4-D 5.00 + GA_3 10.00 + BA 0	26.67	13.33	18.25	11.25
T_{20}	Control (No PGS)	13.33	0	6.0	0

a. The data represent the average of 11 replications
b. The data represent the average of 4 replications

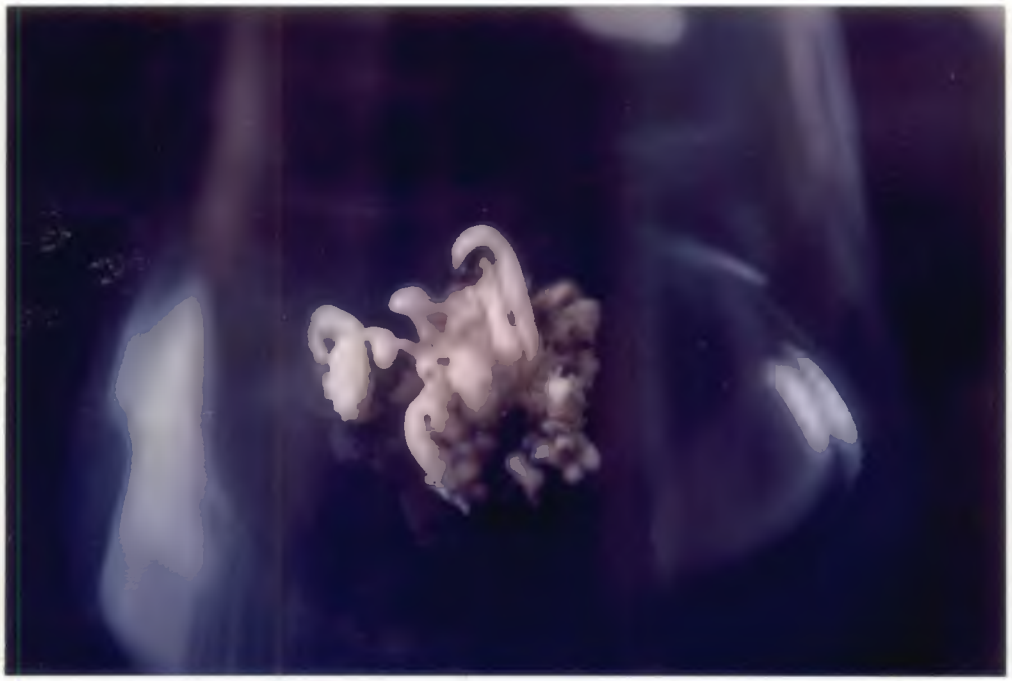
Culture medium - Half strength MS basal medium + sucrose 60.0g/l + glutamine 400.0mg/l + CH 600.0 mg/l + agar 5.5 g/l + CW 200.0 ml/l + AC 2.5g/l

Plate 7

Somatic embryoids initiated from the embryo mass of Neelum

Plate 8

Somatic embryoids initiated from the embryo mass of Vellari Manga



The highest number of embryoids per culture were produced in T_4 (20.25), followed by T_{12} (15.25) and T_8 media (13.75).

b. Basal media

In Neelum, half strength MS basal medium was the best in initiating somatic embryoids from nucellus and embryo mass. In half strength MS media, somatic embryoids were initiated in 56.56 per cent cultures (Table 24). In full strength MS and B_5 basal media somatic embryoids were initiated in 33.33 per cent in cultures. The embryogenic callus derived from embryo mass of Neelum initiated somatic embryoids in 22.22 per cent of cultures only in half strength MS basal medium (Plate 7). Full strength MS, B_5 and SH basal media did not support the initiation of somatic embryoids from embryogenic callus of embryo mass in the mango variety Neelum.

The number of embryoids per culture from the embryogenic callus of nucellus in Neelum was significantly the highest in half strength MS basal medium (48.80). The least response was observed when subcultured in SH basal medium (11.11 embryoids / culture). The embryogenic callus obtained from embryo mass of Neelum when subcultured in half strength MS basal medium gave 7.50 somatic embryoids/culture. None of the cultures in other media responded.

In Vellari Manga, half strength MS basal medium initiated somatic embryoids from the embryogenic callus of nucellus in 77.78 per cent cultures which was the highest (Table 25). The per cent cultures initiating somatic

embryoids when cultured in full strength MS basal medium was 55.56. The per cent cultures initiating somatic embryoids in B_5 and SH basal media were 33.33 and 22.22 respectively.

Table 24. Effect of basal media on the initiation of somatic embryoids from nucellus and embryo mass of the mango variety Neelum

Basal medium	Cultures initiating somatic embryoids (%)		Number of embryoids / culture	
	Nucellus	Embryo mass	Nucellus	Embryo mass
1/2 MS	56.56	22.22	48.80	7.50
MS	33.33	0	30.80	0
B5	33.33	0	29.00	0
SH	11.11	0	10.20	0
CD (5%)			13.09	

The data represent the average value of 12 replications

Supplements in culture medium - 2,4-D 2.0mg/l + GA_3 5.0mg/l + BA 1.0mg/l + Sucrose 60.0g/l + glutamine 400.mg/l + CH 500.0mg/l + agar 5.0g/l + CW 200.0ml/l + AC 2.5g/l

Table 25. Effect of basal media on the initiation of somatic embryoids from nucellus and embryo mass the mango variety Vellari Manga

Basal medium	Cultures initiating somatic embryoids (%)		Number of embryoids / culture	
	Nucellus	Embryo mass	Nucellus	Embryo mass
1/2 MS	77.78	33.33	63.20	17.80
MS	55.56	0	66.20	0
B5	33.33	11.11	16.60	8.10
SH	22.22	0	14.80	0
CD (1%)			16.77	

The data represent the average value of 12 replications

Supplements in culture medium - 2,4-D 0.5mg/l + GA_3 5.0mg/l + BA 1.0mg/l + sucrose 60.0g/l + glutamine 400.mg/l + CH 600.0mg/l + agar 5.5g/l + CW 200.0ml/l + AC 2.5g/l

The highest number of embryoids per culture obtained from nucellus was when cultured on full strength MS basal medium (66.20), which was on par with half strength MS basal medium (63.20). The number of embryoids/ culture produced from the embryogenic callus of embryo mass in half strength MS basal medium was 17.80 (Table 25; Plate 8), whereas, in full strength MS basal medium, somatic embryoids did not initiate.

c. Sucrose

In Neelum, sucrose 60.0g/l in the medium favoured the initiation of somatic embryoids. Eighty per cent cultures initiated somatic embryoids from the embryogenic callus of nucellus (Table 26). The Sucrose levels, 50.0g/l and 60.0g/l were found to be the best in initiating somatic embryoids from the embryogenic callus of embryo mass in Neelum (40.0 per cent). Number of embryoids/culture was highest at 50.0g/l from the embryogenic callus of both nucellus and embryo mass of Neelum (58.50 and 10.60, respectively) (Fig. 4).

Table 26. Effect of sucrose on the initiation of somatic embryoids from nucellus and embryo mass of the mango variety Neelum

Sucrose (g/l)	Cultures initiating somatic embryoids (%)		No. of embryoids per culture	
	Nucellus	Embryo mass	Nucellus	Embryo mass
40.0	40.00	20.00	45.83	2.80
50.0	60.00	40.00	58.50	10.60
60.0	80.00	40.00	57.66	8.50
CD			NS	5.14

The data represent the average value of 5 replications

NS - Not significant

Culture medium - Half strength MS basal medium + 2,4-D 2.0mg/l + GA_3 5.0mg/l + BA 1.0mg/l + glutamine 400.0mg/l + CH 500.0mg/l + CW 200.0ml/l + agar 5.0g/l + AC 2.5g/l

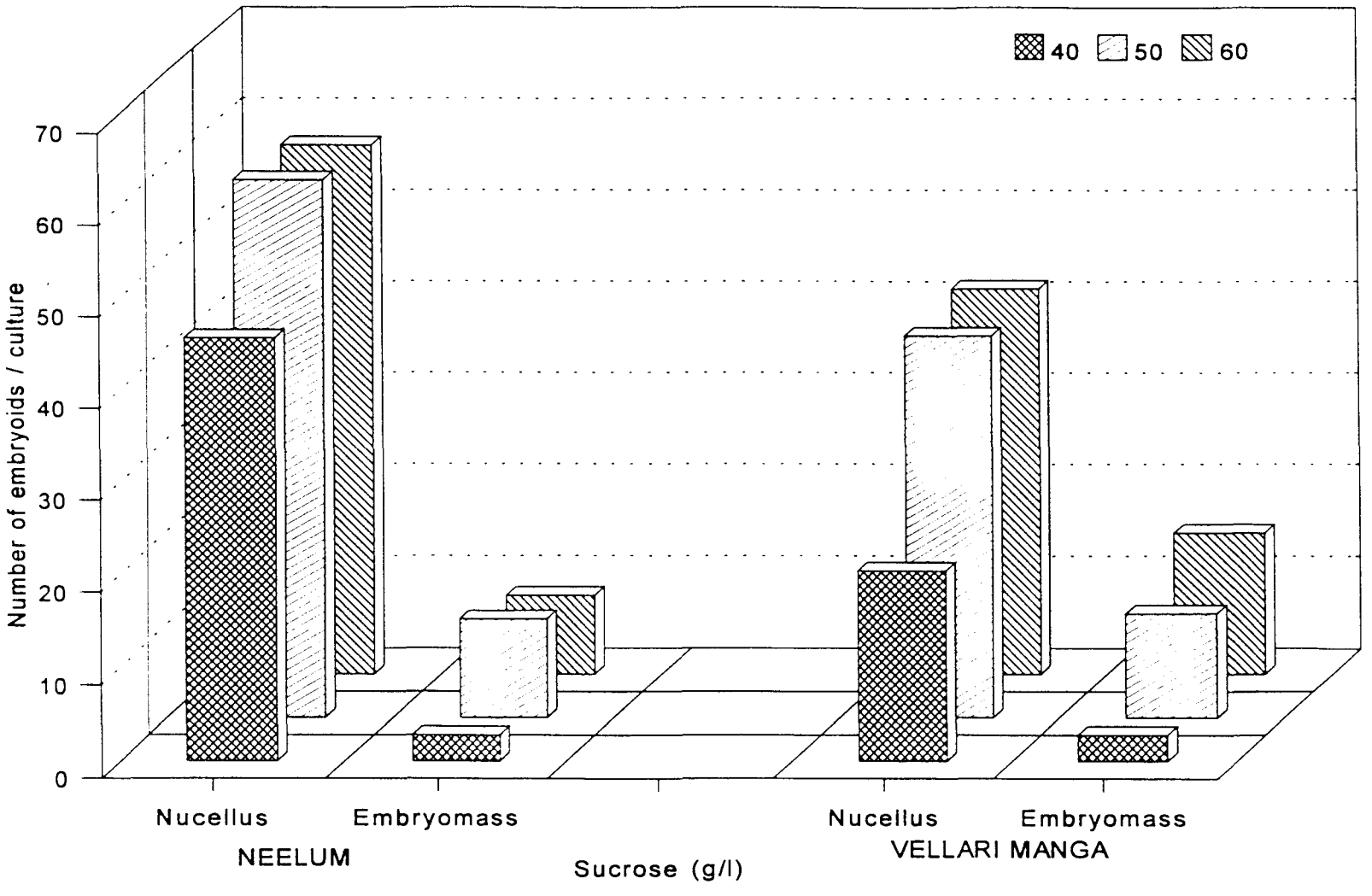


Fig. 4. Effect of sucrose on the number of somatic embryoids per culture from nucellus and embryo mass of the mango varieties Neelum and Vellari Manga

Sucrose 50.0 g/l and 60.0 g/l favoured the initiation of somatic embryoids from embryogenic callus of nucellus in Vellari Manga (60.0 per cent cultures each). The highest number of embryoids per culture was 42.00 when sucrose 60.0g/l was used in the medium (Table 27). However, the effect was on par with sucrose 50.0g/l producing 41.50 embryoids / culture. Sucrose 40.0g/l and 60.0g/l produced the highest per cent cultures initiating somatic embryoids from the embryogenic callus of embryo mass (20.00). However, the number of embryoids produced per culture was highest at sucrose 60.0g/l (15.30) which was on par with sucrose 50.0g/l (11.20) (Fig. 4)

Table 27. Effect of sucrose on the initiation of somatic embryoids from nucellus and embryo mass of the mango variety Vellari Manga

Sucrose (g/l)	Cultures initiating somatic embryoids (%)		No. of embryoids per culture	
	Nucellus	Embryo mass	Nucellus	Embryo mass
40.0	40.00	20.00	20.50	2.80
50.0	60.00	10.00	41.50	11.20
60.0	60.00	20.00	42.00	15.30
CD (1%)			13.18	7.01

The data represent the average value of 5 replications

Culture medium - Half strength MS basal medium + 2,4-D 0.5mg/l + GA_3 5.0mg/l + BA 1.0mg/l + glutamine 400.0mg/l + CH 600.0mg/l + CW 200.0ml/l + agar 5.5g/l + AC 2.5g/l

d. Casein hydrolysate

Casein hydrolysate 500.0mg/l initiated somatic embryoids from embryogenic callus of nucellus in 66.67 per cent cultures of Neelum. The same treatment initiated the highest number of embryoids per culture (54.00) which was on par with casein hydrolysate 600.0mg/l (45.60). From the embryogenic callus of embryo mass in Neelum, 16.67 per cent cultures initiated somatic embryoids when casein hydrolysate 500.0mg/l was used in the initiation medium (Table 28). But the number of embryoids produced per culture from the embryogenic callus of embryo mass was not significant. When no casein hydrolysate was used somatic embryoids were not initiated.

Table 28. Effect of casein hydrolysate on the initiation of somatic embryoids from nucellus and embryo mass of the mango variety Neelum

CH (mg/l)	Cultures initiating somatic embryoids (%)		No. of embryoids per culture	
	Nucellus	Embryo mass	Nucellus	Embryo mass
400.0	33.33	8.33	32.20	18.20
500.0	66.67	16.67	54.00	21.35
600.0	50.00	8.33	45.60	25.38
0	8.33	0	8.80	0
CD (5%)			18.76	NS

The data represent the average value of 7 replications

NS - Not significant

Culture medium - Half strength MS basal medium + 2,4-D 2.0mg/l + GA_3 5.0mg/l + BA 1.0mg/l + sucrose 60.0g/l + glutamine 400.0mg/l + CW 200.0mg/l + agar 5.0g/l + AC 2.5g/l

In Vellari Manga, CH 500.0mg/l was found to be the best in initiating somatic embryoids from nucellus and embryogenic callus of embryo mass (Table 29). The highest per cent cultures initiating somatic embryoids from nucellus was at casein hydrolysate 500.0mg/l (85.71). At casein hydrolysate 600.0mg/l, the per cent cultures initiating of somatic embryoids was 71.43.

Table 29. Effect of casein hydrolysate on the initiation of somatic embryoids from nucellus and embryo mass of the mango variety Vellari Manga

CH (mg/l)	Cultures initiating somatic embryoids (%)		No. of embryoids per culture	
	Nucellus	Embryo mass	Nucellus	Embryo mass
400	57.14	14.29	52.80	13.0
500	85.71	28.57	72.80	14.50
600	71.43	0	79.80	0
0	14.29	14.29	23.60	5.75
CD (5%)			19.48	10.13

The data represent the average value of 7 replications

NS - Not significant

Culture medium - Half strength MS basal medium + 2,4-D 0.5mg/l + GA_3 5.0mg/l + BA 1.0mg/l + sucrose 60.0g/l + glutamine 400.0mg/l + CW 200.0mg/l + agar 5.5g/l + AC 2.5g/l

The percentage was 14.29, when no CH was used. The per cent cultures initiating somatic embryoids from the embryogenic callus of embryo mass was highest at CH 500.0mg/l (28.57). At CH 600.0mg/l, somatic embryoids from embryogenic callus of embryo mass did not initiate. The highest number of embryoids from nucellus was obtained at CH 600.0mg/l (79.80). The treatment was on par with CH 500.0mg/l producing 72.80 embryoids per culture. The highest number of embryoids produced from the embryogenic callus of embryo mass was at CH 500.0mg/l (14.50).

e. Glutamine

Glutamine 400.0mg/l was the best in initiating somatic embryoids from the embryogenic callus of nucellus and embryo mass in Neelum. Embryogenic callus of nucellus initiated somatic embryoids in 62.50 per cent cultures (Table 30). The embryogenic callus of embryo mass initiated somatic embryoids in 25.00 per cent cultures. At glutamine 600.0mg/l, embryogenic callus of nucellus initiated somatic embryoids in 50.00 per cent cultures and the embryogenic callus of embryo mass did not initiate somatic embryoids. The number of embryoids produced per culture from nucellus was significantly the highest at glutamine 400.0mg/l (81.83).

Table 30. Effect of glutamine on the initiation of somatic embryoids from nucellus and embryo mass of the mango variety Neelum

Glutamine (mg/l)	Cultures initiating somatic embryoids (%)		Number of embryoids/ culture	
	Nucellus	Embryo mass	Nucellus	Embryo mass
400.0	62.50	25.00	81.83	10.75
500.0	50.00	12.50	59.16	8.11
600.0	50.00	0	46.00	0
CD (5%)			22.64	

The data represent the average value of 8 replications

Culture medium - Half strength MS basal medium + 2,4-D 2.0mg/l + GA_3 5.0mg/l + BA 1.0mg/l + CH 500.0mg/l + sucrose 60.0g/l + CW 200.0ml + agar 5.5g/l + AC 2.5g/l

In Vellari Manga, glutamine 400.0mg/l and 600.0mg/l initiated somatic embryoids in 87.50 per cent cultures and 62.50 per cent cultures from nucellus (Table 31). The embryogenic callus from embryo mass did not initiate somatic embryoids either at glutamine 400.0mg/l, 500.0mg/l or 600.0mg/l. The number of embryoids produced per culture from nucellus was not significant.

Table 31. Effect of glutamine on the initiation of somatic embryoids from nucellus and embryo mass of the mango variety Vellari Manga

Glutamine (mg/l)	Cultures initiating somatic embryoids (%)		Number of embryoids/ culture	
	Nucellus	Embryo mass	Nucellus	Embryo mass
400.0	87.50	0	105.83	0
500.0	25.00	0	80.83	0
600.0	62.50	0	85.83	0
CD			NS	

The data represent the average value of 8 replications

NS - Not significant

Culture medium - Half strength MS basal medium + 2,4-D 0.5mg/l + GA_3 5.0mg/l + BA 1.0mg/l + sucrose 60.0g/l + CH 600.0mg/l + CW 200.0mg/l + agar 5.5g/l + AC 2.5g/l

f. Coconut water

Coconut water 200.0 ml/l was found to the best in initiating somatic embryoids from nucellus and embryogenic callus of Neelum. The treatment recorded 60.00 per cent cultures initiating somatic embryoids and produced 56.00 number of embryoids per culture from the embryogenic callus of nucellus (Table 32). Coconut water 200.0ml/l initiated somatic embryoids in 10.00 per cent cultures and produced 12.0 embryoids per culture from embryogenic callus of embryo mass.

Table 32. Effect of coconut water on the initiation of somatic embryoids from nucellus and embryo mass of the mango variety Neelum

Coconut water (ml/l)	Cultures initiating somatic embryoids (%)		Number of embryoids/ culture	
	Nucellus	Embryo mass	Nucellus	Embryo mass
150.0	50.00	0.0	38.40	0
200.0	60.00	10.00	56.00	12.00
250.0	50.00	10.00	44.00	9.25
0	10.00	0	18.00	0
CD (5%)			14.26	

The data represent the average value of 10 replications

Culture medium - Half strength MS basal medium + 2,4-D 2.0mg/l + GA_3 5.0mg/l + BA 1.0mg/l + sucrose 60.0g/l + CH 500.0mg/l + agar 5.0g/l + AC 2.5g/l + glutamine 400.0mg/l

When coconut water was not used in the culture media, none of the cultures initiated somatic embryoids from the embryogenic callus of embryo mass in Neelum. However, the embryogenic callus of nucellus initiated somatic embryoids in 10.00 per cent cultures in the absence of coconut water. This treatment resulted in 18.00 embryoids per culture.

In Vellari Manga, coconut water 200.0ml/l and 250.0ml/l initiated somatic embryoids from nucellus in 80.00 per cent cultures. However, the number of embryoids produced per culture was not significant (Table 33).

Table 33. Effect of coconut water on the initiation of somatic embryoids from nucellus and embryo mass of the mango variety Vellari Manga

Coconut water (ml/l)	Cultures initiating somatic embryoids (%)		Number of embryoids/ culture	
	Nucellus	Embryo mass	Nucellus	Embryo mass
150.0	50.00	10.00	30.20	10.80
200.0	80.00	20.00	36.00	12.50
250.0	80.00	10.00	35.00	10.10
CD			NS	NS

The data represent the average value of 10 replications

NS - Not significant

Culture medium - Half strength MS basal medium + 2,4-D 0.5mg/l + GA_3 5.0mg/l + BA 1.0mg/l + sucrose 60.0g/l + CH 600.0mg/l + agar 5.5g/l + AC 2.5g/l + glutamine 400.0mg/l

The embryogenic callus of embryo mass also showed the highest response at coconut water 200.0ml/l. None of the cultures initiated somatic embryoids when coconut water was not added to the initiation medium.

g. Solidifying agent (agar)

Agar 5.0g/l and 5.5g/l when added to the medium were found to be best in initiating somatic embryoids in 70.00 per cent cultures from the embryogenic callus of Neelum (Table 34). But the highest number of

embryoids per culture was recorded when agar 5.0g/l was added to the medium (44.33) which was on par with medium containing agar 4.5g/l (38.33) and agar 5.5g/l (36.33). In liquid media, only 10.00 per cent cultures initiated somatic embryoids from embryogenic callus of nucellus, producing 19.67 embryoids per culture. In liquid media, embryogenic callus from embryo mass did not initiate somatic embryoids. The embryogenic callus of embryo mass initiated somatic embryoids in 30.00 per cent cultures which was the highest and produced 12.30 embryoids per culture when agar in the medium was 5.0g/l.

Table 34. Effect of agar on the initiation of somatic embryoids from nucellus and embryo mass of the mango variety Neelum

Agar (g/l)	Cultures[a] initiating somatic embryoids (%)		Number of[b] embryoids / culture	
	Nucellus	Embryo mass	Nucellus	Embryo mass
4.5	60.00	0	38.33	0
5.0	70.00	30.00	44.33	12.30
5.5	70.00	20.00	36.33	7.80
6.0	50.00	10.00	22.33	9.00
6.5	20.00	10.00	17.00	0
0	10.00	0	19.67	0
CD (1%)			13.44	

a - The data represent the average value of 10 replications

b - The data represent the average value of 8 replications

Culture medium - Half strength MS basal medium + 2,4-D 2.0mg/l + GA_3 5.0mg/l + BA 1.0mg/l + Sucrose 60.0g/l + CW 200.0 mg/l + AC 2.5g/l + CH 500.0mg/l + glutamine 400.0mg/l

In Vellari Manga, agar 5.5g/l and 4.5g/l was the best in initiating somatic embryoids from nucellus (50.00 per cent). However, the highest number of embryoids per culture (53.33) was observed in the treatment having agar 5.5g/l. The treatment with agar 4.5g/l produced only 23.33 number of embryoids per culture. The treatment with agar 5.0g/l initiated somatic embryoids in 10.00 per cent cultures of the embryogenic callus of embryo mass. There were 12.50 embryoids per culture (Table 35). In liquid media, both nucellus and embryogenic calli of embryo mass initiated somatic embryoids, the per cent initiation being 30.00 and 10.00 respectively. The number of embryoids per culture were also less with 16.00 and 6.90 from nucellus and embryogenic calli of embryo mass, when cultured in liquid media.

Table 35. Effect of agar on the initiation of somatic embryoids from nucellus and embryo mass of the mango variety Vellari Manga

Agar (g/l)	Cultures initiating[a] somatic embryoids (%)		Number of embryoids/[b] culture	
	Nucellus	Embryo mass	Nucellus	Embryo mass
4.5	50.00	10.00	23.33	11.80
5.0	40.00	10.00	49.67	12.50
5.5	50.00	0	53.33	0
6.0	40.00	0	50.00	0
6.5	30.00	0	25.33	0
0	30.00	10.00	16.00	6.90
CD (1%)			15.65	

a - The data represent the average value of 10 replications
b - The data represent the average value of 8 replications

Culture medium - Half strength MS basal medium + 2,4-D 0.5mg/l + GA_3 5.0mg/l + BA 1.0mg/l + Sucrose 60.0g/l + CH 600.0mg/l + CW 200.0ml/l + AC 2.5g/l + glutamine 400.0mg/l

h. Activated charcoal

Activated charcoal when used at the rate of 2.5g/l in the media, initiated somatic embryoids in 50.00 per cent cultures from the embryogenic callus of nucellar explants of Neelum (Table 36). The highest number of embryoids per culture was also observed at charcoal 2.5 g/l (28.67).

In Vellari Manga, the highest per cent initiation of somatic embryoids from nucellus (43.80) was at activated charcoal 1.5g/l in the medium. The number of embryoids produced per culture was not significant.

Table 36. Effect of activated charcoal on the initiation of somatic embryoids from nucellus of the mango varieties Neelum and Vellari Manga

Activated charcoal (g/l)	Cultures initiating somatic embryoids (%)		Number of embryoids/ culture	
	Neelum	Vellari Manga	Neelum	Vellari Manga
1.5	33.33	43.80	16.67	19.50
2.5	50.00	40.00	28.67	19.17
5.0	33.33	20.00	10.33	15.67
CD (5%)			12.55	NS

The data represent the average value of 8 replications

NS - Not significant

Culture medium for Neelum - Half strength MS basal medium + 2,4-D 2.0mg/l + GA_3 5.0mg/l + BA 1.0mg/l + sucrose 60.0g/l + CH 500.0mg/l + CW 200.0ml/l + agar 5.0g/l + glutamine 400.0mg/l

Culture medium for Vellari Manga - Half strength MS basal medium + 2,4-D 0.5mg/l + GA_3 5.0mg/l +BA 1.0mg/l + sucrose 60.0g/l + CH 600.0mg/l + CW 200.0ml/l + agar 5.5g/l + glutamine 400.0mg/l

i. Culture conditions

In both Neelum and Vellari Manga, when the cultures were incubated in darkness and low temperature at (26°C ± 2°C) somatic embryoids were initiated at higher rate as compared to light (3000 lux; 16h photoperiod and ambient temperature at (32° ± 2°C) from nucellar explants (Table 37). In darkness plus low temperature (at 26°C ± 2°C), cultures of Neelum initiated somatic embryoids in 66.67 per cent of cultures and produced 50.80 embryoids/culture. In light plus low temperature (at 26°C ± 2°C), the per cent cultures initiating somatic embryoids was only 16.67 with 13.15 number of somatic embryoids / culture. In light at room temperature somatic embryoids did not initiate from the embryogenic callus of Neelum nucellus.

In Vellari Manga somatic embryoids were observed in 83.33 per cent cultures in darkness plus low temperature (26° ± 2°C). There were 60.10 embryoids/culture. In a culture condition of darkness and room temperature, the nucellus of Vellari Manga initiated somatic embryoids in 66.67 per cent cultures and produced 50.60 embryoids per culture.

j. Frequency of subculturing

Of the four frequencies of subculturing tested, the cultures of both Neelum and Vellari Manga when subcultured at five days and ten days interval, gave the best responses in the initiating somatic embryoids (Table 38).

Table 37. Effect of culture conditions on the initiation of somatic embryoids from the nucellus of the mango varieties Neelum and Vellari Manga

Culture conditions	Cultures initiating somatic embryoids (%)		Number of embryoids/ culture	
	Neelum	Vellari Manga	Neelum	Vellari Manga
Light (3000 lux; 16h photoperiod) + Low temperature (26 ± 2°C)	16.67	16.67	13.15	20.0
Darkness + Low temperature (26 ± 2°C)	66.67	83.33	50.80	60.10
Darkness + Room temperature (32 ± 2°C)	50.00	66.67	18.35	50.60
Light (3000 lux; 16h photoperiod) + Room temperature (32 ± 2°C)	0	16.67	0	5.10

The data represent the average value of 6 replications

Culture medium - for Neelum: Half strength MS basal medium + 2,4-D 2.0mg/l + GA_3 5.0mg/l + BA 1.0mg/l + sucrose 60.0g/l + CH 500.0mg/l + CW 200.0ml/l + agar 5.0g/l + AC 2.5g/l + glutamine 400.0m/l

Culture medium - for Vellari Manga: Half strength MS basal medium + 2,4-D 0.5mg/l + GA_3 5.0mg/l + BA 1.0mg/l + sucrose 60.0g/l + CH 600.0mg/l + CW 200.0ml/l + agar 5.5g/l + AC 2.5g/l + glutamine 400.0mg/l

Table 38. Effect of frequency of subculture on the initiation of somatic embryoids from nucellus of the mango varieties Neelum and Vellari Manga

Subculturing intervals (days)	Cultures survived (%)		Cultures initiating embryoids (%)	
	Neelum	Vellari Manga	Neelum	Vellari Manga
5	77.78	77.78	44.44	55.56
10	77.78	88.89	66.67	55.56
15	33.33	66.67	33.33	50.00
0	33.33	55.56	22.22	33.33

The data represent the average value of 9 replications

Culture medium for Neelum - Half strength MS basal medium + 2,4-D 2.0mg/l + GA_3 5.0mg/l + BA 1.0mg/l + sucrose 60.0g/l + CH 500.0mg/l + CW 200.0ml/l + agar 5.0g/l + AC 2.5g/l + glutamine 400.0mg/l

Culture medium for Vellari Manga - Half strength MS basal medium + 2,4-D 0.5mg/l + GA_3 5.0mg/l + BA 1.0mg/l + sucrose 60.0g/l + CH 600.0mg/l + CW 200.0ml/l + agar 5.5g/l + AC 2.5g/l + glutamine 400.0mg/l

When subcultured at five days interval the embryogenic callus of Neelum nucellus initiated somatic embryoids in 44.44 per cent cultures and Vellari Manga in 55.56 per cent cultures. In Neelum, when the embryogenic calli were subcultured at 10 days interval, 66.67 per cent cultures initiated somatic embryoids. In Vellari Manga, the corresponding value was 55.56 per cent. When no subculturing was done, only less per cent cultures initiated somatic embryoids from nucellus in both Neelum (22.22 per cent) and Vellari Manga (33.33 per cent).

6. Maturation of somatic embryoids

a. Abscisic acid (ABA)

Different levels of ABA were tried to study its effect on the maturation of somatic embryoids. In Neelum, when ABA 5.0 mg/l was used in the maturation media 75.0 per cent cultures survived (Table 39). This treatment had a low frequency of secondary somatic embryoid production. The treatment produced large sized (1.0-2.0cm) light brown somatic embryoids (Plate 9). When ABA 4.22mg/l was used in the maturation media, 87.50 per cent cultures survived and the size was 1.0-1.5cm. At the lowest concentration of ABA (0.50 mg/l) tried, a high frequency of secondary somatic embryoid production was observed. This treatment produced small somatic embryoids (< 0.5cm). The colour of embryoids was cream. At the highest level of ABA (10.57mg/l), the size of embryoids were 0.5cm and only 12.50 per cent cultures survived.

The size of somatic embryoids of Vellari Manga when ABA 4.22 mg/l and 5.0 mg/l were added in the maturation medium was 1.5-2.0 cm and 1.0-2.0cm, respectively (Table 39). At ABA 4.22mg/l, the frequency of secondary somatic embryo production was low. The colour of embryoids varied from cream to light brown. At the highest level of ABA (10.57 mg/l), the size of embryoids ranged from 0.5-1.0cm. They were cream in colour.

Table 39. Effect of abscisic acid on the maturation of somatic embryoids of the mango varieties Neelum and Vellari Manga

ABA (mg/l)	Neelum				Vellari Manga			
	[a]Cultures survived (%)	Secondary somatic embryo-genesis	[b]Size of embryoids (length in cm)	Colour of embryoids	[a]Cultures survived (%)	Secondary somatic embryo-genesis	[b]Size of embryoids (length in cm)	Colour of embryoids
0.50	25.0	High	< 0.5	Cream	37.50	High	0.5	Cream
1.00	37.5	High	0.5-10	Cream	50.00	Medium	< 0.5	Cream
1.50	12.5	Medium	< 0.5	Cream	37.50	Medium	0.5-1.0	Brown
2.11	50.0	High	0.5	Light brown	100.0	Medium	0.5	Cream
3.17	75.0	Medium	0.5-1.0	Cream	37.50	Low	1.0-1.5	Cream
4.22	87.5	Medium	1.0-1.5	Brown	50.00	Low	1.5-2.0	Light brown
5.00	75.0	Low	1.0-2.0	Light brown	62.50	Medium	1.0-2.0	Cream
8.44	50.0	Low	0.5-1.0	Cream	37.50	Low	1.0-1.5	Cream
10.57	12.5	Low	0.5	Cream	37.50	Low	0.5-1.0	Cream

a. The data represent the average value of 8 replications

b. The data represent the average value of 10 replications

Low 0-5 secondary embryoids

Medium 5-25 secondary embryoids

High 25 and above secondary embryoids

Culture medium - for Neelum: Basal medium with B5 major salts + MS minor salts + sucrose 50.0g/l + CH 100.0mg/l + CW 200.0ml/l + PVP 10.0g/l + agar 4.5g/l

Culture medium - for Vellari Manga: Basal medium with B5 major salts + MS minor salts + sucrose 40.0g/l + CH 100.0mg/l + CW 200.0ml/l + PVP 10.0g/l + agar 6.0g/l

Plate 9

Somatic embryoids of Neelum in maturation medium
(Basal medium containing of B_5 major salts and MS minor salts
supplemented with abscisic acid 5.0 mg/l)

Plate 10

Vellari Manga somatic embryoids in basal medium containing B_5 major salts and
MS minor salts supplemented with abscisic acid 4.22 mg/l

b. Basal medium

In both Neelum and Vellari Manga, basal medium consisting of B_5 major salts and MS minor salts was the best for the maturation of somatic embryoids (Table 40). In Neelum, 51.14 per cent cultures survived in this medium. The embryoids were cream and large sized (1.0-1.5cm in length). In full strength and half strength MS basal medium, though a higher per cent survival could be observed (71.43 and 85.71 respectively), the size of the embryoids was below 0.5cm. B_5 and SH basal media also did not support the maturation of somatic embryoids producing under sized embryoids (< 0.5cm).

In Vellari Manga, basal medium consisting of B_5 major salts and MS minor salts supported, 85.71 per cent survival of cultures and produced cream coloured somatic embryoids of 1.0-1.5cm size (Plate 10). When full strength MS as well as B_5 basal media were used, the size of the somatic embryoids was less than 0.5cm (Table 40).

c. Sucrose

When sucrose 40.0 g/l or 50.0 g/l was added in the maturation media, the somatic embryoids produced were 1.0-1.5cm in length, in Neelum (Table 41). With 40.0 g/l sucrose, 80.0 per cent cultures survived, whereas with 50.0 g/l, only 20.0 per cent of the cultures survived. Sucrose 15.0 g/l, used in the medium could not increase the size of embryoids. All the embryoids were below 0.5cm and were cream in colour.

Table 40. Effect of basal media on the maturation of somatic embryoids of the mango varieties Neelum and Vellari Manga

Basal medium	Neelum			Vellari Manga		
	Cultures survived (%)	Size of embryoids (length in cm)	Colour of embryoids	Cultures survived (%)	Size of embryoids (length in cm)	Colour of embryoids
MS	71.43	< 0.5	Creamy white	85.71	< 0.5	Cream
1/2 MS	85.71	< 0.5	Cream	100.00	1.0	Cream
B_5	42.86	< 0.5	Cream	42.86	< 0.5	Light brown
1/2 B_5	42.86	0.5-1.0	Brown	85.71	0.50	Cream
B_5 major + salts MS minor salts	51.14	1.0-1.5	Cream	85.71	1.0-1.5	Cream
MS major + salts B5 minor salts	42.86	0.5-1.0	Cream	51.14	0.5	Cream
SH	28.57	< 0.5	Brown	42.86	0.5	Cream

The data represent the average value of 7 replications

Supplements in the culture medium for Neelum - Sucrose 50.0g/l + ABA 5.0mg/l + CH 100.0 mg/l + CW 200.0ml/l + PVP 10.0g/l + agar 4.5g/l

Supplements in the culture medium for Vellari Manga - Sucrose 40.0g/l + ABA 4.22mg/l + CH 100.0 mg/l + CW 200.0ml/l + PVP 10.0g/l + agar 6.0g/l

Table 41. Effect of sucrose on the maturation of somatic embryoids of the mango varieties Neelum and Vellari Manga

Sucrose (g/l)	Neelum			Vellari Manga		
	Cultures survived (%)	Size of embryoids (length in cm)	Colour of embryoids	Cultures survived (%)	Size of embryoids (length in cm)	Colour of embryoids
15.0	40.00	< 0.5	Cream	20.00	< 0.5	Cream
20.0	40.00	0.5	Cream	20.00	< 0.5	Cream
30.0	80.00	0.5-1.0	Light brown	60.00	< 0.5	Cream
40.0	80.00	1.0-1.50	Cream	40.00	1.0-2.0	Cream
50.0	20.00	1.0-1.50	Cream	60.00	0.5-1.0	Light brown
60.0	60.00	0.5	Light brown	20.00	0.5-1.0	Light brown

The data represent the average value of 10 replications

Culture medium - for Neelum: Basal medium with B_5 major salts + MS minor salts + ABA 5.0mg/l + CH 100.0mg/l + CW 200.0ml/l + PVP 10.0g/l + agar 4.5g/l

Culture medium - for Vellari Manga: Basal medium with B_5 major salts + MS minor salts + ABA 4.22mg/l + CH 100.0mg/l + CW 200.0ml/l + PVP 10.0g/l + agar 6.0g/l

In Vellari Manga, sucrose 40.0 g/l in the maturation medium was the best which produced cream coloured somatic embryoids of 1.0-2.0cm size (Table 41). With 50.0 g/l and 60.0 g/l sucrose in the medium, the somatic embryoids produced were 0.5-1.0cm in size and were light brown.

d. Solidifying agent (agar)

Agar 4.5 g/l, 5.0 g/l and 5.5 g/l favoured maturation and yielded somatic embryoids of 0.5-1.0cm size, in Neelum (Table 42). When the somatic embryoids were cultured in maturation medium with 4.5 g/l agar, 80.0 per cent of the cultures survived. The treatments with 5.0 g/l and 5.5 g/l agar supported 60.0 per cent survival of the cultures. The colour of embryoids were cream and light brown. The somatic embryoids in the treatment with 7.0 g/l agar, the size of embryoids was below 0.5cm and were brown coloured. In liquid medium, 20.0 per cent of cultures survived. The embryoids were large sized (1.5-2.0cm). However, vitrification of the embryoids was observed (Plate 11).

In Vellari Manga, agar 5.0 g/l favoured the production of the largest embryoids (1.5-2.0cm). The embryoids were cream coloured (Table 42). Agar, 4.5g/l favoured the production of cream embryoids of size 0.5-1.0cm. In Vellari Manga also, in liquid medium, light brown somatic embryoids of 1.0-1.5cm size were obtained. But the resultant embryoids were vitrified and gradually necrosis developed.

Plate 11

Somatic embryoids of Neelum in liquid maturation media

Plate 12

A matured somatic embryo of Vellari Manga in germination medium containing B_5 major salts and MS minor salts supplemented with BA 1.0 mg/l

Table 42. Effect of agar on the maturation of somatic embryoids of the mango varieties Neelum and Vellari Manga

Agar (g/l)	Neelum			Vellari Manga		
	Cultures survived (%)	Size of embryoids (length in cm)	Colour of embryoids	Cultures survived (%)	Size of embryoids (length in cm)	Colour of embryoids
1.5	40.00	< 0.5	Cream	20.00	< 0.5	Cream
4.5	80.00	0.5-1.0	Cream	60.00	0.5-1.0	Cream
5.0	60.00	0.5-1.0	Cream	60.00	1.5-2.0	Cream
5.5	60.00	0.5-1.0	Light brown	60.00	0.5	Cream
6.0	60.00	0.5	Cream	40.00	0.5	Cream
7.0	40.00	< 0.5	Brown	20.00	0.5	Light brown
0	20.00	1.5-2.0	Cream	40.00	1.0-1.5	Light brown

The data represent the average value of 5 replications

Culture medium - for Neelum: Basal medium with B_5 major salts + MS minor salts + sucrose 50.0g/l + ABA 5.0mg/l + CH 100.0mg/l + CW 200.0ml/l + PVP 10.0g/l

Culture medium - for Vellari Manga: Basal medium with B_5 major salts + MS minor salts + sucrose 40.0g/l + ABA 4.22mg/l + CH 100.0mg/l + CW 200.0ml/l + PVP 10.0g/l

e. Casein hydrolysate (CH)

Casein hydrolysate 100.0mg/l in the maturation medium was found to be the best in getting mature somatic embryoids of 0.5-1.0cm size in Neelum (Table 43). When CH was used at higher quantities (200.0mg/l, 300.0mg/l and 400.0mg/l), the size of embryoids was below 0.5cm. When no casein hydrolysate was used, the size of embryoids was only 0.5cm and was cream coloured.

In Vellari Manga, with CH, 100.0mg/l in the maturation medium, brown coloured somatic embryoids could be obtained with a size ranging from 1.0 to 1.5cm. With 150.0 mg/l of CH, the size of embryoids was 0.5-1.0cm and without CH, the size was below 0.5cm (Table 43). In both cases the colour of the embryoids was cream.

f. Polyethylene glycol (PEG)

Maturation media with and without PEG did not increase the size of embryoids in Neelum as well as Vellari Manga. The size of embryoids in all treatments was below 0.5cm (Table 44).

g. Coconut water

In Neelum, when coconut water was not added in the maturation medium, the embryoids were under-sized (< 0.50cm) (Table 45). But when 200.0 ml/l or 250.0 ml/l coconut water was added in the maturation medium, size of the embryoids could be increased to 0.50-1.0cm. At 200.0 ml/l coconut water, the per cent survival was 66.67, whereas at 250.0 ml/l, 50.00 per cent of cultures survived.

Table 43. Effect of casein hydrolysate on the maturation of somatic embryoids of the mango varieties Neelum and Vellari Manga

CH (mg/l)	Neelum			Vellari Manga		
	Cultures survived (%)	Size of embryoids (length in cm)	Colour of embryoids	Cultures survived (%)	Size of embryoids (length in cm)	Colour of embryoids
100.00	80.00	0.5-1.0	Cream	100.00	1.0-1.5	Brown
150.00	80.00	0.5	Cream	100.00	0.5-1.0	Cream
200.00	80.00	< 0.5	Cream	60.00	0.5-1.0	Cream
300.00	60.00	< 0.5	Cream	40.00	0.5	Cream
400.00	40.00	< 0.5	Cream	70.00	0.5	Cream
500.00	60.00	0.5	Brown	50.00	0.5-1.0	Cream
0	40.00	0.5	Cream	30.00	< 0.5	Cream

The data represent the average value of 10 replications

Culture medium for Neelum - Basal medium with B_5 major salts + MS minor salts + sucrose 50.0g/l + ABA 5.0mg/l + CW 200.0ml/l + PVP 10.0g/l + agar 4.5g/l

Culture medium for Vellari Manga - Basal medium with B_5 major salts + MS minor salts + sucrose 40.0g/l + ABA 4.22mg/l + CW 200.0ml/l + PVP 10.0g/l + agar 6.0g/l

Table 44. Effect of polyethylene glycol (PEG) on the maturation of somatic embryoids of the mango varieties Neelum and Vellari Manga

PEG (g/l)	Neelum			Vellari Manga		
	Cultures survived (%)	Size of embryoids (length in cm)	Colour of embryoids	Cultures survived (%)	Size of embryoids (length in cm)	Colour of embryoids
50.0	50.0	< 0.5	Cream	60.0	0.5	Cream
80.0	50.0	< 0.5	Cream	70.0	< 0.5	Cream
100.0	50.0	< 0.5	Cream	70.0	< 0.5	Cream
0	60.0	< 0.5	Cream	60.0	< 0.5	Light brown

The data represent the average value of 6 replications

Culture medium for Neelum - Basal medium with B_5 major salts + MS minor salts + sucrose 50.0g/l + ABA 5.0mg/l + CH 100.0ml/l + CW 200.0g/l + PVP 10.0g/l + agar 4.5g/l

Culture medium for Vellari Manga - Basal medium with B_5 major salts + MS minor salts + sucrose 40.0g/l + ABA 4.22mg/l + CH 100.0ml/l + CW 200.0ml/l + PVP 10.0g/l + agar 6.0g/l

Table 45. Effect of coconut water on the maturation of somatic embryoids of the mango varieties Neelum and Vellari Manga

CW (ml/l)	Neelum			Vellari Manga		
	Cultures survived (%)	Size of embryoids (length in cm)	Colour of embryoids	Cultures survived (%)	Size of embryoids (length in cm)	Colour of embryoids
150.0	66.67	0.50	Cream	83.33	< 0.50	Cream
200.0	66.67	0.5-1.0	Cream	100.00	0.5-1.5	Cream
250.0	50.00	0.50-1.0	Light brown	83.33	0.5	Cream
0	50.00	< 0.50	Cream	50.00	< 0.50	Light brown

The data represent the average value of 6 replications.

Culture medium - for Neelum: Basal medium with B_5 major salts + MS minor salts + sucrose 50.0g/l + ABA 5.0mg/l + CH 100.0ml/l + PVP 10.0g/l + agar 4.5g/l

Culture medium - for Vellari Manga: Basal medium with B_5 major salts + MS minor salts + sucrose 40.0g/l + ABA 4.22mg/l + CH 100.0ml/l + PVP 10.0g/l + agar 6.0g/l

In Vellari Manga also, when coconut water was not added in the media, the size of embryoids was below 0.50cm. With 200.0ml/l coconut water, the size was 0.5-1.5cm. The embryoids were cream coloured and cent per cent of the cultures survived (Table 45).

h. Activated Charcoal

The different levels of charcoal tried did not influence the size of embryoids in Neelum and Vellari Manga. When charcoal was not added in the maturation medium, none of the cultures of Neelum or Vellari Manga survived. At all the levels of activated charcoal in the medium, the size of embryoids of Neelum were 0.5cm and Vellari Manga 0.5-1.0cm (Table 46).

i. Polyvinyl pyrrolidone (PVP)

The different levels of PVP did not increase the size of embryoids. But the highest per cent survival was with PVP 1.0 per cent in Neelum (80.00) and Vellari Manga (70.00). The size of embryoids was between 0.5 and 1.0cm in all cases. When PVP was not included in the maturation medium none of the cultures of Neelum and Vellari Manga survived (Table 47).

j. Light

Darkness was better for the maturation of somatic embryoids than light. In darkness, 83.33 per cent of Neelum cultures survived and the size of embryoids was 0.5-1.0cm (Table 48). Cent per cent of Vellari Manga cultures survived producing cream coloured somatic embryoids of size 1.0-1.5cm, in darkness. Under light (3000 lux; 16h photoperiod) 66.67 per cent cultures of Neelum survived producing very small sized embryoids ($\leq$ 0.5cm).

Table 46. Effect of activated charcoal on the maturation of somatic embryoids of the mango varieties Neelum and Vellari Manga

AC (%)	Neelum			Vellari Manga		
	Cultures survived (%)	Size of embryoids (length in cm)	Colour of embryoids	Cultures survived (%)	Size of embryoids (length in cm)	Colour of embryoids
0.1	10.0	< 0.5	Cream	20.0	0.5-1.0	Cream
0.15	10.0	0.5	Cream	20.0	0.5-1.0	Cream
0.2	40.0	0.5	Cream	60.0	0.5-1.0	Cream
0.25	50.0	0.5	Cream	80.0	0.5-1.0	Cream
0	0	0	0	0	0	0

The data represent the average value of 10 replications

Culture medium - for Neelum: Basal medium with B_5 major salts + MS minor salts + sucrose 50.0g/l + ABA 5.0mg/l + CH 100.0ml/l + CW 200.0g/l + agar 4.5g/l

Culture medium - for Vellari Manga: Basal medium with B_5 major salts + MS minor salts + sucrose 40.0g/l + ABA 4.22mg/l + CH 100.0ml/l + CW 200.0ml/l + agar 6.0g/l

Table 47. Effect of Polyvinyl pyrrolidone (PVP) on the maturation of somatic embryoids of the mango varieties Neelum and Vellari Manga

PVP (%)	Neelum			Vellari Manga		
	Cultures survived (%)	Size of embryoids (length in cm)	Colour of embryoids	Cultures survived (%)	Size of embryoids (length in cm)	Colour of embryoids
0.7	50.0	0.5-1.0	Cream	60.0	0.5-1.0	Cream
0.8	60.0	0.5-1.0	Light brown	70.0	0.5-1.0	Cream
1.0	80.0	0.5-1.0	Cream	70.0	0.5-1.0	Cream
0	0	0	0	0	0	0

The data represent the average value of 10 replications

Culture medium - for Neelum: Basal medium with B_5 major salts + MS minor salts + sucrose 50.0g/l + ABA 5.0mg/l + CH 100.0ml/l + CW 200.0g/l + agar 4.5g/l

Culture medium - for Vellari Manga: Basal medium with B_5 major salts + MS minor salts + sucrose 40.0g/l + ABA 4.22mg/l + CH 100.0ml/l + CW 200.0ml/l + agar 6.0g/l

Table 48. Effect of light on the maturation of somatic embryoids of the mango varieties Neelum and Vellari Manga

Culture conditions	Neelum			Vellari Manga		
	Cultures survived (%)	Size of embryoids (length in cm)	Colour of embryoids	Cultures survived (%)	Size of embryoids (length in cm)	Colour of embryoids
Light (3000 lux; 16hr photoperiod)	66.67	< 0.5	Cream	66.67	0.5-1.0	Light brown
Darkness	83.33	0.5-1.0	Cream	100.00	1.0-1.5	Cream

The data represent the average value of 6 replications

Culture medium - for Neelum: Basal medium with B_5 major salts + MS minor salts + sucrose 50.0g/l + ABA 5.0mg/l + CH 100.0ml/l + CW 200.0g/l + PVP 10.0g/l + agar 4.5g/l

Culture medium - for Vellari Manga: Basal medium with B_5 major salts + MS minor salts + sucrose 40.0g/l + ABA 4.22mg/l + CH 100.0ml/l + CW 200.0ml/l + PVP 10.0g/l + agar 6.0g/l

In Vellari Manga, the size of embryoids was 0.5-1.0cm under light with the same per cent survival (Table 48).

k. Frequency of subculturing

Subculturing at an interval ten days was good in increasing the size of somatic embryoids (1.0-1.5cm) for Neelum (Table 49). In Vellari Manga subculturing at an interval of 15 days produced cream coloured embryoids of size 0.5-1.5cm. When no subculturing was done, the embryoids were small (0.5cm). In both cases, 50.0 per cent of the cultures survived in the maturation media.

7. Germination of somatic embryoids

a. Plant growth substances

Of the 49 combinations of PGS tried (Table 3), none of the treatments favoured normal germination of somatic embryoids of Neelum and Vellari Manga. However, the presence of BA in the germination medium was found to influence the germination of somatic embryoids in Neelum and Vellari Manga. The percentage germination of somatic embryoids of Neelum and Vellari Manga varied with different levels of BA (Table 50). BA 0.10mg/l was significantly superior to all other levels of BA in the germination of somatic embryoids of Neelum except BA 0.05mg/l and BA 0.50mg/l which were on par. BA 0.10mg/l recorded a per cent germination of 41.60 (Fig. 5). BA 10.00mg/l and 50.00mg/l were not useful in the germination of somatic embryoids of Neelum. BA 0.10mg/l produced near-normal growth of the somatic embryoids and the length of embryoids varied from 2.0-3.0cm.

Table 49. Effect of frequency of subculture on the maturation of somatic embryoids of the mango varieties Neelum and Vellari Manga

Sub culturing interval (days)	Neelum			Vellari Manga		
	Cultures survived (%)	Size of embryoids (length in cm)	Colour of embryoids	Cultures survived (%)	Size of embryoids (length in cm)	Colour of embryoids
5	41.67	0.5-1.0	Cream	66.67	0.5-1.0	Cream
10	50.00	1.0-1.5	Cream	50.00	0.5-1.0	Cream
15	58.33	0.5-1.0	Cream	50.00	0.5-1.5	Cream
20	50.00	0.5	Cream	41.67	0.5-1.0	Cream
0	41.67	< 0.5	Cream	58.33	0.5	Light brown

The data represent the average value of 12 replications

Culture medium - for Neelum: Basal medium with B_5 major salts + MS minor salts + sucrose 50.0g/l + ABA 5.0mg/l + CH 100.0ml/l + CW 200.0g/l + PVP 10.0g/l + agar 4.5g/l

Culture medium - for Vellari Manga: Basal medium with B_5 major salts + MS minor salts + sucrose 40.0g/l + ABA 4.22mg/l + CH 100.0ml/l + CW 200.0ml/l + PVP 10.0g/l + agar 6.0g/l

Table 50. Effect of benzyl adenine (BA) on the germination of somatic embryoids of the mango variety Neelum

BA (mg/l)	Cultures[a] survived (%)	Cultures with[b] germinating embryoids (%)	Size of embryoids (length in cm)	Growth of embryoids
0.05	30.00	33.33(35.08)	1.5-2.0	Well developed root system
0.10	50.00	41.60 (40.49)	2.0-3.0	Near normal
0.50	20.00	25.00 (31.35)	1.5-2.0	Near normal
1.00	40.00	25.00 (31.35)	1.0-1.5	Enlargement and progressive greening of embryoids
2.00	10.00	8.33 (14.50)	1.0-1.5	Development of secondary somatic embryoids
4.00	50.09	16.67 (22.11)	0.5-1.0	Malformed
6.00	60.00	8.33 (14.50)	0.5-1.0	Fused cotyledons
8.00	40.00	8.33 (14.50)	0.5-1.0	Malformed
10.00	30.00	0	—	—
50.00	10.00	0	—	—
CD (5%)		9.80		

a - The data represent the average value of 10 replications

b - The data represent the average value of 12 replications

Values in parenthesis are the transformed values

Culture medium - Basal medium with B_5 major salts + MS minor salts + BA 0.1mg/l + sucrose 40.0g/l + agar 5.0g/l + PVP 10.0g/l + agar 4.5g/l

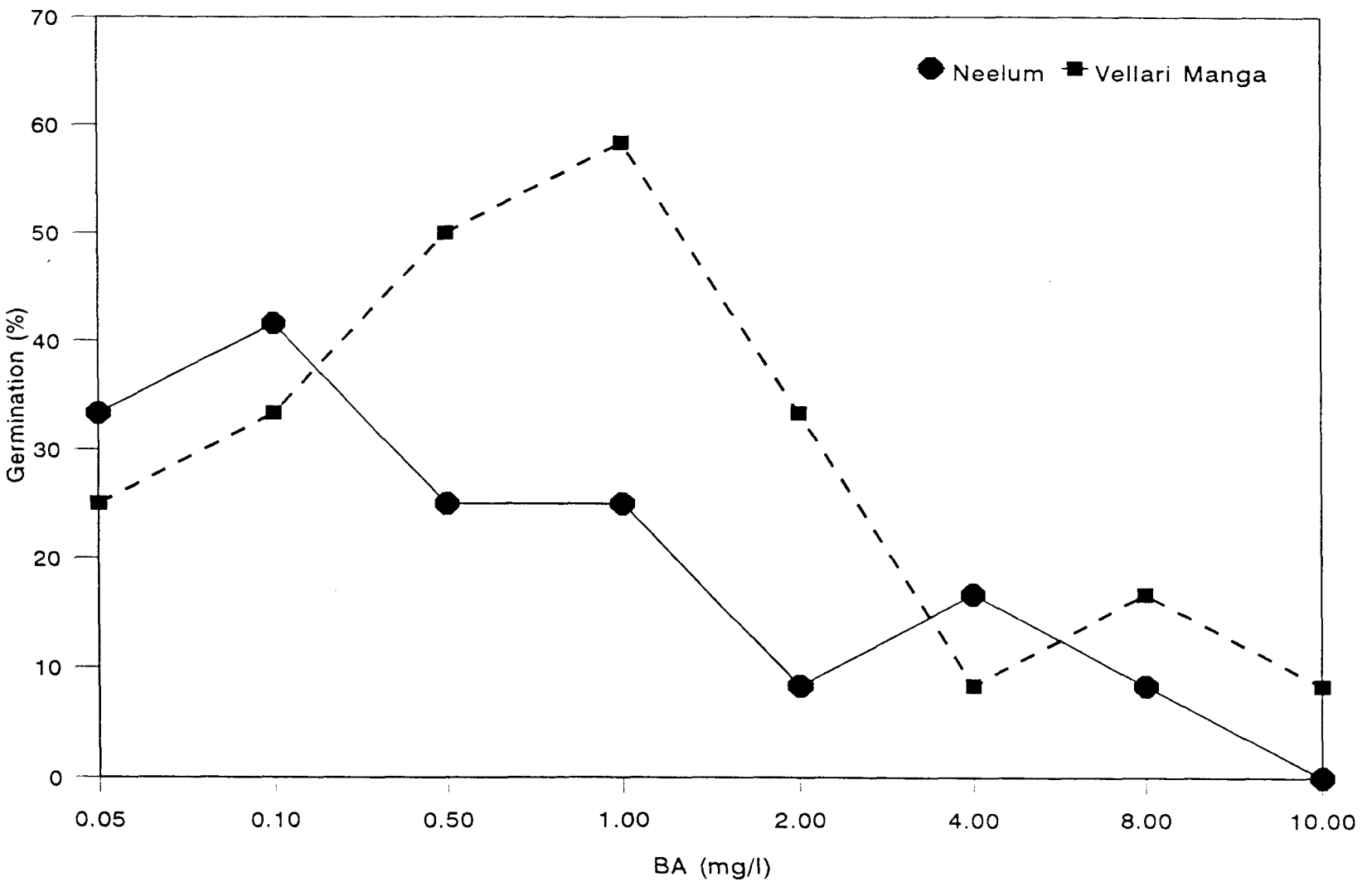


Fig. 5. Effect of BA on germination of somatic embryoids from nucellus of the mango varieties Neelum and Vellari Manga

Table 51. Effect of benzyl adenine (BA) on the germination of somatic embryoids of the mango variety Vellari Manga

BA (mg/l)	Cultures[a] survived (%)	Cultures with[b] germinating embryoids (%)	Size of embryoids (length in cm)	Growth of embryoids
0.05	50.00	25.00 (31.35)	0.5-1.0	Malformed
0.10	40.00	33.33 (35.08)	2.0-2.5	Root well developed
0.50	40.00	50.00 (44.17)	2.5-3.0	Root well developed
1.00	30.00	58.33 (50.56)	3.0-3.5	Near normal growth with a root and shoot pole
2.00	20.00	33.33 (35.08)	2.5-3.0	Fused cotyledons
4.00	30.00	8.33 (14.50)	3.0-3.5	Malformed
6.00	10.00	8.33 (14.50)	1.5-2.0	Shoot not well developed
8.00	10.00	16.67 (22.11)	2.0-2.5	Lack of shoot pole
10.00	20.00	8.33 (14.50)	1.5-2.0	Normal growth of root system
50.00	20.00	0	—	—
CD (5%)		9.80		

a - The data represent the average value of 10 replications

b - The data represent the average value of 12 replications

Values in parenthesis are the transformed values

Culture medium - Basal medium with B_5 major salts + MS minor salts + BA 1.0mg/l + sucrose 50.0g/l + agar 5.5g/l + PVP 10.0g/l + cobalt chloride 10.0mg/l

Table 52. Effect of basal media on the germination of somatic embryoids of the mango variety Neelum

Basal medium	Cultures[a] survived (%)	Cultures with[b] germinating embryoids (%)	Size of embryoids (length in cm)	Growth of embryoids
MS	50.00	22.22 (25.91)	1.0-1.5	Roots well developed
1/2 MS	50.00	16.67 (22.11)	0.5	Roots well developed
1/2 MS with $NH_4 NO_3$ 1/2	40.00	22.22 (25.91)	< 0.5	Roots well developed
1/2 MS with $NH_4 NO_3$ 1/4	20.00	16.67 (22.11)	0.5-1.0	Shoots fused and secondary somatic embryogenesis observed
1/2 MS with KNO_3 1/2	30.00	22.22 (25.91)	1.5-2.0	Precocious germination
1/2 MS with KNO_3 1/4	40.00	5.55 (7.92)	0.5	Abnormal growth
B5	60.00	16.67 (22.91)	0.5-1.0	Shoot pole lacking
B5 major salts + MS minor salts	60.00	38.89 (39.33)	2.0-2.5	Near normal growth
MS major salts + B5 minor salts	50.00	33.33 (35.56)	1.5-2.0	Malformed
SH	0	0	—	—
CD (5%)		9.89		

a - The data represent the average value of 10 replications
b - The data represent the average value of 18 replications

Values in parenthesis are the transformed values

Supplements - BA 0.1mg/l + sucrose 40.0mg/l + agar 5.0g/l + PVP 10.0g/l

Table 53. Effect of basal media on the germination of somatic embryoids of the mango variety Vellari Manga

Basal medium	Cultures[a] survived (%)	Cultures with[b] germinating embryoids (%)	Size of embryoids (length in cm)	Growth of embryoids
MS	20.00	27.78 (31.29)	0.5-1.0	Roots well developed but cotyledons fused
1/2 MS	70.00	33.33 (35.56)	1.0-1.5	Precocious germination
1/2 MS with $NH_4 NO_3$ 1/2	80.00	27.78 (31.29)	2.0-2.5	Malformed
1/2 MS with $NH_4 NO_3$ 1/4	40.00	38.89 (38.23)	1.5-2.0	Malformed
1/2 MS with KNO_3 1/2	30.00	22.22 (25.91)	1.0-1.5	Malformed
1/2 MS with KNO_3 1/4	30.00	16.67 (22.11)	0.5	Tricotyly
B_5	50.00	11.11 (19.26)	1.5-2.0	Abnormal shoot growth and normal root growth
B_5 major salts + MS minor salts	50.00	55.56 (47.85)	2.0-3.0	Near normal growth
MS major salts + B_5 minor salts	40.00	27.78 (31.29)	2.0-3.0	Well developed root system
SH	10.00	0	—	—
CD (5%)		11.23		

a - The data represent the average value of 10 replications
b - The data represent the average value of 18 replications

Values in parenthesis are the transformed values

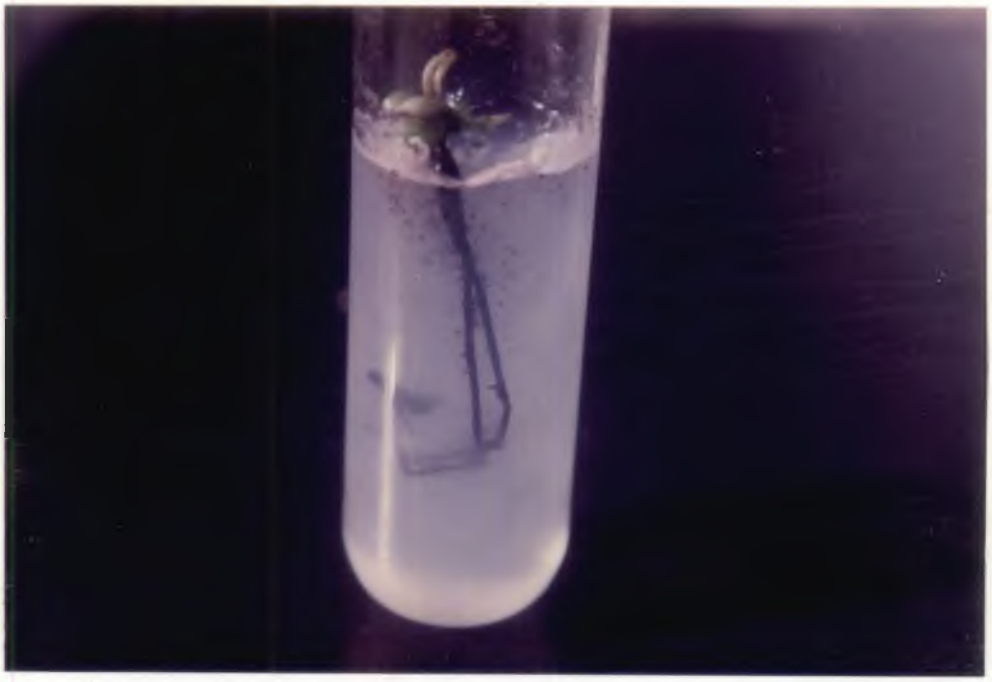
Supplements - BA 1.0mg/l + sucrose 50.0g/l + agar 5.5g/l + PVP 10.0g/l

Plate 13

A germinating somatic embryoid of Vellari Manga in basal medium containing B_5 major salts and MS minor salts supplemented with BA 1.0 mg/l and sodium chloride 0.5 g/l

Plate 14

Abnormal growth of a germinating somatic embryoid of Vellari Manga when sucrose was reduced in the germination medium



c. Sucrose

In Neelum, sucrose 40.0 g/l and 50.0 g/l could initiate near normal growth of the somatic embryoids. These treatments could produce 20.0 per cent germination (Table 54). The germinated embryoids were 2.0-2.5cm in length. At the lowest level of sucrose (20.0g/l), though 10.00 per cent cultures survived, none germinated. At the highest level (60.0g/l), 10.00 per cent germinated. But they were malformed.

Table 54. Effect of sucrose on the germination of somatic embryoids of the mango variety Neelum

Sucrose (g/l)	Cultures[a] survived (%)	Cultures with[b] germinating embryoids (%)	Size of embryoids (length in cm)	Growth of embryoids
20.0	10.0	0	—	—
30.0	20.0	10.0	1.5-2.0	Near normal
40.0	40.0	20.0	2.0-2.5	Near normal
50.0	60.0	20.0	2.0-2.5	Near normal
60.0	100.0	10.0	2.0-2.5	Cotyledons fused

The data represent the average value of 10 replications

Culture medium - Basal medium with B_5 major salts + MS minor salts + BA 0.1mg/l + agar 5.0g/l + CW 200.0ml/l + PVP 10.0g/l

In Vellari Manga, sucrose 50.0 g/l was the best in the germination of the somatic embryoids with a germination percentage of 40.0 (Table 55). The germinated embryoids showed near normal growth and were 2.5-3.0cm in length. When sucrose 20.0g/l was used in the germination medium, only 10.00 per cent germinated with malformed shoot (Plate 14).

Table 55. Effect of sucrose on the germination of somatic embryoids of the mango variety Vellari Manga

Sucrose (g/l)	Cultures[a] survived (%)	Cultures with[b] germinating embryoids (%)	Size of embryoids (length in cm)	Growth of embryoids
20.0	50.0	10.0	1.5-2.0	Malformed
30.0	50.0	10.0	1.5-5.0	Malformed
40.0	60.0	30.0	2.0-2.5	Near normal
50.0	60.0	40.0	2.5-3.0	Near normal
60.0	80.0	20.0	1.5-2.0	Near normal

The data represent the average value of 20 replications

Culture medium - Basal medium with B_5 major salts + MS minor salts + BA 0.1mg/l + agar 5.5g/l + CW 200.0ml/l + PVP 10.0g/l

d. Thidiazuron

Thidiazuron did not help in the germination of somatic embryoids of Neelum and Vellari Manga. Only swelling of the embryoids was observed. No proper germination was observed (Table 56). The germinated embryoids were malformed. In the germination medium without thidiazuron, 20.00 per cent cultures of Neelum and 10.00 per cent cultures of Vellari Manga germinated with near normal growth.

Table 56. Effect of thidiazuron (TDZ) on the germination of somatic embryoids of the mango varieties Neelum and Vellari Manga

TDZ (mg/l)	Cultures with germinating embryoids (%)		Growth of embryoids	
	Neelum	Vellari Manga	Neelum	Vellari Manga
Control	20.0	10.0	Near normal	Near normal
1.0	10.0	0	Malformed	—
2.0	20.0	0	Malformed	—
4.0	10.0	10.0	Malformed	Malformed

The data represent the average value of 10 replications

Culture medium for - Neelum: Basal medium with B_5 major salts + MS minor salts + BA 0.1 mg/l + agar 5.0g/l + PVP 10.0g/l + Sucrose 40.0g/l

Culture medium for - Vellari Manga: Basal medium with B_5 major salts + MS minor salts + BA 1.0 mg/l + agar 5.5g/l + PVP 10.0g/l + Sucrose 50.0g/l

e. Polyethylene glycol

The influence of polyethylene glycol (PEG) in the germination of somatic embryoids was studied by incorporating 5.0 and 10.0 per cent PEG to the germination medium. The different levels of PEG did not support the germination of somatic embryoids of Neelum and Vellari Manga.

f. Sodium butyrate

The effect of sodium butyrate in the germination medium (0.55, 1.10 and 1.65mg/l) were observed. Sodium butyrate was not useful in inducing the germination of the somatic embryoids of Neelum and Vellari Manga.

g. Sodium chloride

Sodium chloride at 0.05 per cent could initiate germination of Neelum and Vellari Manga somatic embryoids. In Neelum, 50.0 per cent germination and in Vellari Manga 40.0 per cent germination could be obtained (Table 57). Near normal growth of the embryoids could be observed in both cases (Plate 13). In the germination medium without sodium chloride, though normal growth could be observed, the per cent germination was 20.0 in Neelum and 10.0 in Vellari Manga. At the highest levels of sodium chloride tried (0.40 and 0.80 per cent) the somatic embryoids of both varieties did not germinate.

Table 57. Effect of sodium chloride on the germination of somatic embryoids of the mango varieties Neelum and Vellari Manga

Sodium chloride (%)	Cultures with germinating embryoids (%)		Growth of embryoids	
	Neelum	Vellari Manga	Neelum	Vellari Manga
Control	20.0	10.0	Near normal	Near normal
0.05	50.0	40.0	Near normal	Near normal
0.10	10.0	10.0	Near normal	Malformed
0.20	0	10.0	—	Malformed
0.40	0	0	—	—
0.80	0	0	—	—

The data represent the average value of 10 replications

Culture medium - for Neelum: Basal medium with B_5 major salts + MS minor salts + BA 0.1mg/l + agar 5.0g/l + PVP 10.0g/l + Sucrose 40.0g/l

Culture medium - for Vellari Manga: Basal medium with B_5 major salts + MS minor salts + BA 1.0mg/l + agar 5.5g/l + PVP 50.0g/l + Sucrose 50.0g/l

h. Ethylene inhibitors

Different levels of silver nitrate and cobalt chloride were tried as ethylene inhibitors, which can promote germination. Silver nitrate did not support the germination of somatic embryoids of Neelum and Vellari Manga (Table 58). Cobalt chloride supported germination of both Neelum and Vellari Manga somatic embryoids (Plate 17). In Neelum, cobalt chloride 10.0mg/l, 15.0mg/l and 5.0mg/l were found effective in the normal germination of a few somatic embryoids. Cobalt chloride 10.0 mg/l could initiate germination in 40.0 per cent cultures of Neelum somatic embryoids and 20.0 per cent of Vellari Manga. Cobalt chloride 15.0 mg/l when added to the germination medium could initiate germination in 10.0 per cent cultures of Neelum and Vellari Manga (Fig. 6) When no ethylene inhibitor was used, the per cent germination was reduced to half, although the embryoids were near normal.

i. Coconut water

Coconut water 150.0ml/l and 200.0ml/l in the medium was effective in the germination of somatic embryoids than in medium without coconut water. Coconut water 150.0ml/l and 200.0ml/l could germinate 40.0 per cent cultures of Neelum (Table 59). Coconut water 150.0ml in the medium produced malformed growth whereas 200.0ml of coconut water induced near normal growth of embryoids and the length ranged from 3.0-3.5cm (Plate 18). When no coconut water was used, though 50.0 per cent cultures survived, none germinated.

Plate 15

Developmental stages of somatic embryoids initiated from nucellus of Vellari Manga

Plate 16

A typical bipolar somatic embryoid of Vellari Manga

Plate 17

Normal growth of Vellari Manga somatic embryoid in germination medium supplemented with BA 1.0 mg/l, sodium chloride 0.5 g/l and cobalt chloride 10.0 mg/l

Plate 18

A germinated somatic embryoid of Neelum in medium supplemented with coconut water 200.0 ml/l

Table 58. Effect of ethylene inhibitors on the germination of somatic embryoids of the mango varieties Neelum and Vellari Manga

Treatment (mg/l)	Cultures with germinating embryoids (%)		Growth of embryoids	
	Neelum	Vellari Manga	Neelum	Vellari Manga
Control	20.0	10.0	Near normal and weak	Near normal
Silver nitrate				
5.0	0	0	—	—
10.0	0	0	—	—
15.0	0	0	—	—
Cobalt chloride				
5.0	5.0	0	Near normal	—
10.0	40.0	20.0	Near normal	Near normal
15.0	10.0	10.0	Near normal	Near normal

The data represent the average value of 20 replications

Basal medium for - Neelum: Basal medium with B_5 major salts + MS minor minor salts + BA 0.1mg/l + sodium chloride 0.5g/l + agar 5.0g/l + PVP 10.0g/l + sucrose 40.0g/l

Culture medium for - Vellari Manga: Basal medium with B_5 major salts + MS minor salts + BA 1.0 mg/l + sodium chloride 0.5g/l + agar 5.5g/l + PVP 10.0g/l + sucrose 50.0g/l

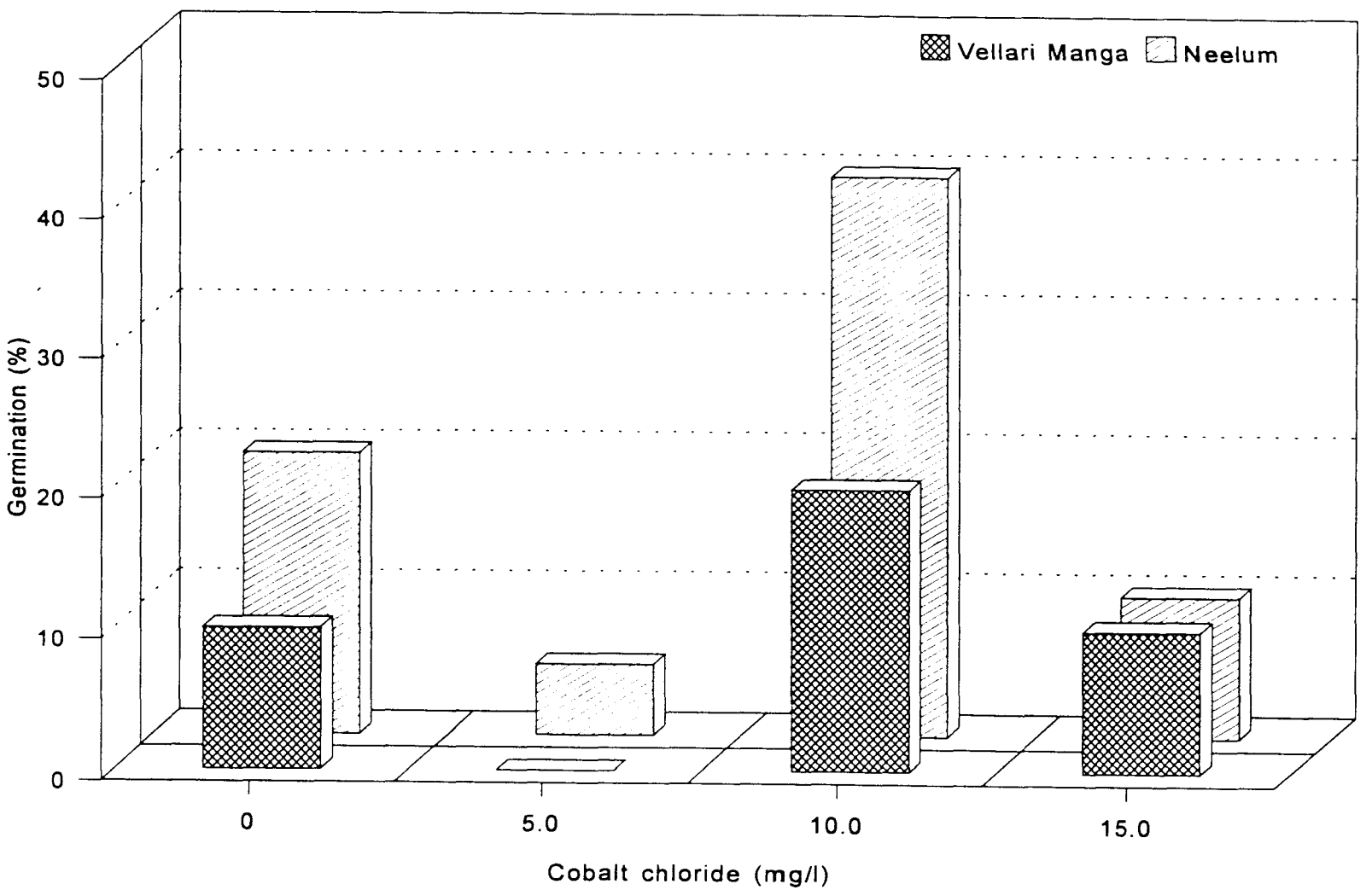


Fig. 6. Effect of cobalt chloride on germination of somatic embryoids from nucellus of the mango varieties Neelum and Vellari Manga

Table 59. Effect of coconut water on the germination of somatic embryoids of the mango variety Neelum

CW (ml/l)	Cultures survived (%)	Cultures with germinating embryoids (%)	Size of embryoids (length in cm)	Growth of embryoids
150.0	60.0	40.0	2.0-2.5	Malformed
200.0	60.0	40.0	3.0-3.5	Near normal
0	50.0	—	—	—

The data represent the average value of 10 replications

Culture medium - Basal medium with B_5 major salts + MS minor salts + BA 0.1mg/l + agar 5.0g/l + PVP 10.0g/l + Sucrose 40.0g/l

In Vellari Manga, coconut water 200.0ml/l effected germination in 60.0 per cent cultures, producing 2.0-2.5cm sized embryoids of near normal growth (Table 60). When no coconut water was used, only 10.0 per cent cultures germinated. They were small sized (0.5cm) and malformed.

j. Solidifying agent (agar)

Agar 5.0g/l in the germination medium favoured the germination of somatic embryoids of Neelum. It supported germination in 75.0 per cent cultures, producing near normal germination of the embryoids (Table 61).

Table 60. Effect of coconut water on the germination of somatic embryoids of the mango variety Vellari Manga

CW (ml/l)	Cultures survived (%)	Cultures with germinating embryoids (%)	Size of embryoids (length in cm)	Growth of embryoids
150.0	50.0	50.0	2.0-2.5	Near normal
200.0	60.0	60.0	2.0-2.5	Near normal
0	30.0	10.0	0.5	Fused cotyledons

The data represent the average value of 10 replications

Culture medium - Basal medium with B_5 major salts + MS minor salts + BA 1.0mg/l + agar 5.0g/l + PVP 10.0g/l + Sucrose 50.0g/l

Table 61. Effect of agar on the germination of somatic embryoids of the mango variety Neelum

Agar (g/l)	Cultures[a] survived (%)	Cultures with[b] germinating embryoids (%)	Size of embryoids (length in cm)	Growth of embryoids
4.5	60.0	50.0	1.0-2.0	Near normal
5.0	70.0	75.0	2.0-2.5	Near normal
5.5	60.0	50.0	2.0-2.5	Near normal
6.0	50.0	25.0	1.0-1.5	Malformed
0	40.00	25.0	2.0-2.5	Vitrified shoot

a - The data represent the average value of 10 replications
b - The data represent the average value of 4 replications

Culture medium - Basal medium with B_5 major salts + MS minor salts + BA 1.0mg/l + agar 5.0g/l + PVP 10.0g/l + Sucrose 40.0g/l

Germination medium with agar 4.5g/l and 5.5g/l gave 50.0 per cent germination of Neelum somatic embryoids. The size of embryoids ranged from 2.0-2.5cm. In liquid medium, 25.0 per cent germination could be observed. But the germinated embryoids produced vitrified shoot.

In Vellari Manga, agar 5.5 g/l was the best in supporting 50.0 per cent germination of somatic embryoids. A well developed root system and near-normal shoot growth resulted (Table 62; Plate 19). The embryoids were 2.5 to 3.0cm in length. Agar 4.5g/l and 5.0g/l were also good in inducing germination (50.0 per cent). But the size of embryoids was little reduced (2.0-2.5cm). Liquid medium favoured germination in 50.0 per cent cultures of Vellari Manga somatic embryoids. Though, the size of embryoids increased (1.5-2.0cm), the embryoids in liquid medium, became vitrified and gradually became necrotic.

k. Activated charcoal and PVP

Different levels of activated charcoal and PVP were tried in eliminating the problem of phenolic interference and there by getting proper germination of somatic embryoids of Neelum and Vellari Manga. PVP 1.0 per cent was found better than all the levels of activated charcoal in obtaining higher percentage of germination (30.0) in Neelum (Table 63). However, activated charcoal 0.10 per cent was the best in initiating root development in 20.0 per cent cultures of Neelum somatic embryoids (Table 63).

Plate 19

Well developed root and shoot system of a germinated somatic embryoid of Vellari Manga

Plate 20

A germinated somatic embryoid of Vellari Manga planted out in potting media (sterilized sand)

Table 62. Effect of agar on the germination of somatic embryoids of the mango variety Vellari Manga

Agar (g/l)	Cultures[a] survived (%)	Cultures with[b] germinating embryoids (%)	Size of embryoids (length in cm)	Growth of embryoids
4.5	50.0	50.0	2.0-2.5	Near normal
5.0	60.0	50.0	2.0-2.5	Near normal
5.5	60.0	50.0	2.5-3.0	Well developed root system and near normal shoot growth
6.0	60.0	25.0	1.5-2.0	Fused cotyledons
0	70.0	50.0	1.5-2.0	Swelling but no normal growth

a - The data represent the average value of 10 replications

b - The data represent the average value of 4 replications

Culture medium - Basal medium with B_5 major salts + MS minor salts + BA 1.0mg/l + agar 5.5g/l + PVP 10.0g/l + Sucrose 50.0g/l

Table 63. Effect of activated charcoal and PVP on the germination of somatic embryoids of the mango variety Neelum

Treatment (%)	Cultures survived (%)	Cultures with germinating embryoids (%)	Size of embryoids (length in cm)	Growth of embryoids
Activated charcoal				
0.1	30.0	20.0	1.0-2.0	Well developed root system and malformed shoot
0.15	30.0	20.0	2.0-2.5	Well developed root system
0.2	20.0	20.0	1.5-2.0	Well developed root system
0.25	40.0	10.0	1.5-2.0	Weak
PVP				
0.7	50.0	10.0	1.5-2.0	Root system developed
0.8	50.0	10.0	1.5-2.0	No shoot pole
1.0	60.0	30.0	1.5-2.0	No shoot pole

The data represent the average value of 10 replications

Culture medium - Basal medium with B_5 major salts + MS minor salts + BA 0.1mg/l + agar 5.0g/l + PVP 10.0g/l + Sucrose 40.0g/l

In Vellari Manga, PVP 1.0 mg/l effected germination of somatic embryoids in 30.0 per cent cultures. The embryoids were having near normal growth (Table 64). In Vellari Manga, activated charcoal 0.10 per cent also supported 30.0 per cent germination of somatic embryoids and produced 2.0-2.5cm sized embryoids (Table 64). However, the per cent survival of the somatic embryoids was the highest (70.00) in the medium with PVP 1.00 per cent. The germinated embryoids had better root growth than shoot growth.

8. Histological studies

Histological observations of embryogenic callus showed that competent cells were isodiametric and easily distinguished from non-embryogenic cells. The competent cells were darkly stained (Plate 21). At the time of culture, the parenchyma was filled with food reserves (Plate 22). Globular embryos protruded from the callus surface, during development (Plates 23 & 24). A prominent epidermal layer, formed on the globular embryo was soon followed by vascular strand development at the heart shaped stage. Darkly stained cells extending from the base of the embryo to the cotyledonary regions represent the initiation of vascular strands (Plate 25 & 26). Mature somatic embryoids were bipolar structures and the root and shoot meristems were connected by vascular strands (Plate 27). Longitudinal section of a germinated somatic embryoid had a meristematic growing tip (Plate 28). However, no histological variation in the basic characters could be observed between the somatic embryoids of Neelum and Vellari Manga.

Table 64. Effect of activated charcoal and PVP on the germination of somatic embryoids of the mango variety Vellari Manga

Treatment (%)	Cultures survived (%)	Cultures with germinating embryoids (%)	Size of embryoids (length in cm)	Growth of embryoids
Charcoal				
0.1	60.0	30.0	2.0-2.5	Better root growth than shoot growth
0.15	50.0	10.0	1.0-1.5	Malformed
0.2	50.0	10.0	2.5-3.0	Fused cotyledons
0.25	40.0	20.0	1.5-2.0	Fused cotyledons
PVP				
0.7	50.0	20.0	2.0-2.5	Malformed
0.8	60.0	20.0	2.0-2.5	Near normal
1.0	70.0	30.0	1.5-2.0	Near normal

The data represent the average value of 10 replications

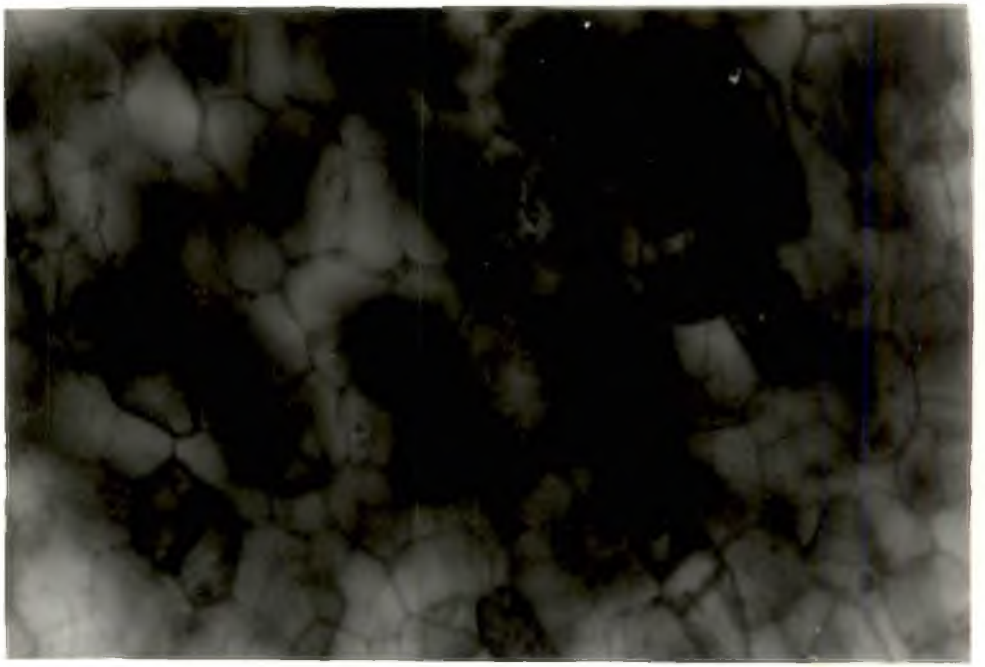
Culture medium - Basal medium with B_5 major salts + MS minor salts + BA 1.0mg/l + agar 5.5g/l + PVP 10.0g/l + Sucrose 50.0g/l

Plate 21

L.S. of embryogenic cells of Neelum nucellus distinguished by their darkly stained cells

Plate 22

L.S. of embryogenic cells of Vellari Manga nucellus distinguished by their darkly stained cells with reserved food materials



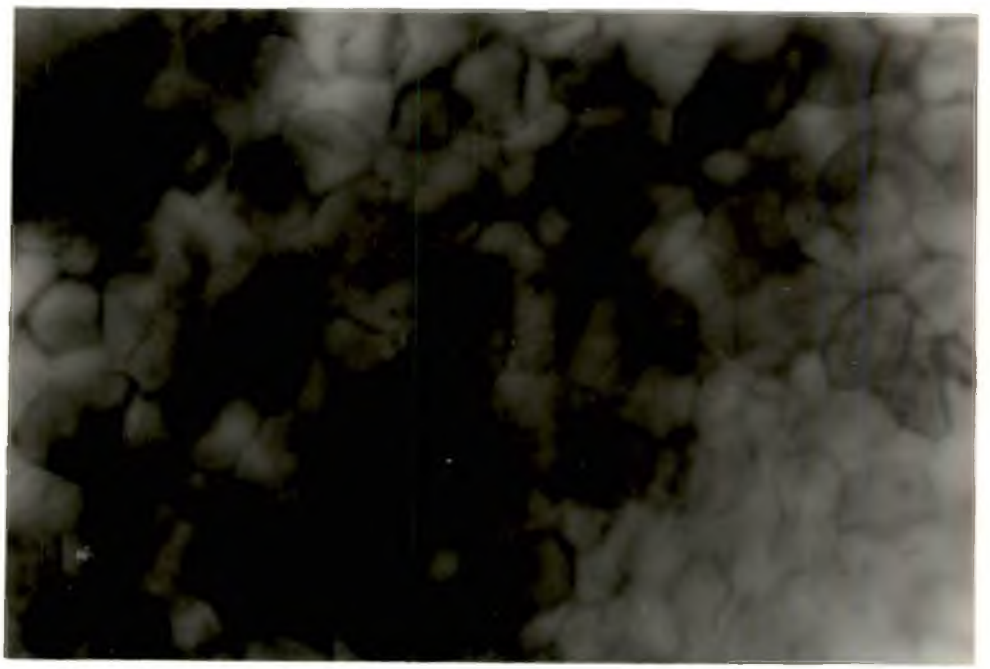
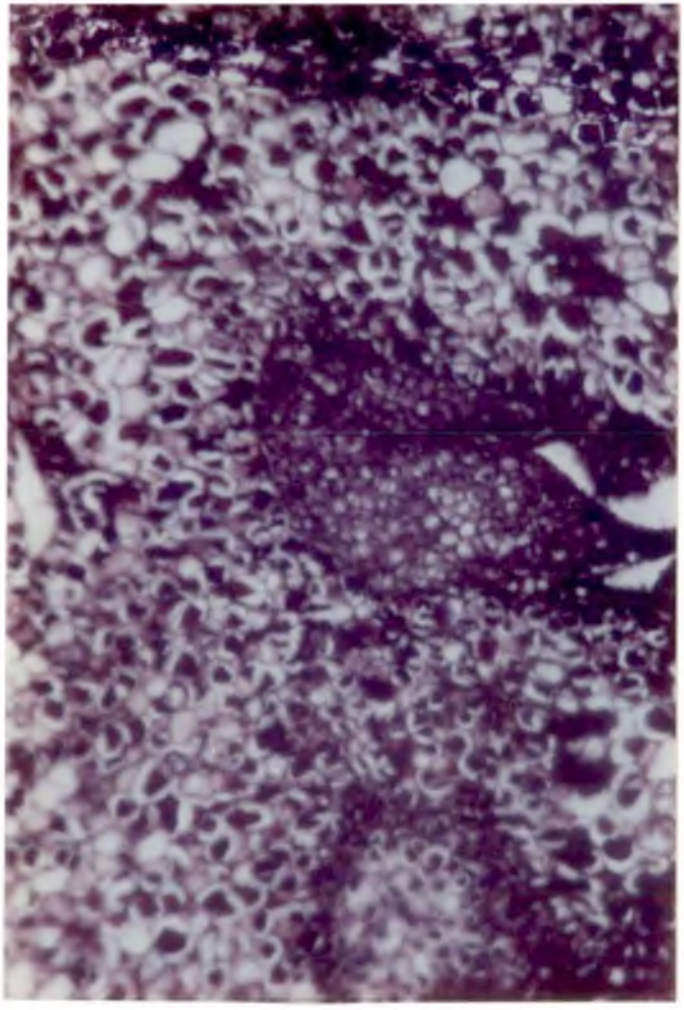


Plate 23

Section of an embryogenic callus of Neelum
showing initiation of globular somatic embryoids

Plate 24

Origin of a somatic embryoid from the nucellar tissue of Vellari Manga



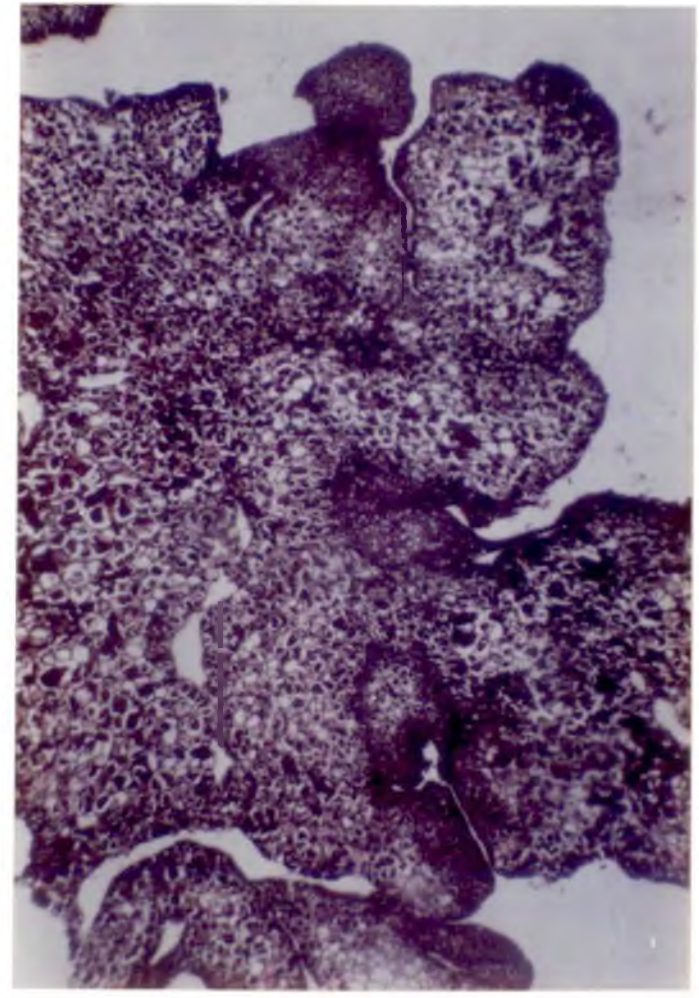
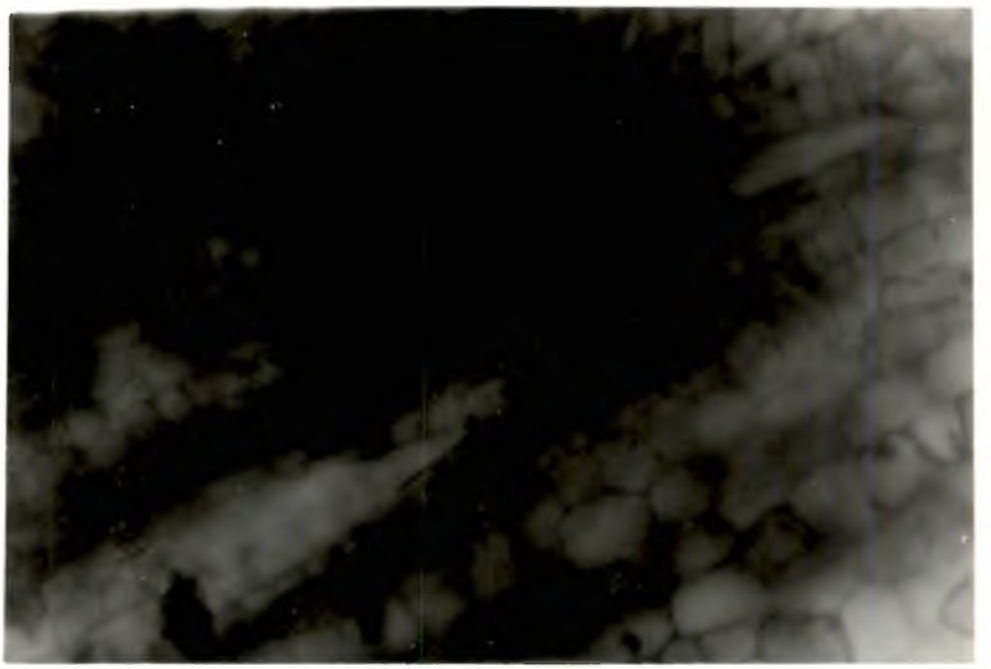


Plate 25

L.S. of a somatic embryoid of Vellari Manga with vascular strand development

Plate 26

L.S. of a matured somatic embryoid of Neelum showing the development of vascular strands



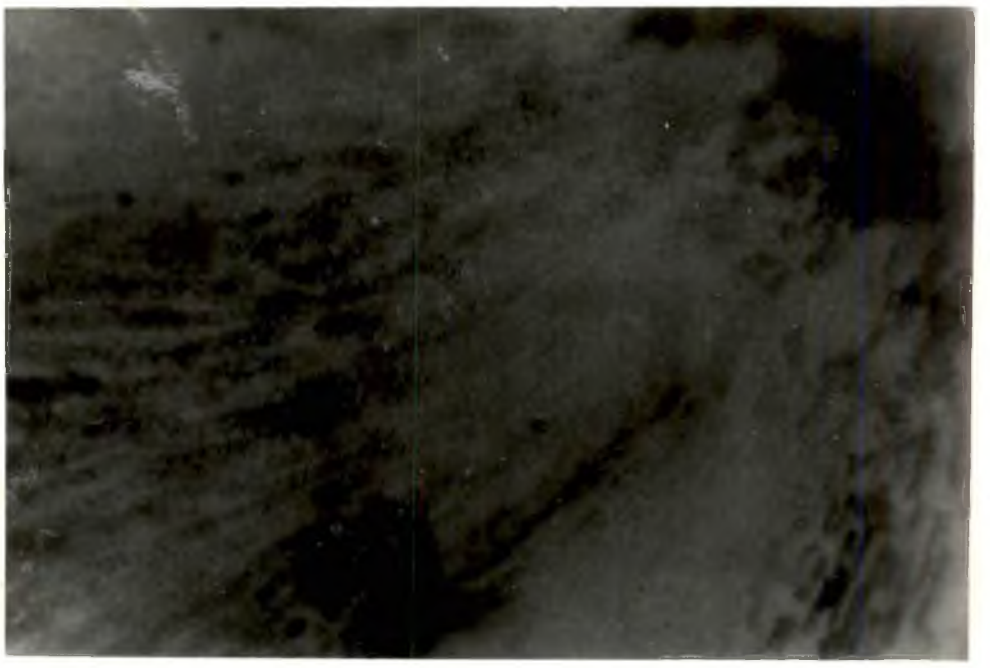
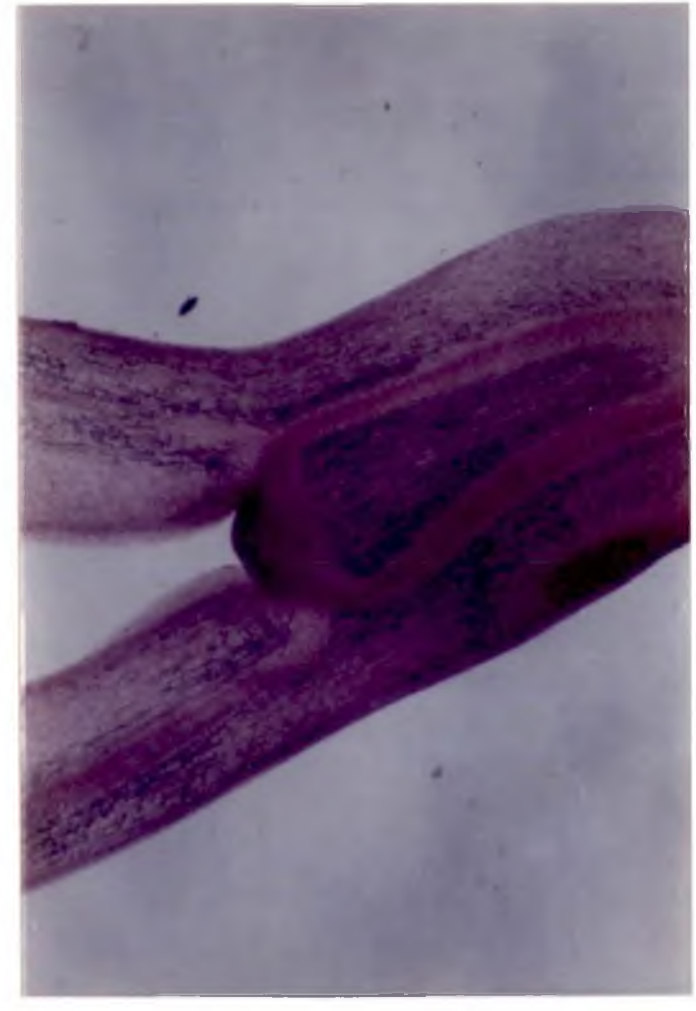


Plate 27

L.S. of a typical bipolar somatic embryoid of Vellari Manga. The darkly stained cells shows the vascular connection between the root and shoot

Plate 28

L.S. of a germinated somatic embryoid of Vellari Manga showing the meristematic growing tip



9. Morphological studies

Using scanning electron microscope, the morphological characters of the different somatic embryoids of Neelum and Vellari Manga, were studied. The examination of the the embryogenic callus, showed the presence of lobed, more or less individualized and organized globular structures (Plate 29 & 30). The pro-embryogenic cell cluster composed of early stages of globular embryos and pro-embryogenic cell aggregates (Plates 29 & 30). In a mature somatic embryoid, cotyledons were well developed (Plate 31). A germinated somatic embryoid with a well developed root and shoot primordium could be observed for both Neelum and Vellari Manga (Plate 32). Abnormalities such as fused cotyledons and secondary somatic embryoids were seen (Plates 33 & 34).

The studies could reveal little variation in morphological characters, of the different stages of somatic embryoids formed from the embryogenic calli, of Neelum and Vellari Manga.

B. *IN VITRO* PROPAGATION *VIA* ENHANCED RELEASE OF AXILLARY BUDS

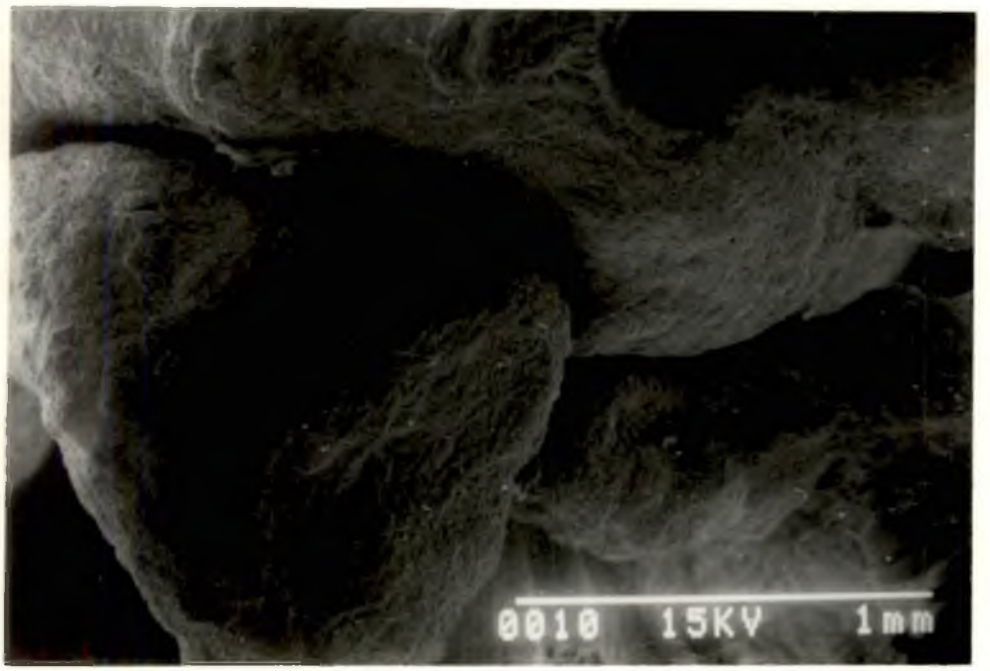
Of the various surface sterilization treatments tried, sodium hypochlorite, 1.0 and 2.0 per cent for 20.0, 30.0 and 40.0 min was not effective. All the cultures got contaminated. Mercuric chloride (0.05, 0.08 and 0.10 per cent) for 5.0, 8.0, 10.0, 12.0, 15.0 and 20.0 min was also tried. The per cent contamination was very high in all treatments, except in mercuric chloride 0.05 per cent for 15.0 min. When the explants were surface sterilized with

Plate 29

Pro-embryogenic cell cluster composed of early stages ofglobular embryos from nucellar explants of Vellari Manga (SEM photograph)

Plate 30

Globular somatic embryoids initiated from the embryogenic callus of Neelum.nucellus (SEM photograph)


0010 15KV 1mm


0011 15KV 1mm

Plate 31

A matured Vellari Manga somatic embryoid with well developed cotyledons (SEM photograph)

Plate 32

A germinated somatic embryoid of Vellari Manga (SEM photograph)

0025 15KV 1mm

0020 15KV 1mm

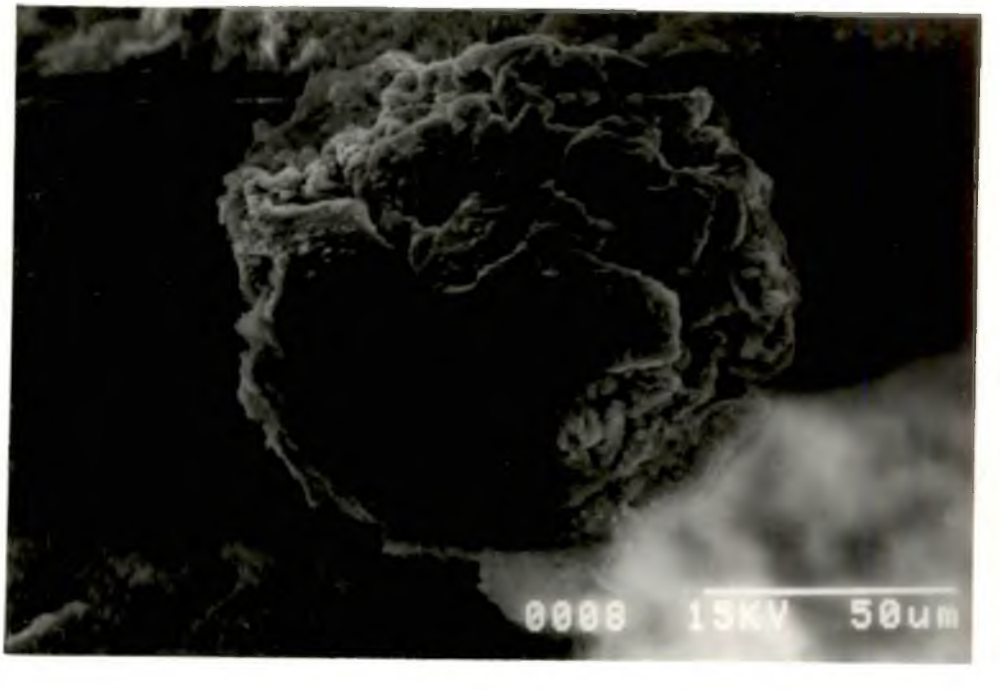
Plate 33

Malformed shoot with fused cotyledons of a Neelum somatic embryoid (SEM photograph)

Plate 34

Secondary somatic embryoid originated from the cotyledon of a primary somatic embryoid as an abnormality (SEM photograph)


0006 15KV 500um


0008 15KV 50um

0.05 per cent mercuric chloride for 15.0 min, 40.0 per cent cultures of Neelum, 26.67 per cent of Mulgoa and 53.33 per cent of Vellari Manga survived (Table 65). But no further response was observed in all the varieties tried. The problem of phenolic interference was severe. Both the tissue and media finally became brown.

Explants (shoot tips and nodal segments) of both monoembryonic (Neelum and Mulgoa) and polyembryonic varieties of mango (Vellari Manga and Pulichi) did not respond to *in vitro* propagation *via* enhanced release of axillary buds. Of the twenty five treatments tried for enhanced release of axillary buds (Table 5), none was effective. However, the nodal segments of *in vitro* zygotic plant-lets of Vellari Manga when cultured on MS medium supplemented with BA 5.0 mg/l, NAA 0.2 mg/l, Sucrose 30.0 g/l, activated charcoal 1.0 g/l and agar 5.5 g/l, responded. Sprouting of axillary buds was observed (Plate 35 & 36). However, multiple shoot formation could not be obtained. When subcultured, the tissue browned and the shoots gradually became necrotic.

C. *IN VITRO* PROPAGATION *VIA* SOMATIC ORGANOGENESIS

Sodium hypochlorite and mercuric chloride were used as surface sterilants. Sodium hypochlorite (1.0 and 2.0 per cent for 20.0, 30.0 and 40.0 min) was not effective. All the cultures were contaminated (Table 66). Treatment with mercuric chloride 0.05 per cent for 8.0 min was effective and resulted in 50.0 per cent contamination free cultures in Neelum, 30.0 per cent in Mulgoa, 10.0 per cent in Vellari Manga and 10.0 per cent in Pulichi (Table 66).

Plate 35

Elongation of axillary bud from the nodal segment of *in vitro* shoot of Vellari Manga

Plate 36

Further growth of the elongated axillary bud

Table 66. Effect of mercuric chloride on the surface sterlization of leaf segments of the mango varieties Neelum, Mulgoa, Vellari Manga and Pulichi

Mercuric chloride		Cultures survived (%)			
(%)	Time (min)	Neelum	Mulgoa	Vellari Manga	Pulichi
0.05	5.0	30.0	0	0	10.0
0.05	8.0	50.0	30.0	10.0	10.0
0.05	10.0	10.0	10.0	10.0	0
0.05	12.0	10.0	0	0	0
0.05	15.0	0	0	0	0
0.05	20.0	0	0	0	0
0.08	5.0	10.0	10.0	10.0	20.0
0.08	8.0	10.0	0	0	0
0.08	10.0	0	0	0	0
0.08	12.0	0	0	0	0
0.08	15.0	0	0	0	0
0.08	20.0	0	0	0	0
0.10	5.0	0	0	0	0
0.10	8.0	0	0	0	0
0.10	10.0	0	0	0	0
0.10	12.0	0	0	0	0
0.10	15.0	0	0	0	0
0.10	20.0	0	0	0	0

The data represent the average value of 10 replications

Culture medium - MS basal medium + BA 5.0 mg/l + NAA 0.24mg/l + BA 2.0mg/l + Sucrose 30.0g/l + agar 6.0g/l

Of the eleven treatments tried (Table 6), one was effective in inducing indirect organogenesis. The treatment (MS + NAA 0.24mg/l + BA 2.0mg/l + sucrose 30.0g/l + agar 6.0 gm/l, with initial culturing of the explant in liquid MS basal medium for 24h and subculturing in solid MS basal medium at a subculturing frequency of 24h for two days) was effective in inducing callus growth from the leaves of Neelum and Mulgoa (Plates 37 & 38). But the calli when transferred to medium for shoot regeneration no response was observed. Proliferation of calli could only be observed in the case of both Neelum and Mulgoa.

Plate 37

Callus production from leaf segment of Neelum

Plate 38

Callus production from leaf segment of Mulgoa

DISCUSSION

DISCUSSION

D, which favoured induction of somatic embryogenesis. Jana *et al.* (1994), in the mango variety Alphonso and Bindu (1995), in the mango varieties Vellari Manga and Pulichi, observed that somatic embryogenesis could be induced only in a medium supplemented with 2,4-D and GA_3.

Sucrose is an ideal carbon / energy source and an osmoticum for supporting somatic embryogenesis. Induction of somatic embryogenesis and the early stages of somatic embryo development often require moderate to high concentrations of sucrose (Dewald *et al.*, 1989a). In the present studies, per cent induction of somatic embryogenesis was the highest at sucrose 60.0g/ l, from nucellus in the monoembryonic variety Neelum, as well as in the polyembryonic variety Vellari Manga (66.54 per cent). Sucrose 30.0g/l and 40.0g/l produced only less per cent induction (Table 16). According to Dewald *et al.* (1989a), five to six per cent sucrose was required to maximize. somatic embryo production in mango varieties James Saigon amd Parris and for the production of normally differentiated somatic embryos. This requirement may be related to the specific carbohydrate metabolism through which water relations and endogenous phytohormones are regulated (Chong and Pua, 1985). The findings of Bindu (1995) that sucrose 60.0g/l was beneficial in inducing somatic embryogenesis in the variety Vellari Manga and Pulichi supports the result of the present investigations. In citrus also, sucrose stimulated somatic embryo development, according to Kochba *et al.* (1978).

For somatic embryogenesis, the optimal concentration and form of nitrogen appears to be critical (Sharp *et al.*, 1980). The benefits of reduced

DISCUSSION

Mango (*Mangifera indica* L.) is a leading fruit crop of Kerala. It occupies an area of 75,000 ha in the State and has an annual production of 2,50,000 t (FIB, 1996). There are over 350 mango varieties in South India (Naik, 1963). The varieties are either monoembryonic or polyembryonic.

Mango is cross pollinated and is generally propagated by seeds. This has led to variability among the progeny. However, only limited variability occurs in the case of polyembryonic varieties. Conventional vegetative propagation methods like inarching and stone grafting are quite successful (Singh, 1996). However, the rate of multiplication is not sufficient to meet the demand for superior planting materials.

Evolving techniques for the *in vitro* propagation of mango varieties will help rapid clonal propagation and early establishment of trees having superior traits. Rapid multiplication of scion material and large scale production of clonal root-stocks are also possible. As the root-stock influences the growth and quality of the scion, clonal root-stocks would be helpful in ensuring uniform performance of the grafts. Rare and endangered cultivars can be multiplied and prevented from becoming extinct. Success has been reported on the *in vitro* propagation of mango varieties (Litz, 1984a and 1986a;

Litz and Gray, 1992; Jaiswal, 1990; Jana *et al.*, 1994). However, several problems have to be tackled before commercially viable protocols could be developed. The present studies were conducted for evolving protocols for the *in vitro* propagation of monoembryonic and polyembryonic mango varieties of Kerala. The results of the investigations are discussed in the following pages.

Standardisation of techniques for the *in vitro* clonal propagation of mango was done *via* somatic embryogenesis, somatic organogenesis and enhanced release of axillary buds. The best response was observed for *in vitro* propagation *via* somatic embryogenesis, whereas the other two routes showed only an initial response.

Somatic embryogenesis is the process by which somatic cells develop into plants through a series of stages characteristic of zygotic embryo development (Plates 15 & 16). Somatic embryogenesis has been reported in many higher plants (Evans *et al.*, 1981) and represents the most striking confirmation of totipotency.

Six monoembryonic and six polyembryonic mango varieties of Kerala were screened for their initial response in inducing somatic embryogenesis. The monoembryonic varieties were Neelum, Bangalora, Banganpalli, Mundappa, Mulgoa and Prior. The highest per cent (66.67) cultures initiating embryogenic callus from nucellus and embryo mass (75.00) was in the variety Neelum. The least response (25.00 per cent) from nucellus and embryomass

(16.67 per cent) was observed for Mundappa. The six polyembryonic varieties tried were Olour, Kurukkan, Vellari Manga, Panchara Varikka, Kilichundan and Vellayani Varikka. The variety Vellari Manga showed the best response. This variety initiated somatic embryoids from nucellus in 83.33 per cent cultures and embryogenic callus from embryo mass in 66.67 per cent cultures. The variety Vellayani Varikka did not respond to any of the induction treatments when nucellus was used as explant. The ability to form somatic embryoids, in most cases, is not merely an intrinsic property of a species. Instead, it is a property under genetic control such that individual genotypes within a species can differ in their ability to undergo somatic embryogenesis (Litz and Gray, 1992). This type of genotypic variation has been reported in other crops. James *et al.* (1984) demonstrated that *Malus* root-stocks M.25 and M.27 could be regenerated on the same medium; however, M.9 and M.26 could not be regenerated using the same protocol. In mango, 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 (Litz *et al.*, 1984). They observed somatic embryogenesis from nucellar explants in both monoembryonic and polyembryonic varieties. Although monoembryonic and polyembryonic cultivars appeared to respond equally well, somatic embryogenesis was observed to be cultivar dependent (Litz *et al.*, 1991). Mathews and Litz (1992) reported that certain cultivars like Red Itamaraca did not respond to the treatment for inducing somatic embryogenesis.

Based on the initial response studies, Neelum from among the monoembryonic varieties and Vellari Manga from among the polyembryonic varieties were selected for further detailed studies.

Embryogenic potential is largely a function of the explant, its stage of development and the interaction of the explant with the growth medium. Choice of appropriate explant is, therefore, critical for morphogenesis. Of the different explants used for inducing somatic embryogenesis, only nucellus and embryo mass responded. In the variety Neelum, nucellar tissue initiated embryogenic callus in 66.67 per cent cultures. Embryo mass of Neelum initiated embryogenic callus in 75.00 per cent cultures. But in Vellari Manga, nucellus was the better explant, recording 83.33 per cent cultures initiating somatic embryoids. The embryo mass of Vellari Manga initiated callus in 66.67 per cent cultures. The present studies showed the superiority of nucellus for inducing somatic embryogenesis. Nucellus represents the wall of the megasporangium and is a nourishing tissue for the developing embryo (Plate 2). As it is part of the mother plant and has the same ploidy, it can be used for clonal propagation. 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 the nucellus in *Pyrus communis* (Janick, 1982), *Syzigium* spp. (Litz, 1984b),*Eriobotrya japonica* (Litz, 1985) and *Theobroma cacao* (Sondahl, 1991). These studies confirmed the morphogenic potential of the nucellus for a broad range of woody plant species. In mango also, nucellus has been identified as the best explant for inducing somatic embryogenesis by Litz

et al. (1982, 1984 and 1992) and Dewald *et al.* (1989a and 1989b). The present study revealed that embryo mass could also be used as explant for inducing somatic embryogenesis. But the propagules obtained cannot be used for clonal propagation since variability may occur. Somatic embryogenesis has also been reported from embryo mass explant in *Theobroma cacao* (Pence *et al.*, 1979) and *Persea americana* (Pliego-Alfaro and Murashige, 1988).

According to Sharp *et al.* (1982), there are two patterns of somatic embryogenesis. In the first, embryogenesis proceeds from cells that are already determined for embryogenesis prior to culturing the explants. Embryogenesis from these pre-embryogenic determined cells (PEDC) requires only an *in vitro* environment to release them into the requisite pattern of cell division. In contrast, induced embryogenic determined cells (IEDC) require an *in vitro* environment not only to re-enter the mitotic cycle, but also for re-determination of the formerly quiescent cells to an embryogenic state. Embryogenesis from both PEDCs and IEDCs are inductive, the former being permissive while the latter is directive. Direct and indirect embryogenesis are used to describe PEDC and IEDC embryogenesis, respectively. In direct embryogenesis, somatic embryoids appear directly from the explant, whereas,in indirect embryogenesis a highly organized callus phase is observed (Sharp *et al.*, 1982). In the present instance also, similar results could be obtained. In the monoembryonic variety Neelum, indirect embryogenesis was observed from both the nucellus and embryo mass. In the polyembryonic variety Vellari Manga, on the other hand, direct embryogenesis occurred from the nucellus and an indirect one from the embryo mass. Somatic

embryogenesis can occur directly from the explant without an intermediate callus phase, as observed in the nucellus of polyembryonic *Citrus* sp. (Rangan *et al.*, 1968) and *Malus domestica* (Eichholtz *et al.*, 1979). In mango, an embryogenic callus is induced in nucellar explants of the monoembryonic type, although somatic embryos developed permissively from nucellar explants of the polyembryonic type. Litz *et al.* (1982) reported that in polyembryonic mango varieties,somatic embryogenesis occurred directly from nucellar explant. Embryogenic cells do not occur naturally in the nucellus of monoembryonic mango (Litz *et al.*, 1982). It is evident, therefore, that embryogenic cells must be induced *in vitro* from the nucellus of monoembryonic types of mango. For that purpose, growth substances are essential in the medium, especially auxins or auxins in combination with cytokinins (Fujimura and Komamine, 1980).

The various steps involved in somatic embryogenesis are induction of embryogenic callus/somatic embryoids, initiation, maturation and germination of somatic embryoids. The influence of culture medium, culture condition (light and temperature) and frequency of subculture on the *in vitro* response of explants was studied.

Kohlenbach (1978) views the induction of embryogenesis as the previous transformation of vacuolated parenchymatical cells into densely cytoplasmic cells with an embryogenic determination. These embryogenic determined cells later lead to embryo development by polarized cell divisions, typical of the embryogenic developmental sequence (globular, heart, torpedo and cotyledonary stages) (Plate 15).

Choice of the basal medium can influence the induction of embryogenic callus / somatic embryoids in the cultures. Of the different basal media such as MS, 1/2 MS, 1/2 B_5 and SH studied, MS basal medium was found better for the induction of somatic embryogenesis in the monoembryonic variety Neelum and in the polyembryonic variety Vellari Manga, when both nucellus and embryo mass were used as explants. In the basal media B_5 and SH, somatic embryogenesis was not induced in Neelum (Table 10). The strength of MS basal medium was also studied in relation to induction of somatic embryogenesis. Half strength MS basal medium was found better than the full strength medium. When nucellus and embryo mass of Neelum were cultured in half strength MS basal medium, 66.67 per cent and 20.00 per cent cultures initiated embryogenic callus, respectively. In full strength MS basal medium, nucellus and embryo mass explants of Neelum initiated embryogenic callus only in 26.67 and 13.33 per cent cultures, respectively. In half strength MS basal medium, 80.00 per cent cultures induced somatic embryoids from nucellus and 30.00 per cent cultures induced embryogenic callus from embryo mass explants of Vellari Manga. In full strength MS basal medium, 60.00 per cent cultures induced somatic embryoids from nucellus and 20.00 per cent induced embryogenic callus from embryo mass. In SH and B_5 basal media, 10.00 per cent cultures induced somatic embryoids from nucellus, whereas, embryo mass did not respond. Growth and morphogenesis of plant tissues *in vitro* are largely governed by the composition of culture media. Although the basic requirements of cultured plant tissues are similar to those of whole plants, in practice, nutritional components promoting optimal growth of a tissue may vary with respect to the particular species.

Media compositions are therefore, formulated considering specific requirements of a particular culture system. Evans *et al.* (1981) noted that 70.00 per cent of the explants of various crops were cultured on MS medium or a modification of MS. A key element of the MS medium is the presence of high levels of nitrogen in the form of ammonium nitrate. A substantial amount of nitrogen, usually in reduced form such as ammonium salts is required for somatic embryogenesis. The superiority of half strength MS basal medium may be due to the low ionic strength which was found beneficial in inducing somatic embryogenesis. The same strength of MS basal medium was identified as the best in inducing embryogenic callus in mono and polyembryonic varieties of mango (Litz *et al.*, 1982; Litz, 1984a; Litz, 1984b; Bindu, 1995).

Somatic embryogenesis in both mono and polyembryonic mango varieties is found to be auxin - dependent. The presence of an auxin, mostly 2,4-D, in the medium is generally essential for inducing somatic embryogenesis (Litz *et al.*, 1982). In the monoembryonic variety Neelum, 2,4-D 5.0mg/l supplemented with GA_3 5.0mg/l in the medium was found to be the best in inducing embryogenic callus from the nucellar tissue (62.50 per cent) and embryo mass (20.83 per cent). When 2,4-D 5.0mg/l alone was used in the induction medium without GA_3, the percentage induction of embryogenic callus was reduced to 20.83 in nucellus and 12.50 in embryo mass (Table 12). When no plant growth substances were supplemented in the induction medium, induction of embryogenic callus did not take place. This showed that 2,4-D along with GA_3 was essential for induction of somatic embryogenesis in the present instance. Growth regulator concentrations in the culture medium are

critical to the control of growth and morphogenesis (Skoog and Miller, 1957). In the present studies, induction of somatic embryogenesis in the polyembryonic variety occurred at lower levels of 2,4-D along with GA_3. The combination of 2,4-D (2.0mg/l and 4.0mg/l) with GA_3 (5.0mg/l) was the best for the induction of somatic embryoids from the nucellar tissue of Vellari Manga (83.33 per cent). When 2,4-D 5.0mg/l alone was used, induction did not take place (Table 13). When no plant growth substances were added in the induction medium also, induction did not happen as in the case of the monoembryonic variety. Hammerschlag *et al.* (1985) reported that in *Prunus persica*, 2,4-D is the only auxin which induced somatic embryogenesis. Embryogenic callus of woody angiosperm species was obtained on a medium containing 2,4-D or other synthetic auxins (Hammerschlag and Litz, 1992). The mechanism by which 2,4-D operates in achieving embryogenesis is not well understood. In PEDC system, the role of auxin is to clone PEDCs; whereas, in IEDC systems, it is the mitogenic substance that results in re-determination (Wann, 1990). Morphogenesis in polyembryonic mangoes conforms with the pattern of direct somatic embryogenesis from predetermined embryogenic callus (Sharp *et al.*, 1980), whereas, somatic embryogenesis from explanted monoembryonic nucellus involves the induction of embryogenically determined cells. In the present studies, GA_3 was also found to be essential along with 2,4-D in inducing somatic embryogenesis. Gibberellins promote cell division and elongation. In tissue culture media, GA_3 is used to stimulate new growth (Danielle and William, 1989). It can promote the growth of callus in combination with auxin and low rates of cytokinin (Engelke *et al.*, 1973). In the present instance, GA_3 might have caused a synergistic effect with 2,4-

nitrogen in addition to nitrate nitrogen for induction of somatic embryogenesis has already been established (Evans *et al.*, 1983). In the present studies, reduced nitrogen was provided in the form of amino acids and coconut water.

Amino acids provide plant cells with an immediately available source of nitrogen and their uptake can also be much more rapid than that of inorganic nitrogen in the same medium (Thom *et al.*, 1981). In the present studies, of the three types of amino acids used, namely, glutamine, arginine and asparagine, only glutamine was found promising in the induction of somatic embryogenesis of both monoembryonic and polyembryonic varieties. Glutamine 400.0mg/l when added to the medium, induced somatic embryogenesis in 48.26 per cent cultures of Neelum. A higher concentration (600.0mg/l) was found to induce the response in 69.39 per cent cultures of Vellari Manga from nucellar explants. When embryo mass was used as explant, the same trend could be observed for both varieties (Table 18). The results were similar to the findings of Jana *et al.* (1994) and Bindu (1995) who observed glutamine to be the best amino acid in inducing somatic embryogenesis in different mango varieties. In carrot also, Kamada and Harada (1979) observed that glutamine was necessary for inducing somatic embryogenesis.

Coconut water 200.0ml/l in the medium was found to induce somatic embryogenesis in 45.08 per cent cultures from nucellar explants of Neelum. In Vellari Manga, coconut water 150.0ml/l and 200.0ml/l in the medium were good in inducing somatic embryogenesis in 38.36 per cent and 37.64 per cent

cultures, respectively, from nucellus. When coconut water was not added, the percentage induction was only 5.28 from the nucellus of both Neelum and Vellari Manga, whereas, the embryo mass did not respond. This shows that coconut water is essential in the induction medium. Coconut water acts as a source of reduced nitrogen in the medium (Tulecke *et al.*, 1961). Steward *et al.* (1964) claimed that coconut water in the medium was required for cell division and embryo formation in carrot cell cultures. In mango, the importance of coconut water in inducing somatic embryogenesis was reported in the varieties Cambodiana, Carabao, Ruby and Kent (Litz *et al.*, 1982 and 1984) and James Saigon and Parris varieties (Dewald, 1989a). According to Mathews and Litz (1992), coconut water can even replace plant growth substances in inducing somatic embryogenesis in PEDCs. Jana *et al.* (1994) and Bindu (1995) also concluded that coconut water 200.0ml/l was essential in inducing somatic embryogenesis in the mango varieties Alphonso, Mundan, Beneshan, Pulichi, Kilichundan and Vellari Manga.

Agar is used as a gelling agent for plant tissue culture media. In the present instance, agar 5.0g/l in the medium recorded significantly higher per cent cultures inducing embryogenic callus in Neelum (55.56 per cent). Agar 6.0g/l induced maximum somatic embryoids in Vellari Manga (42.86 per cent). Intrestingly, in liquid medium, induction of somatic embryogenesis could not be obtained for both Neelum and Vellari Manga. Rao and Narayanaswami (1972) noted that asexual embryos produced from agar gelled media appeared more normal than those in liquid media. Physiologically, agar is not a completely inert material and is a source of various types of substances which

may influence growth in sensitive species (Hu and Wang, 1983). According to Dewald *et al.* (1989 a & b), developmental anomalies were less when mango somatic embryos were cultured on medium containing agar. Bindu (1995) also stressed the importance of agar in the medium in inducing somatic embryogenesis in polyembryonic mango varieties.

The addition of activated charcoal in the medium is useful for the induction of somatic embryogenesis. Activated charcoal is commonly used to remove inhibitory substances of tissue or medium origin. It has the ability to modify medium composition by adsorbing a wide range of compounds. It can prevent unwanted callus growth and promote morphogenesis, particularly, embryogenesis. In the present studies, activated charcoal was found essential for the induction of somatic embryogenesis. Browning of tissue and media were severe when cultured in media without activated charcoal, in the case of both Neelum and Vellari Manga. Such cultures did not show further response. Activated charcoal 2.5g/l registered the highest per cent induction of embryogenic callus from nucellus of Neelum (78.24) and Vellari Manga (66.13) and from embryo mass of Neelum (58.57) and Vellari Manga (62.33). At the lower levels of activated charcoal (0.5g/l and 1.5g/l), the percentage induction was reduced. Earlier reports showed that activated charcoal hastened the differentiation of somatic embryos in mango (Litz *et al.*, 1984; Bindu, 1995). Bindu (1995) observed that activated charcoal 2.5g/l was effective in inducing somatic embryogenesis in the polyembryonic mango varieties Vellari Manga and Pulichi. Hu and Wang (1983) found that treating the explants with PVP, washing them with sterile water and inclusion of

activated charcoal in the medium reduced the oxidation of polyphenols because of the adsorption of the oxidation products by these chemicals. The importance of activated charcoal in the induction of somatic embryogenesis, even by replacing auxin, have also been reported in carrot (Fridborg and Eriksson, 1975), *Carica stipulata* (Litz and Conover, 1982) and date palm (Tisserat, 1982).

Along with the culture media, culture conditions also influence somatic embryogenesis. When the cultures were incubated in induction medium and kept under darkness at low temperature (26 ± 2°C), 57.14 per cent cultures induced embryogenic callus from the nucellus of Neelum and 85.71 per cent induced somatic embryoids from Vellari Manga. Keeping the cultures under light (3000 lux; 16h photoperiod) at low temperature and at ambient temperature (32 ± 2°C) did not induce embryogenic callus in Neelum and Vellari Manga, from nucellar explants. Similar results were obtained by early workers in mango. Induction and growth of mango callus were better in dark than under a normal photoperiod (Rao *et al.*, 1982). Litz *et al.* (1992) maintained the nucellar cultures in the induction medium in darkness at 25°C. Jana *et al.* (1994) observed that dark culture condition was better for induction of somatic embryogenesis than light, for the varieties Alphonso, Mundan and Beneshan. Bindu (1995) found that darkness combined with a temperature of 26 ± 2°C favoured the induction of somatic embryogenesis of Vellari Manga, Pulichi and Kilichundan. The probable reason may be that under darkness, photo-oxidation of auxin in the medium is reduced and hence the potentiality for inducing somatic embryogenesis is increased. Dark culture conditions can also reduce polyphenol oxidation which may accelerate the response.

Many plants are rich in poly-phenolic compounds. After tissue injury during dissection, such compounds will be oxidized in the presence of polyphenol oxidases and the tissue will turn brown or black. The oxidation products (orthoquinones) are known to inhibit enzyme activities, kill the explants and darken the tissues and culture media, thereby reducing the *in vitro* response. Such phenomena impose a serious block on the establishment of primary cultures, especially in woody plants (Hu and Wang, 1983). Frequency of subculture can overcome this problem and influence the effectiveness of somatic embryogenesis. In the present study, when the initial explants were subcultured at five days interval, 30.0 per cent cultures initiated embryogenic callus from the nucellar explants of Neelum and 40.0 per cent from Vellari Manga. As the interval of subculturing was reduced, the phenolic interference was minimised. When the cultures were kept without subculturing, there was a serious problem of phenolics interference and the percentage response was reduced to 10.0 in Neelum and 20.0 in Vellari Manga (Table 21). Litz (1986a) reported on the use of frequent subculturing to counteract polyphenol interference in mango somatic embryogenesis and thereby to increase the response. Litz *et al.* (1991) got similar response when mango explants were subcultured daily for the first week. Frequent subculturing was also found to be beneficial in inducing somatic embryogenesis in apple (Paul *et al.*, 1994).

After four to five weeks in the induction medium, the embryogenic calli were transferred to the initiation or expression medium. The choice of basal medium is critical for the initiation of somatic embryoids. The composition of basal media has been shown to influence specific *in vitro*

responses and their stages. Different basal media such as MS (full and half strength), B_5 and SH were tried. MS basal medium was found to be better than B_5 and SH media. The concentration of inorganic salts in the MS basal medium also seemed to influence the per cent initiation of somatic embryoids, since half strength MS medium was found to be the best for Neelum (56.56 per cent) and Vellari Manga (77.78 per cent). The number of embryoids produced per culture was also the highest (48.80) in this concentration for Neelum. In Vellari Manga, the highest number of embryoids per culture was produced in full strength MS basal medium (66.20) which was on par with half strength MS basal medium (63.20). Litz *et al.* (1982) reported that 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 varieties Cambodiana, Carabao and Sabre. Bindu (1995) could also identify half strength MS basal media as the best in initiating somatic embryoids in the varieties Vellari Manga, Pulichi and Kilichundan. However, Jana *et al.* (1994) obtained the initiation of somatic embryoids of mango varieties Alphonso, Mundan and Beneshan in full strength MS basal medium. This difference can be attributed to the genotypic variations among varieties.

It is well known that plant growth substances influence the developmental stages in somatic embryogenesis. The requirement can vary with plant species and varieties. In the present instance, the highest per cent cultures (72.72) initiating somatic embryoids from embryogenic callus of nucellus in Neelum was in half strength MS basal medium supplemented with 2,4-D 2.0mg/l, GA_3 5.0mg/l and BA 1.0mg/l. The highest number of

embryoids (46.25) was obtained when subcultured in the medium containing 2,4-D 5.0mg/l, GA_3 5.0mg/l and BA 0.05mg/l. In Vellari Manga, the highest per cent cultures initiating somatic embryoids (86.67) from nucellus was when subcultured in media supplemented with 2,4-D 0.5mg/l, GA_3 5.0mg/l and BA 1.0 mg/l. The highest number of embryoids (90.50) was also obtained in the same combination of plant growth substances. The number of embryoids produced per culture was 1.96 times higher in Vellari Manga than in Neelum. The highest number of embryoids produced in Vellari Manga, a polyembryonic variety, may be due the pre-embryogenically determined state of the cells of its nucellus. When 2,4-D was excluded from the media, the percentage initiation was drastically reduced in both the varieties (Tables 22 & 23). When no plant growth substances were included in the medium, initiation of somatic embryoids did not take place in Neelum. And in Vellari Manga, the percentage initiation was reduced to 13.33. In usual practice, the embryogenic callus growing on induction media with relatively high 2,4-D concentrations does not initiate somatic embryoids until after transfer to a medium lacking 2,4-D or to one with a substantially lower 2,4-D concentration (Fujimura and Komamine, 1980). Litz *et al.* (1982) reported that somatic embryoids did not develop beyond the globular stage on initiation medium containing 2,4-D. After subculture on medium without 2,4-D, advanced stages of somatic embryo development were observed. In cashew, Jha (1988) observed that removal of 2,4-D in the initiation medium enhanced the number of embryoids produced; but they failed to develop plantlets. Vieitez and Barciela (1990) observed that globular and heart shaped embryoid structures of *Camellia japonica* differentiated following transfer to medium without 2,4-D. But in the present

studies, lower levels of 2,4-D along with $GA_3$5.0mg/l and BA 1.0mg/l was found to be best in initiating somatic embryoids. Cytokinins stimulate cell division and gibberellin induces cell elongation and enlargement. GA_3 and BA might have caused a synergistic effect with 2,4-D and favoured the initiation of somatic embryoids. This has been supported by the findings of Atree and Fowke (1991), who also reported that somatic embryoids initiated on a medium containing auxin and cytokinin. The beneficial effects of GA_3 in the initiation medium was observed by Nitsch and Nitsch (1969). They recorded that the presence of gibberellin in the initiation medium affects the rate of embryo development but has no effect on the frequency of embryogenesis. Bindu (1995) reported the use of 2,4-D along with GA_3 for initiating somatic embryoids in the mango varieties Pulichi and Kilichundan.

Sucrose has been reported to be the most effective reduced carbon source for the initiation of somatic embryoids (Verma and Dougall, 1977). This requirement may be related to the specific carbohydrate metabolism through which water relations and endogenous phytohormones are regulated (Chong and Pua, 1985). In the present studies, sucrose 60.0g/l in the medium favoured the initiation of somatic embryoids in 80.0 per cent cultures of embryogenic callus of Neelum and produced 57.66 embryoids per culture. At sucrose 40.0g/l, the per cent initiation was reduced to half (Table 26). In Vellari Manga, sucrose 50.0g/l and 60.0g/l were effective in initiating somatic embryoids in 60.0 per cent cultures and produced 41.50 and 42.00 embryoids, respectively. Most of the other reports also show that a higher concentration of sucrose was necessary for somatic embryo initiation. According to Dewald

et al. (1989a), five to six per cent sucrose was required to maximise somatic embryo production in mango and for the production of normally differentiated somatic embryos. Bindu (1995) also reported that sucrose 60.0 g/l is the most ideal for the initiation of somatic embryoids in the polyembryonic mango varieties Vellari Manga and Pulichi. It should also be noted that raising the sucrose concentration in the primary medium to 12 per cent benefitted the formation of embryogenic callus from the scutellum of immature embryos of *Zea mays* (Lu and Ozias, 1982). The high response might have been due to increased osmotic potential provided by higher concentration of sucrose. It should also be noted that sucrose acts as a source of carbon and energy.

Reduced nitrogen in the basal medium is necessary for the initiation of somatic embryoids (Evans *et al.*, 1983). Reduced nitrogen was available in the form of glutamine, casein hydrolysate and coconut water which may be more readily metabolised than inorganic nitrogen.

The amino acid, glutamine supplements the existing ammonium to nitrate ratio in the medium, thereby influencing initiation of somatic embryogenesis and morphogenesis (Litz and Gray, 1992). Glutamine 400.0 mg/l was identified to be the best in initiating somatic embryoids (62.50 per cent in Neelum and 87.50 per cent in Vellari Manga). The highest number of embryoids produced per culture was also recorded in glutamine 400.0 mg/l (81.83 in Neelum and 105.83 in Vellari Manga). The results are in agreement with the findings of Bindu (1995), who also identified glutamine 400.0mg/l to be the best in initiating somatic embryoids in the mango varieties Pulichi,

Kilichundan and Vellari Manga. However, Jana *et al.* (1994) obtained the initiation of somatic embryoids at a higher concentration of glutamine (600.0mg/l) in the varieties Alphonso and Beneshan. This variation might be due to varietal differences.

Inclusion of casein hydrolysate (CH) at a concentration of 500.0 mg/l in the initiation medium significantly enhanced the per cent initiation and number of embryoids in Neelum and Vellari Manga (Tables 28 & 29). Jana *et al.* (1994) and Bindu (1995) got similar results which justified the present findings. In their studies, CH (500.0mg/l) was identified as the best for initiating somatic embryoids in different varieties of mango. Casein hydrolysate is a non-specific organic nitrogen source and serves as an amino acid supplement (Skoog and Miller, 1957).

Coconut water was beneficial in initiating somatic embryoids. Coconut water acts as a source of reduced nitrogen in the medium (Tulecke *et al.*, 1961). Coconut water 200.0 ml/l in the initiation medium could produce significant response in the initiation of somatic embryoids (60.0 per cent in Neelum and 80.0 per cent in Vellari Manga). When coconut water was not added in the medium, the per cent initiation was reduced to 8.33 in Neelum with only 8.80 embryoids per culture. However, in Vellari Manga, initiation did not occur. Dewald *et al.* (1989a) reported that coconut water (20.0 % v/v) added to the basal medium enhanced somatic embryo production in both James Saigon and Parris mango varieties. Jana *et al.* (1994) and Bindu (1995) also reported that coconut water 200.0ml/l was best in initiating somatic embryoids in

different mango varieties. According to Steward *et al.* (1964), coconut water in the initiation medium was required for cell division and embryo formation in carrot cell cultures. All these showed that coconut water is essential for the initiation of somatic embryoids.

Gelling agents are reported to influence somatic embryogenesis, especially initiation. Agar 5.0 g/l and 5.5 g/l could initiate somatic embryoids in 70.0 per cent cultures of Neelum. The highest number of embryoids was produced when agar 5.0 g/l was used (44.33). In Vellari Manga, agar 5.5 g/l initiated somatic embryoids in 50.0 per cent cultures and produced 53.33 emryoids per culture. As the concentration of agar was increased, the number of embryoids produced was found to decrease (Table 34 & 35) . This can be due to the reduced availability of nutrient ions as the osmotic potential in the medium increased. When agar was not used the per cent initiation and number of embryoids produced were very low (Tables 34 & 35). In addition, the embryoids produced in liquid medium were vitrified. Gradually necrosis developed and the embryoids died eventually. According to Dewald *et al.* (1989a), developmental anomalies were less when mango somatic embryos were cultured on medium containing agar. The optimum concentration of gelling agent was found to influence the initiation of somatic embryoids. Jana *et al.* (1994) reported that the initiation of somatic embryoids took place when agar 4.2g/l was used in the medium for the mango varieties Alphonso and Beneshan. Bindu (1995) observed that agar 6.0g/l was the best in initiating somatic embryoids in Pulichi and Vellari Manga varieties. These variations might be due to varietal differences.

Activated charcoal at 2.5 g/l was found ideal in initiating somatic embryoids in both Neelum and Vellari Manga (Table 36). In this concentration, 50.00 per cent initiation with 28.67 embryoids per culture was observed in Neelum and 40.00 per cent initiation with 19.17 embryoids per culture in Vellari Manga. When activated charcoal was not used in the medium, the cultures turned brown and no response could be obtained. This may be due to the ability of activated charcoal in removing substances of tissue or medium origin that are growth inhibitory. It has the ability to modify medium composition by adsorbing a wide range of compounds. Jana *et al.* (1994) and Bindu (1995) supported the finding. They observed that somatic embryogenesis could be initiated in mono and polyembryonic varieties of mango in MS basal medium with supplements and activated charcoal 2.5 g/l.

Frequent subculturing onto fresh medium is essential to overcome the problem of phenolics interference and thereby to increase the *in vitro* response. In the present studies, subculturing at five days were ideal in initiating somatic embryoids than when kept without subculturing. Subculturing at five days produced 44.44 per cent initiation of somatic embryoids in Neelum and 55.56 per cent in Vellari Manga. Subculturing at ten days produced 66.67 per cent initiation in Neelum and 55.56 per cent in Vellari Manga. When the cultures were kept without subculturing, it produced only 22.22 per cent initiation in Neelum and 33.33 per cent in Vellari Manga (Table 38). Litz (1986a) reported that frequent subculturing was necessary to counteract polyphenol interference in mango somatic embryogenesis. Bindu (1995) observed that frequent subculturing was necessary to increase *in vitro* response of mango somatic

embryoids. Subculturing at shorter intervals was also found to be beneficial during somatic embryogenesis in apple (Paul *et al.*, 1994).

Culture conditions (light and temperature) influenced the initiation of somatic embryoids. When kept under darkness at a temperature of 26 ± 2°C, 66.67 per cent of the cultures of Neelum and 83.33 per cent cultures of Vellari Manga initiated somatic embryoids from embryogenic callus of nucellus (Table 37). The highest number of embryoids were also produced at the same culture conditions (50.80 in Neelum and 60.10 in Vellari Manga). None of the cultures initiated somatic embryoids when kept under light either at room temperature or at low temperature. These results are supported by earlier reports. Dewald *et al.* (1989a) kept mango cultures in the initiation medium only in dark. Litz *et al.* (1991) also maintained the cultures at 24-27°C in darkness and subcultured at two weeks interval. Bindu (1995) could initiate somatic embryoids from the nucellus of polyembryonic varieties of mango only when the cultures were kept under darkness at a temperature of 26 ± 2°C. The increased *in vitro* response under dark condition might be due to the reduced polyphenol content and changes in the endogenous ratio of plant growth substances as the photo-oxidation of auxin is reduced.

Somatic embryoids obtained in the initiation medium were transferred to the maturation medium. According to Litz *et al.* (1992), control of somatic embryo maturation was the most critical and difficult process in mango regeneration. If the somatic embryos are not physiologically mature, they cannot germinate normally and usually cannot survive. Size of embryoids

(length in cm) can be taken as an indication of maturity (Litz *et al.*, 1982); the larger the size the more mature they will be. Mango embryos are very large at the time of physiological maturity *in vivo*. Generally they will be 3.0 to 4.0cm long and require several months *in ovulo* to attain the required size (Janick, 1984). With this objective in mind, in the present investigations, different treatments were given to initiate maturation. The treatments included plant growth substance (ABA), basal media, media composition, osmotic regulations, culture conditions and frequency of subculturing.

The plant growth substance (ABA) is a growth retardant, and has been demonstrated to inhibit somatic embryo development beyond the early stages and initiate maturation (Nadel *et al.*, 1990). ABA influenced the quality of somatic embryoids produced. It increases the uniformity of somatic embryoids and reduces the development of abnormal forms (Ammirato, 1983). In the present studies, various concentrations of ABA were tried for obtaining physiogically mature somatic embryoids. For obtaining maturation of Neelum somatic embryoids, ABA 5.0 mg/l in the maturation medium was effective, the size being 1.0 - 2.0 cm (Plate 9). In Vellari Manga; the requirement of ABA was slightly less. The largest size of embryoids (1.5 - 2.0 cm) was attained when the somatic embryoids were subcultured onto maturation media supplemented with 4.22 mg/l ABA. At the highest (10.57mg/l) and lowest (0.50mg/l) levels of ABA, the size was 0.5 cm and below (Table 39). Earlier, Litz *et al.* (1992) reported that 3.00 µM (0.79mg/l) ABA had a significant effect on the appearance of mango somatic embryoids. According to Bindu (1995), maturation of somatic embryoids of

polyembryonic mango varieties (Pulichi, Kilichundan and Vellari Manga) was attained when ABA 4.22 mg/l was supplemented in the medium. But Jana *et al.*(1994) could obtain matured somatic embryos of the mango varieties Alphonso, Mundan and Beneshan, when ABA 1.0mg/l was used in the medium. This might be due to the varietal differences. The normalizing effect of ABA on embryo maturation has also been observed with *Daucus carota* (Kamada and Harada, 1981) and *Pennistum americanum* (Vasil and Vasil, 1981). Kochba *et al.* (1978) also reported that ABA promoted somatic embryo development in citrus ovular callus. All these findings revealed that ABA might serve to promote somatic embryo maturation by countering the effects of growth promoters. Hence its addition to culture medium may permit somatic embryo maturation to proceed under conditions where it normally would not occur. In the present studies, though some extent of maturation of somatic embryoids could be observed, physiologically mature large sized somatic embryoids were difficult to obtain. Hence attempts are being made to ensure proper maturation of the somatic embryoids before transferring to the germination medium for effecting normal germination of somatic embryoids.

Various types of basal media and the concentration of inorganic and organic salts in the basal medium have been shown to influence the maturation of somatic embryoids. In the present studies, modified basal medium containing B_5 major salts and MS minor salts and organics was identified as the best for the maturation of Neelum and Vellari Manga somatic embryoids. This modified basal medium produced the largest sized somatic embryoids (1.0-1.5 cm length). When full strength MS basal medium and B_5 medium

were used separately, the somatic embryoids produced were below 0.5 cm (Table 40). Dewald *et al.* (1989 b) obtained mature embryoids on incubation of the cultures in a maturation medium consisting of B_5 major salts, MS minor salts and organics. However, Jana *et al.* (1994) and Bindu (1995) reported that half strength and full strength MS basal media, respectively, were ideal for maturation of somatic embryoids. This might be due to the variation in chemical composition of tissues with respect to varieties (Razdan, 1993).

Sucrose was provided in the medium for optimising the maturation process of somatic embryoids. In the present studies, attempts were made to find out the effects of concentration of sucrose in the maturation medium on the response of the embryoids. The effective concentrations were 40.0 g/l and 50.0 g/l. These concentrations produced 1.0-1.5 cm long embryoids. When the concentration of sucrose was reduced below 30.0 g/l in Neelum and 40.0 g/l in Vellari Manga, the length of embryoids was below 0.5cm. At the highest level of sucrose also (60.0g/l), the length of embryoids was much reduced (0.5cm) in Neelum (Table 41). This indicates that a moderately high level of sucrose is necessary during the maturation period. Previous reports have also shown that a moderately higher concentration of sucrose is necessary for embryo maturation. According to Dewald *et al.* (1989 b), sucrose 60.0 g/l was essential for the maturation of mango somatic embryoids. This might be due to varietal differences. Litz *et al.* (1993) also reported that a moderately high level of sucrose should be maintained in order to prevent precocious germination and control the development of mango somatic embryos to physiological maturity in the varieties Sensation, Golden Brooks and Brooks.

Jana *et al.* (1994) could get matured somatic embryos of Alphonso, Mundan and Beneshan when 40.0g/l sucrose was used in the medium. The increased osmotic potential provided by moderately higher concentration of sucrose might have contributed to the increased size of the embryoids. Sucrose can also act as the carbon and energy source causing increased size (length in cm) of embryoids.

A substantial amount of reduced form of nitrogen is required for embryo maturation (Razdan, 1993). Casein hydrolysate (100.0 to 500.0 mg/l) and coconut water (150.0 ml/l to 250.0 ml/l) were tried in the maturation media. Casein hydrolysate 100.0 mg/l in the medium produced 0.5-1.0 cm long somatic embryoids in Neelum and 1.0-1.5cm long somatic embryoids in Vellari Manga. At higher levels of CH tried (300.0, 400.0, 500.0 and 600.0mg/l), the size of embryoids produced were 0.5 cm or below (Table 43). At zero level also, the length of embryoids was 0.5cm contributing to poor germination. Hence optimum concentration of CH was standardised. In earlier reports also, CH (100.0mg/l) was found to be the best in initiating maturation of mango somatic embryoids of different varieties (Jana *et al.*, 1994; Bindu, 1995). Casein hydrolysate is a non-specific source of organic nitrogen and serves as an amino acid supplement (Skoog and Miller, 1957).

Optimum quantity of coconut water for the maturation of somatic embryoids could be standardised. Coconut water 200.0 ml/l produced 0.5-1.0cm long somatic embryoids in Neelum and 0.5-1.5 cm long embryoids in Vellari Manga. When coconut water 150.0ml/l and 250.0ml/l were used in

the maturation medium, the size of Vellari Manga somatic embryoids was 0.5cm or below. Hence coconut water 200.0ml/l is recommended to be supplemented in the maturation media. Coconut water, when not added in the medium, resulted in somatic embryoids of less than 0.5 cm length in both varieties (Table 45). The result emphasised the importance of coconut water in the maturation of somatic embryoids, since it is a source of reduced nitrogen, minerals, vitamins, plant growth substances and sugars. Coconut water was identified as an essential component in the maturation of somatic embryoids by various workers in mango. According to Dewald *et al.* (1989b), maturation of somatic embryoids of mango variety Parris was achieved by sequential transfer of somatic embryoids into medium containing 200.0 ml/l coconut water and reduced sucrose concentration. The mango somatic embryoids that were produced on media containing coconut water were also larger and had few developmental abnormalities. Jana *et al.* (1994) and Bindu (1995) also identified 200.0ml/l coconut water to be the best in getting matured mango somatic embryoids. Coconut water 150.0 ml/l and 250.0ml/l in the medium might be sub and supra optimal, respectively, for the maturation process of somatic embryoids, as it reduced the size of embryoids.

Osmotic potential can be critical in the maturation process of somatic embryoids. Agar and polyethylene glycol (PEG) were tried in the maturation medium to bring about various ranges of osmotic potential. Agar (4.5, 5.0 and 5.5 g/l) in the medium produced 0.5-1.0 cm long embryoids in Neelum. But in Vellari Manga, agar 5.0 g/l in the medium produced large sized embryoids (1.5-2.0cm). At higher concentration of agar (6.0g/l and 7.0g/l),

the size of embryoids was reduced (0.5cm or below). This might be due to the non availability of nutrients from the medium due to very high osmotie potential. *It* was interesting to note that somatic embryoids of Neelum and Vellari Manga were found to increase in size when cultured in liquid media (1.5-2.0cm and 1.0-1.5cm respectively). But later they became vitrified, decayed and perished. Other scientists who worked on mango could also observe certain barriers in the maturation process in liquid media. According to Dewald *et al.* (1989b), larger embryos were formed in liquid maturation media but developmental abnormalities were more. He observed that somatic embryoids grown on solid maturation medium developed more normally. Jana *et al.* (1994) could obtain matured somatic embryoids of Alphonso and Beneshan when they used 4.2g/l agar in the medium. This might be due to the varietal difference. Bindu (1995) identified agar 5.5g/l to be the best in getting proper maturation of Pulichi and Kilichundan mango somatic embryoids.

In the present studies, it was found that PEG had no effect on the maturation of somatic embryoids in both Neelum and Vellari Manga. At all the levels tried, the size of embryoids were 0.5cm or below (Table 44). Optimum concentration of PEG could not be obtained because the various concentrations tried might be sub or supra optimal. Hence further studies are required.

The effect of charcoal and PVP were also studied in the maturation process of somatic embryoids by reducing the severity of phenolics

interference in the medium and removing inhibitory substances. When charcoal or PVP were not added in the maturation medium, there was a high rate of phenolics interference and none of the somatic embryoids survived (Tables 46 & 47). PVP had a better response than charcoal in adsorbing phenolic substances. PVP 1.0 per cent in the maturation medium produced 0.5 to 1.0 cm long embryoids in Neelum and Vellari Manga (Table 47). At all the levels of activated charcoal tried, the length of Neelum somatic embryoids was much reduced (0.5cm or below). In Vellari Manga, no difference could be observed (Table 46). PVP was reported to remove phenolic substances more efficiently than activated charcoal in mango somatic embryogenesis (Jaiswal, 1990). In the case of teak somatic embryogenesis, Gupta *et al.* (1980) observed PVP to be more effective. Activated charcoal might have adsorbed some of the useful substances needed for growth along with the inhibitory substances (Fridborg *et al.*, 1978). This might be the reason for the comparatively reduced response when compared with PVP. However, Jana *et al.* (1994) and Bindu (1995) obtained matured somatic embryoids of mango when activated charcoal 2.5g/l was used in the medium. This could be attributed to the differences among varieties.

Dark culture condition was found to be beneficial during the process of maturation of mango somatic embryoids. It was found that incubating the cultures in darkness favoured the maturation of embryoids. The size of embryoids ranged from 0.5-1.0cm in Neelum and slightly larger (1.0-1.5cm) in Vellari Manga (Table 48). The importance of darkness have also been tested by other workers who observed favourable responses. Litz *et al.* (1991)

maintained the cultures of mango in maturation media under darkness only. Bindu (1995) reported that in mango somatic embryogenesis, phenolics problem could be minimised if the cultures were kept under darkness till germination. She observed that the somatic embryoids kept under darkness matured early than those kept under light. In addition to maturation, darkness also prevented precocious germination of somatic embryoids (Dewald *et al.*, 1989b; Litz *et al.*, 1992).

Frequent subculturing could be identified as a beneficial step in the process of maturation of somatic embryoids. The present studies revealed that subculturing at 10 days interval produced 1.0-1.5 cm long somatic embryoids in Neelum and 15 days interval produced 0.5-1.5 cm long embryoids in Vellari Manga (Table 49). However, when the cultures were kept without subculturing, embryo size could not be increased (0.5cm or below). Earlier, Litz *et al.* (1991) maintained the cultures of mango in maturation media by subculturing at two weeks interval. Frequent subculturing was also found to be beneficial in the maturation of apple somatic embryoids (Paul *et al.*, 1994).

Germination of somatic embryoids is a critical factor. Poor germination is typical in many embryogenic culture systems (Litz *et al.*, 1982). They reported many developmental abnormalities during the course of germination. In the present instance also, barriers still persisted, even though stray instances of germination were observed. However, the problem can be tackled by optimising the culture media and components (PGS, osmotic

regulants, ethylene inhibitors), maturity associated problems and culture conditions.

The somatic embryoids were transferred to the germination medium after attaining a size of 1.0 to 2.0cm. Treatments involving plant growth substances like BA, GA_3, 2iP, NAA and their combinations in various concentrations were attempted for the germination of somatic embryoids. All PGS except BA could not bring about germination of somatic embryoids. BA 0.1mg/l and 1.0mg/l supplemented in the germination medium were found to produce near normal germination of somatic embryoids of Neelum (41.60 per cent) and Vellari Manga (58.33 per cent), respectively. At higher concentration (10.0mg/l and 50.0mg/l), none of the embryoids of Neelum showed near normal or normal germination. In Vellari Manga, germination did not occur at the highest concentration (50.0mg/l) and at 10.0mg/l, very poor germination (8.33 per cent) was observed. Thus, higher concentration of BA had a suppressive effect on somatic embryo germination. However, Jana *et al.* (1994) reported that BA 5.0mg/l favoured normal germination of mango somatic embryoids of the variety Alphonso. Bindu (1995) observed that 2iP 1.0mg/l and BA 1.0mg/l supported near normal development of Pulichi somatic embryoids. Litz (1984a) could obtain normal growth of the embryoids in a hormone free medium. These variations might have happened since the responses were cultivar-dependent. BA is a synthetic cytokinin analogue. Kavathekar and Johri (1978) reported that cytokinins are required for the growth of embryos and development of the shoot apex. According to Skoog and Miller (1957), a high cytokinin/low auxin favours shoot differentiation, whereas the reverse, root formation.

Thidiazuron, a substituted phenyl urea compound has been demonstrated to stimulate *in vitro* meristem and shoot formation at usually low concentrations (Briggs *et al.*, 1988). These compounds have strong cytokinin like effect on a wide range of species and on species that respond little to conventional cytokinins. However, in the present instance, the use of thidiazuron in the germination medium was not beneficial (Table 56). Abnormal swelling with fused cotyledons and improper shoot and root development of the somatic embryoids resulted. Surprisingly, in the absence of thidiazuron, 20.00 per cent and 10.00 per cent germination could be observed for Neelum and Vellari Manga, respectively. This clearly revealed that thidiazuron was not necessary for the germination of somatic embryoids.

The choice of basal medium could influence the germination of somatic embryoids. The concentration of inorganic and organic salts in the basal medium influenced the germination of somatic embryoids. A basal medium consisting of B_5 major salts and MS minor salts supported the highest percentage germination of somatic embryoids in Neelum (38.89 per cent) and Vellari Manga (55.56 per cent). Near normal growth of the embryoids could be obtained in this basal medium (Plate 13). When full strength MS and B_5 media were used separately, the per cent germination were reduced to 33.33 in Neelum and 27.78 in Vellari Manga, respectively, with abnormal growth. Likewise abnormalities in germination were observed in most of the cases. Earlier reports in mango also showed that modified B_5 medium consisting of half strength major salt formulation supplemented with coconut water and casein hydrolysate resulted in significantly higher germination rate (Dewald

et al., 1989b). He reported that germination was accompanied by slight enlargement and progressive greening of the somatic embryoids when cultured in the above basal medium. However, Jana *et al.* (1994) could get normal growth of Alphonso somatic embryoids in half strength MS basal medium. This might be because the optimum requirement is species or varietal specific (Razdan, 1993).

A correlation existed between high frequency production of normal somatic embryoids and medium composition. Somatic embryogenesis is dependent on the optimal quantity and ratio of nitrogen in the medium (Sharp *et al.*,1980). Among the various treatments tried, it was observed that reduced concentrations of ammonium and nitrate ions did not influence the germination of somatic embryoids (Tables 52 and 53).

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 were tried. In the treatment with coconut water (200.0 ml/l), near normal germination of the embryoids could be achieved in 40.0 per cent cultures of Neelum and 60.0 per cent of Vellari Manga. When coconut water was not used, germination of Neelum somatic embryoids did not happen. However, in Vellari Manga, 10.0 per cent germination was observed. But the resultant plantlets were malformed with fused cotyledons and absence of bipolarity. Previous reports have also

supported the importance of coconut water in the germination of somatic embryoids of mango. Litz *et al.* (1982) reported that normal plantlet development from mature somatic embryoids has not occurred following the transfer of somatic embryos to coconut water free medium. Dewald *et al.* (1989a) observed that the mango somatic embryos that were produced on media containing coconut water were larger and had few developmental abnormalities. Jana *et al.* (1994) and Bindu (1995) had also reported the importance of coconut water (200.0ml/l) in the germination of mango somatic embryoids.

Osmotic potential was found to influence the germination of somatic embryoids. For regulation of osmotic potential, treatments involving sucrose, agar, polyethylene glycol (PEG) and sodium chloride were tried. The stress conditions provided by these chemicalsexcept PEG favoured their near normal germination. However, normal germination could not be obtained except in a few instances. Formation of secondary somatic embryoids from hypocotyls were common. The embryoids germinated precociously and grew as small seedlings instead of completing normal embryonic development. Secondary somatic embryoids from the cotyledons and hypocotyls inhibited growth of the apical meristem and limited the conversion of primary somatic embryoids into normal plantlets (Kim and Janick, 1989).

In the mango variety Neelum, sucrose 40.0 g/l and 50.0 g/l could initiate normal growth of somatic embryoids. 20.0 per cent germination could be effected in both cases (Table 54). At sucrose 60.0 g/l in the medium,

abnormal growth with fused cotyledons were observed in some of the somatic embryoids, whereas, at sucrose 20.0 g/l, no germination was observed. In Vellari Manga, sucrose 50.0 g/l could induce germination in 40.0 per cent cultures with a near normal growth (Table 55). However, many abnormalities such as fasciation and poorly developed apical meristem were noticed. It was observed that somatic embryos that were differentiated in high sucrose concentration were more normal in appearance than those in low sucrose concentration. This shows the superiority of sucrose as the most effective reduced carbon source for somatic embryogenesis, although many other mono and disaccharides can be successfully employed (Verma and Dougall, 1977). In mango,other scientists had reported near normal somatic embryo development and germination when 60.0g/l sucrose was used in the medium (Litz, 1985 and Dewald *et al.*, 1989b). Higher sucrose concentration also prevented precocious germination in carrot (Ammirato and Steward, 1971). However, Jana *et al.* (1994) found that only 20.0 per cent sucrose was necessary for the germination of somatic embryoids of mango variety Alphonso. The requirement of reduced quantity of sucrose in this context might be due to the varietal difference.

Near normal germination of somatic embryoids could be obtained in solid medium than in liquid medium. In Neelum, agar 5.0g/l initiated germination in 75.0 per cent cultures (Table 61). In Vellari Manga, agar 5.5g/l initiated germination in 50.0 per cent cultures (Table 62). In liquid medium, near normal growth with vitrification was observed in Neelum. In Vellari Manga, when the embryoids were cultured in liquid germination media they

underwent significant enlargement in size but normal growth could be observed. Earlier findings support the present investigations. According to Dewald *et al.* (1989a), Jana *et al.* (1994) and Pu (1995), somatic embryos of mango grown on solid maturation medium developed more normally. This might be due to the high osmotic potential provided by agar in the medium which favoured germination. High osmotic levels also prevented the formation of secondary somatic embryos in carrot cultures (Steward *et al.*, 1975).

Sodium chloride was included in the germination medium as an osmoticum to provide stress for the germinating embryoids. Sodium chloride 0.05 per cent supplemented in the germination medium effected 50.0 per cent germination in Neelum and 40.0 per cent germination in Vellari Manga (Table 57). Near normal growth with proper shoot and root development and elongation of hypocotyl could be obtained in a few embryoids. The germinated embryoids had a well developed root system with 4.50 to 5.25cm long tap root and seven to eight laterals. The shoot system consisted of two well developed green leaves (1.50 x 1.0cm) having normal venation and morphology. A well developed apical meristem could be seen in the germinated embryoids. At the higher levels of sodium chloride (0.10 and 0.20 per cent), abnormal growth was observed and at the highest levels (0.40 and 0.80 per cent), none germinated. When the somatic embryoids were grown in culture media without sodium chloride, though near normal growth occurred, the per cent germination was very much reduced (20.0 per cent in Neelum and 10.0 per cent in Vellari Manga). The beneficial effect of osmotic

stress have been reported in carrot by Kamada *et al.* (1986). He concluded that factors which influence the physiology of cultured cells have a positive effect on embryogenesis. The influence of sodium chloride in effecting near normal germination can be attributed to its ability in preventing secondary somatic embryogenesis and reducing developmental abnormalities by regulating the osmotic potential. However, further studies are required to confirm the result.

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). For overcoming the maturity associated problems, if any, and for improving the germination of mango somatic embryoids, sodium butyrate was incorporated in the germination medium. However, no beneficial effect was observed

Ethylene can influence the process of maturation and germination. The endogenous ethylene level from the nucellus of monoembryonic mango has been reported to be higher than from the polyembryonic type of nucellus (Litz *et al.*, 1993). Endogenous ethylene level above the critical concentration can be inhibitory. For addressing this problem, ethylene inhibitors were used. Silver nitrate and cobalt chloride at 5.0, 10.0 and 15.0mg/l were tried. In Neelum and Vellari Manga, cobalt chloride (10.0mg/l) could induce germination in 40.0 per cent cultures of Neelum and 20.0 per cent cultures of Vellari Manga. A near normal growth of the somatic embryoids with a terminal shoot primordium was observed in some of the embryoids. A few plantlets

could be planted out. Silver nitrate was not beneficial (Table 58). According to Tisserat and Murashige (1977), ethylene suppressed embryo germination in *Citrus and Daucus* cultures. According to them, the availability, uptake, evolution and dispersion of various gases can affect somatic embryogenesis. The effect of ethylene inhibitors was studied by Roustan *et al.* (1989). They observed that cobalt chloride at concentrations *10* μM to 50 μM effectively inhibited ethylene production by embryogenic cultures and significantly stimulated somatic embryogenesis in carrot. They also observed that increase in embryo number was proportional to the inhibition of ethylene production. However, further studies are required.

Attempts were made to overcome the problem of polyphenol interference in the germination medium. Of the two agents tried for reducing the severity of phenolics interference, PVP proved more efficient than activated charcoal (Tables 63 and 64). This chemical at 1.0 per cent supported the highest per cent germination (30.00) in Neelum and Vellari Manga somatic embryoids and effected more or less near normal growth. Activated charcoal along with adsorbing inhibitory growth substances can also bind useful hormones and other metabolites (Eriksson, 1978 and Henshaw, 1978) and this lack of selectivity might have been the reason for its less efficient response. PVP was found effective in overcoming the problem of polyphenol interference in various stages of mango somatic embryogenesis (Jaiswal, 1990 and Litz *et al.*, 1993). PVP was also proved most effective in eliminating the problem of phenolics interference in teak, *Tectona grandis* (Gupta *et al.*, 1980).

A few plantlets of Neelum and Vellari Manga that showed near normal germination were planted out in plastic pots containing sterilized sand after treatment with systemic and contact fungicides (Plate 20). The pots were then placed in a mist chamber having 25.0 per cent shade, a relative humidity of 80.0 per cent and a temperature of 26-27°C. The plants survived for a maximum of three weeks. They turned necrotic and finally dried. *Ex-vitro* establishment is a very critical step in somatic embryogenesis. Rajmohan (1985) reported that *ex-vitro* establishment is difficult in woody perennials. Special treatments like inclusion of Vesicular Arbuscular Mycorrhyza in potting medium and carbon dioxide enrichment might become essential in overcoming *ex-vitro* establishment problems.

Histological studies were made to ascertain the status of somatic embryoids formed from nucellus of Neelum and Vellari Manga. A closed vascular system typical of somatic embryoids could be observed. Each embryoid had a bipolar structure and the root and shoot meristems were connected by vascular strands (Plates 25, 26 & 27).

Morphological characters of the embryoids were also studied using scanning electron microscope (SEM). The morphological features of the embryogenic callus and different stages of embryoids (globular, heart-shaped, torpedo and cotyledonary) could be viewed using SEM and were observed to follow the typical developmental sequence of a true somatic embryoid (Plates 29, 30, 31 & 32).

From the present investigations, the culture media and culture conditions for the first two stages of somatic embryogenesis, namely, induction and initiation could be standardised with respect to two varieties of mango (Neelum and Vellari Manga). Proper maturation and normal germination of the embryoids could be successfully induced and near normal germination could be obtained in certain treatments.

Mango somatic embryoids seem to be peculiar in several respects. Size of embryoids appears to be a deciding factor in the maturation and germination process. A genotypic influence is apparent in embryogenesis. The conditions favouring successful maturation and germination of embryoids in the varieties Alphonso, Mundan and Beneshan (Jana *et al.*, 1994) were not useful for Neelum and Vellari Manga. Optimum duration of embryoid maturation also seems important. Since a positive response could be obtained from the ethylene inhibitors studied for germination, this needs special attention, both in the processes of maturation and germination. Different types and concentration of ethylene inhibitors should be resorted to, which might be useful for success. Triazole compounds could also be resorted to, for inducing normal germination of embryoids. A better understanding of the inherent or induced inhibitors during the course of embryogenesis may also be helpful. Methods have to be standardised for obtaining a sequence of growth media to achieve high frequency production of morphologically normal somatic embryos. Method should also be standardised to increase the size of embryoids and to attain correct physiological maturity so that normal germination occurs. Further refining of the maturation and germination media is thus necessary.

The other routes of *in vitro* propagation, namely, somatic organogenesis and enhanced release of axillary buds were also attempted for the propagation of different monoembryonic and polyembryonic varieties of mango. But promising results could not be obtained. Previous reports on *in vitro* somatic organogenesis and enhanced release of axillary buds are lacking.

Microbial contamination has long been a major problem in the culture establishment of explants. Since the plant parts are exposed to the field for a long time, they harbour various micro-organisms, many of which penetrate into the plant tissues and hence get easily contaminated (Chen and Evans, 1990). In the present study also, high rate of fungal contamination was observed. However, the explants (leaf segments) could be successfully made aseptic by treating with mercuric chloride (0.05 per cent) for eight minutes and shoot tips and nodal segments with mercuric chloride (0.05 per cent) for fifteen minutes. But bacterial contamination occurred even at advanced stages of organogenesis, making it difficult to have a systematic study on the *in vitro* propagation *via* somatic organogenesis and enhanced release of axillary buds. Systemic presence of bacteria has been reported to contaminate the cultures at the time of culture establishment or subsequently during the proliferation in many species (Zimmerman, 1985). The same problem was reported in clove by Geetha (1995). Explants from seedlings raised under sterile conditions may solve the problem of systemic bacteria (Rajmohan, 1985). Preliminary studies also pointed out to two other primary problems, polyphenol oxidation and reduced response of the explants. Due to these

problems also, the studies could not be continued. Further refinement of media is thus necessary for standardising *in vitro* propagation of mono and polyembryonic varieties of mango via somatic organogenesis and enhanced release of axillary buds.

SUMMARY

SUMMARY

Attempts were made in the Plant Tissue Culture Laboratory of the Department of Horticulture, College of Agriculture, Vellayani during 1992-96 to standardise *in vitro* techniques for the rapid clonal propagation of mango (*Mangifera indica* L.).

Methods to standardise *in vitro* propagation of mono and polyembryonic varieties of mango grown in Kerala were tried *via* somatic embryogenesis, somatic organogenesis and enhanced release of axillary buds.

Six monoembryonic and six polyembryonic varieties of mango were used for the initial response studies. Different explants such as nucellus and embryo mass from the ovules of tender mango fruits (about 30-45 days after fertilization), floral parts (segments of immature inflorescence) and segments of tender leaves (15-20 days old) were used.

The effects of different basal media, plant growth substances, sucrose, amino acids, casein hydrolysate, sodium chloride, polyethylene glycol, sodium butyrate, silver nitrate, cobalt chloride, coconut water, agar, activated charcoal and polyvinyl pyrrolidone on the *in vitro* response of various stages of somatic embryogenesis (induction, initiation, maturation and germination of somatic embryoids) were studied. The *in vitro* response as influenced by culture

conditions (light and temperature) and frequency of subculture were also studied.

Histological and morphological studies of the somatic embryoids using hand sections and scanning electron microscope were made to ascertain the status of the somatic embryoids.

The salient findings of the above studies are summarised in this chapter.

1. The initial response in inducing somatic embryogenesis of six monocmbryonic mango varieties, namely, Neelum, Bangalora, Prior, Banganpalli, Mundappa and Mulgoa was studied. The highest per cent cultures (66.67) initiating embryogenic callus from nucellus was observed for the variety Neelum. The least response (25.00 per cent) was recorded by Mundappa. Neelum was selected for further detailed studies.

2. Six polyembryonic varieties of mango such as Olour, Kurukkan, Vellari Manga, Panchara Varikka, Kilichundan and Vellayani Varikka were also compared for their initial response. The highest per cent cultures (83.33) initiating somatic embryoids from nucellus was observed for Vellari Manga. The least response from nucellus was recorded by Kurukkan (33.33 per cent), whereas, the nucellus of Vellayani Varikka did not respond. The variety Vellari Manga was selected for more detailed studies.

3. In the monoembryonic varieties of mango, indirect embryogenesis was observed from both nucellus and embryo mass. In the polyembryonic varieties, direct somatic embryogenesis from nucellus and an indirect one from embryo mass were observed.

4. In the variety Neelum, embryo mass recorded the highest response in inducing somatic embryogenesis (75.00 per cent) whereas in Vellari Manga, nucellus was the best explant (83.33 per cent).

5. In all the varieties tried, both leaf segments and immature flower parts did not respond in inducing somatic embryogenesis.

6. Incorporation of 2.5 g/l activated charcoal in the induction medium was the most effective in reducing the problem of phenolics in both Neelum and Vellari Manga.

7. Attempts to reduce phenolic interference with citric acid or ascorbic acid were not effective.

8. Of the different basal media tried, half strength MS basal medium was the best for the induction of embryogenic callus or somatic embryoids from nucellus and embryo mass of Neelum and Vellari Manga.

9. Culturing the explant (nucellus) in induction medium supplemented with 2,4-D 5.0 mg/l and GA_3 5.0 mg/l, recorded the highest per cent cultures

(62.50) initiating embryogenic callus in Neelum. However, in Vellari Manga, the highest per cent induction (83.33) occurred at lower levels of 2,4-D (2.0 and 4.0 mg/l) along with GA_3 5.0 mg/l. When no plant growth substances were used, induction did not take place.

10. Sucrose 60.0 g/l in the induction medium registered significantly higher per cent cultures inducing somatic embryogenesis from nucellar explants of the monoembryonic variety Neelum and polyembryonic variety Vellari Manga (66.54 per cent). The percentage induction decreased with decreasing levels of sucrose.

11. Among the different types of aminoacids tried for inducing somatic embryogenesis, arginine and asparagine were not useful, whereas, glutamine (400.0 mg/l) gave the highest per cent cultures inducing somatic embryogenesis from nucellus of Neelum (60.00 per cent). Glutamine 600.0 mg/l produced the highest per cent cultures initiating somatic embryoids (69.39) in Vellari Manga.

12. Coconut water 200.0 ml/l in the induction medium favoured 45.08 per cent induction from nucellus of Neelum. In Vellari Manga,coconut water 150.0 ml/l and 200.0 ml/l produced the highest per cent cultures inducing somatic embryogenesis from nucellus (38.36 and 37.64, respectively). The treatments without coconut water recorded only 5.28 per cent induction in Neelum as well as Vellari Manga.

13. Agar 5.0 g/l produced the highest per cent cultures (55.56) inducing somatic embryogenesis in Neelum whereas in Vellari Manga the effects were not significant. The highest and lowest levels of agar tried reduced the per cent induction in both the varieties.

14. Induction medium with 2.5 g/l of activated charcoal registered significantly higher per cent initiation of embryogenic callus in Neelum (78.24) and somatic embryoids in Vellari Manga (66.13) from nucellar explants.

15. Incubating the cultures in dark, rather than light at regulated temperature ($26 \pm 2^{0}C$) was ideal for inducing somatic embryogenesis both in Neelum and Vellari Manga.

16. When subcultured at five days interval, nucellar tissue of Neelum recorded 30.0 per cent cultures initiating embryogenic callus and at five to ten days subculture interval, 40.0 per cent in Vellari Manga. The cultures when kept without subculturing, the problem of phenolics interference was very severe and the percentage response was reduced to 10.0 in Neelum and 20.0 in Vellari Manga.

17. Half strength MS basal medium was identified as the best for the initiation of somatic embryoids in Neelum (56.56 per cent) and Vellari Manga (77.78 per cent) from nucellus. The number of embryoids produced per culture was also the highest in half strength MS basal medium for Neelum

(48.80). However, in Vellari Manga, the number of embryoids produced in MS basal medium (66.20) was on par with half strength MS basal medium (63.20).

18. The highest per cent cultures (72.72) initiating somatic embryoids from nucellus of Neelum was in initiation medium supplemented with 2,4-D 2.0 mg/l, GA_3 5.0 mg/l and BA 1.0 mg/l. The highest number of embryoids (46.25) was obtained when subcultured in the medium containing 2,4-D 5.0 mg/l, GA_3 5.0 mg/l and BA 0.05 mg/l. In the case of Vellari Manga, the highest per cent cultures initiating somatic embryoids (86.67) from nucellus was when subcultured in media supplemented with 2,4-D 0.5 mg/l, GA_3 5.0 mg/l and BA 1.0 mg/l. The highest number of embryoids (90.50) was also obtained at the same combination of plant growth substances. The number of embryoids produced per culture was almost double in Vellari Manga than in Neelum. When 2,4-D was excluded from the media, the percentage initiation was drastically reduced in both the varieties.

19. Surcose 60.0 g/l in the medium favoured the initiation of somatic embryoids in 80.0 per cent cultures from embryogenic callus of nucellus in Neelum. At sucrose 40.0 g/l the percentage initiation was reduced to half. However, the number of embryoids produced were not significant. In Vellari Manga, sucrose 50.0 g/l and 60.0 g/l were effective in initiating somatic embryoids in 60.0 per cent cultures and produced 42.00 and 41.50 embryoids per culture, respectively.

20. Glutamine 400.0 mg/l was found to be the best in initiating somatic embryoids (62.50 per cent in Neelum and 87.50 per cent in Vellari Manga). The highest number of embryoids produced per culture was also at glutamine 400.0 mg/l (81.33 in Neelum and 105.83 in Vellari Manga).

21. Inclusion of casein hydrolysate 500.0 mg/l in the initiation medium significantly enhanced the per cent initiation and number of embryoids in Neelum and Vellari Manga.

22. Addition of coconut water 200.0 ml/l in the initiation medium could produce significant response in the initiation of somatic embryoids (60.0 per cent in Neelum and 80.0 per cent in Vellari Manga). The number of embryoids produced per culture from nucellus was 56.0 in Neelum and 36.0 in Vellari Manga, when 200.0 ml/l coconut water was used.

23. Agar 5.0 g/l and 5.5 g/l could initiate somatic embryoids in 70.0 per cent cultures of Neelum. The highest number of embryoids per ceulture was produced when agar 5.0 g/l was used (44.33). In Vellari Manga, agar 5.5 g/l initiated somatic embryoids in 50.0 per cent cultures and produced 53.33 embryoids per culture. As the concentration of agar increased, the number of embryoids produced per culture was found to decrease.

24. Activated charcoal 2.5 g/l was found ideal in initiating somatic embryoids in both Neelum and Vellari Manga. When activated charcoal was not

used in the medium, the cultures turned brown and no response could be obtained.

25. Subculturing at five days (44.44 per cent response in Neelum and 55.56 per cent in Vellari Manga) and ten days (66.67 per cent response in Neelum and 55.56 in Vellari Manga) were ideal in initiating somatic embryoids than when kept without subculturing.

26. Culture conditions (light and temperature) influenced the initiation of somatic embryoids. When kept under darkness at a temperature of 26 $\pm$ 2°C, 66.67 per cent cultures of Neelum and 83.33 per cent cultures of Vellari Manga initiated somatic embryoids from the embryogenic callus of nucellus. The highest number of embryoids per culture were also produced at the same culture conditions (50.80 in Neelum and 60.10 in Vellari Manga). None of the cultures initiated somatic embryoids when kept under light either at room temperature (32 $\pm$ 2°C) or at low temperature (26 $\pm$ 2°C),

27. Maturation of Neelum somatic embryoids was hastened when 5.0 mg/l abscisic acid was supplemented in the maturation medium, producing 1.0-2.0 cm long embryoids. In Vellari Manga the maximum size (length in cm) of embryoids (1.5-2.0 cm) was attained when the somatic embryoids were subcultured into the maturation media supplemented with 4.22 mg/l abscisic acid. At the highest and lowest level of abscisic acid, the length of embryoids was 0.5 cm and below.

28. Modified basal medium containing B5 major salts, MS minor salts and MS organics was identified as the best for the maturation of Neelum and Vellari Manga somatic embryoids and produced 1.0 - 1.5 cm long somatic embryoids.

29. Attempts were made to find out the effects of reduced concentrations of sucrose in the maturation medium. The effective concentrations were 40.0 g/l and 50.0 g/l and produced 1.0-1.5 cm long embryoids.

30. Casein hydrolysate 100.0 mg/l in the maturation medium produced 0.5-1.0 cm long somatic embryoids in Neelum, whereas, casein hydrolysate 100.0 mg/l and 150.0 mg/l produced 0.5-1.5 cm long embryoids in Vellari Manga.

31. Maturation media supplemented with 200.0 ml/l coconut water produced somatic embryoids of 0.5-1.0 cm length in Neelum and 0.5-1.5 cm length in Vellari Manga. When coconut water was not added in the medium, only somatic embryoids of less than 0.5 cm length could be obtained.

32. Agar (4.5, 5.0 and 5.5 g/l) in the maturation medium produced 0.5-1.0 cm long embryoids in Neelum. In Vellari Manga, agar 5.0 g/l in the medium produced 1.5 - 2.0 cm long embryoids. At higher concentration of agar, the size of embryoids was reduced. In liquid medium, though the size of embryoids was found to increase, vitrification was observed later, destroying the embryoids.

33. Polyethylene glycol had no effect on the maturation of somatic embryoids of both Neelum and Vellari Manga.

34. Polyvinyl pyrrolidone had a better response than activated charcoal in adsorbing phenolic substance and thereby contributing to the maturation process. Polyvinyl pyrrolidone 1.0 per cent in the maturation medium produced 0.5 - 1.0 cm long embryoids in Neelum and Vellari Manga.

35. The effect of light on the maturation of somatic embryoids was studied. It was found that incubating the cultures in darkness was beneficial for the maturation to proceed. When kept under darkness, the length of embryoids ranged from 0.5-1.0 cm in Neelum and 1.0-1.5 cm in Vellari Manga.

36. Subculturing at ten days interval produced 1.0-1.5 cm long somatic embryoids in Neelum and at fifteen days interval produced 0.5 - 1.5 cm long embryoids in Vellari Manga. However, when the cultures were kept without subculturing, embryo size could not be increased.

37. The concentration of inorganic and organic salts in the basal medium influenced the germination of somatic embryoids. A basal medium consisting of B5 major salts and MS minor salts supported the highest percentage germination of somatic embryoids in Neelum (38.89 per cent) and Vellari Manga (55.56 per cent). Near normal to normal growth of the embryoids could be obtained in this basal medium.

38. The plant growth substance BA 0.1 mg/l and 1.0 mg/l supplemented in the germination medium were found to give near normal germination of somatic embryoids of Neelum (41.60 per cent) and Vellari Manga (58.33 per cent), respectively.

39. Supplementing thidiazuron in the germination medium was not beneficial in the germination of both Neelum and Vellari Manga somatic embryoids.

40. Coconut water (200.0 ml/l) could effect near normal germination of somatic embryoids of Neelum (40.0 per cent) and Vellari Manga (60.0 per cent). When coconut water was not used, germination of Neelum somatic embryoids did not occur. However, in Vellari Manga, 10.0 per cent germination was observed. But the resultant plantlets were malformed and undersized with fused cotyledons, lacking bipolarity.

41. Polyethylene glycol had no effect in the germination of Neelum and Vellari Manga somatic embryoids.

42. In Neelum, sucrose 40.0 g/l and 50.0 g/l could initiate normal growth of the somatic embryoids (20.0 per cent germination). At sucrose 60.0 g/l in the medium, abnormal growth with fused cotyledons were observed in some of the somatic embryoids whereas at sucrose 20.0 g/l, no germination was observed. In Vellari Manga, sucrose 50.0 g/l could germinate 40.0 per cent cultures with a near normal growth. However, many abnormalities such as fasciation and poorly developed apical meristems were noticed.

43. Near normal germination of somatic embryoids could be obtained in solid medium than in liquid medium. In Neelum, agar 5.0 g/l helped in the germination in 75.0 per cent cultures. In Vellari Manga, agar 5.5 g/l supported germination in 50.0 per cent cultures.

44. Sodium chloride 0.05 per cent supplemented in the germination medium effected germination in 50.0 per cent cultures of Neelum somatic embryoids and in 40.0 per cent cultures of Vellari Manga somatic embryoids. Near normal growth with proper shoot and root development and elongation of hypocotyl could be obtained in a few embryoids.

45. Attempts to find out the effect of sodium butyrate on the germination of somatic embryoids was not fruitful.

46. Cobalt chloride (10.0 mg/l), supplemented in the germination medium could induce germination in 40.0 per cent cultures of Neelum somatic embryoids and 20.0 per cent cultures of Vellari Manga. A near normal growth of the somatic embryoids with a terminal shoot primordium was observed in some of the embryoids. A few plantlets could be planted out. Silver nitrate was not useful in the germination process of mango somatic embryoids.

47. Attempts were made to overcome the problem of polyphenol interference during germination of somatic embryoids. Of the two agents tried for reducing the severity of phenolics interference, polyvinyl pyrrolidone

(1.0 per cent) proved more effective than activated charcoal, supporting 30.0 per cent germination of Neelum and Vellari Manga somatic embryoids.

48. Though a few plantlets of Neelum and Vellari Manga that showed near normal germination and growth were planted out in plastic pots containing sterilized sand, *ex-vitro* establishment was not successful.

49. Histological studies revealed a closed vascular system, typical of a somatic embryoid. Each embryoid had a bipolar structure.

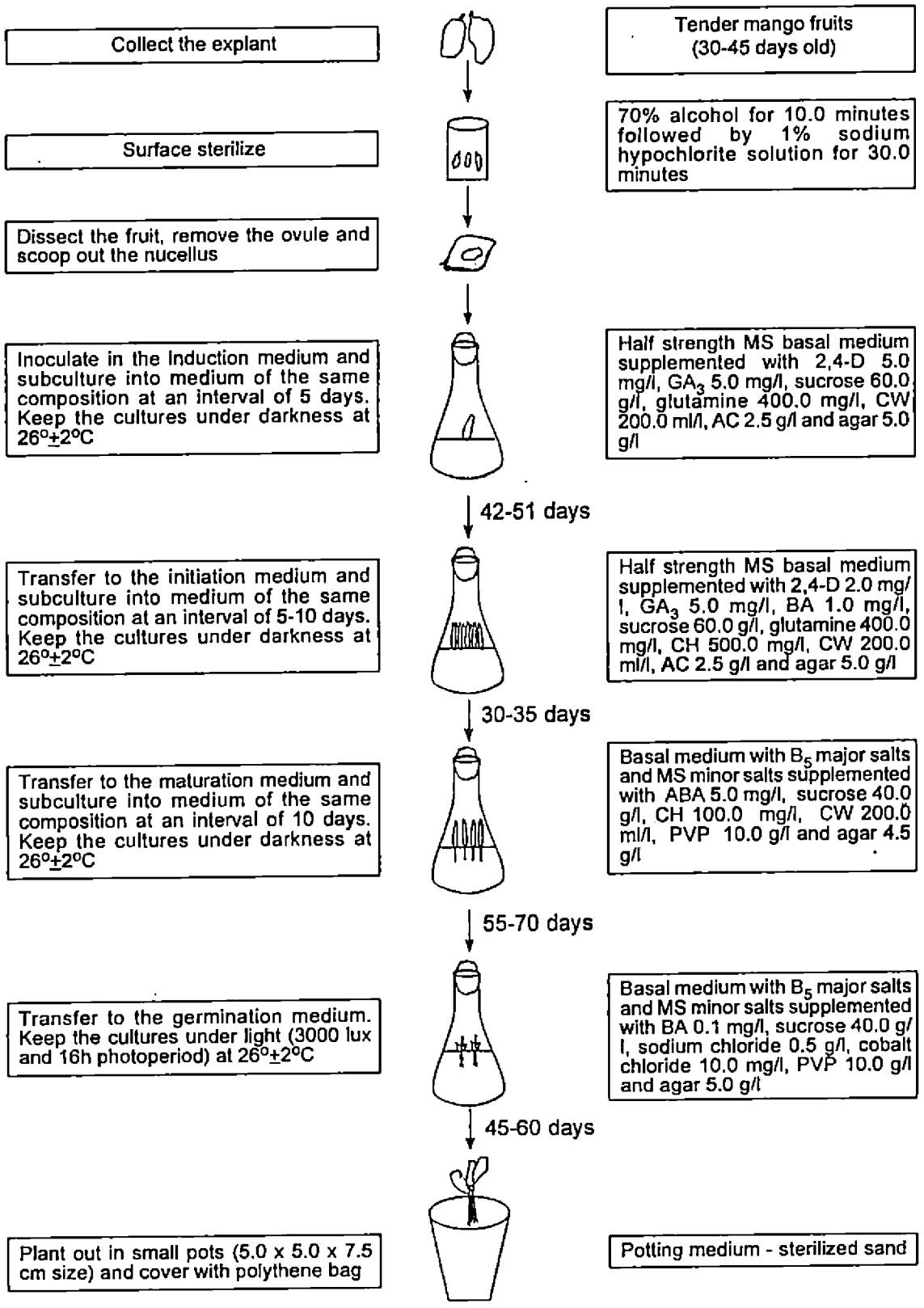
50. Scanning Electron Microscopic studies depicted the morphological features of different stages of somatic embryoids (globular, heart, torpedo and cotyledonary stages) which were typical of the developmental sequence of a true somatic embryoid.

51. Attempts to standaridse *in vitro* propagation *via* somatic organogenesis were not successful. However, de-differentiation could be induced from leaf segment explants of Neelum and Mulgoa.

52. Attempts to induce regeneration *via* enhanced release of axillary buds were not successful.

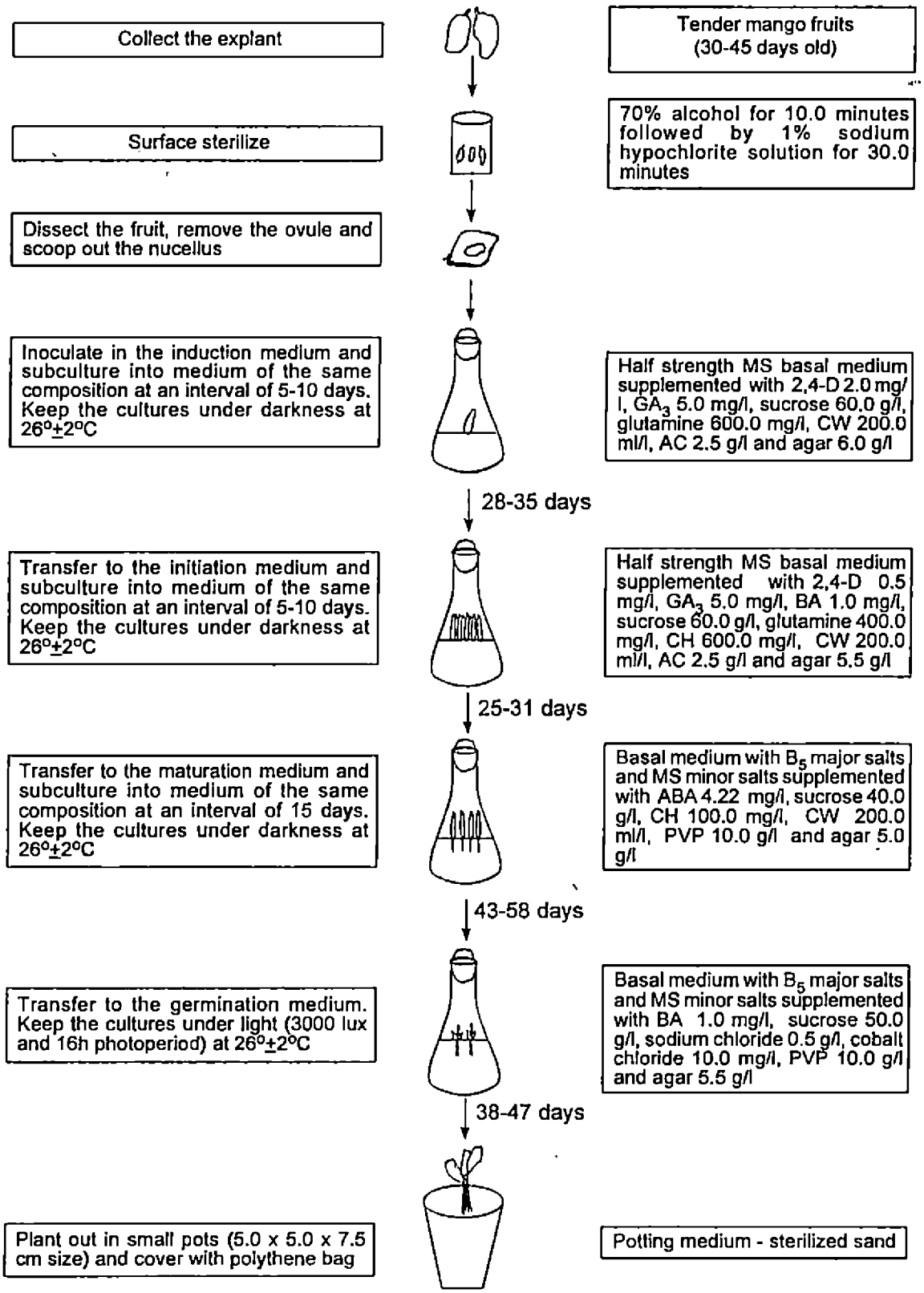
Flow Chart - I

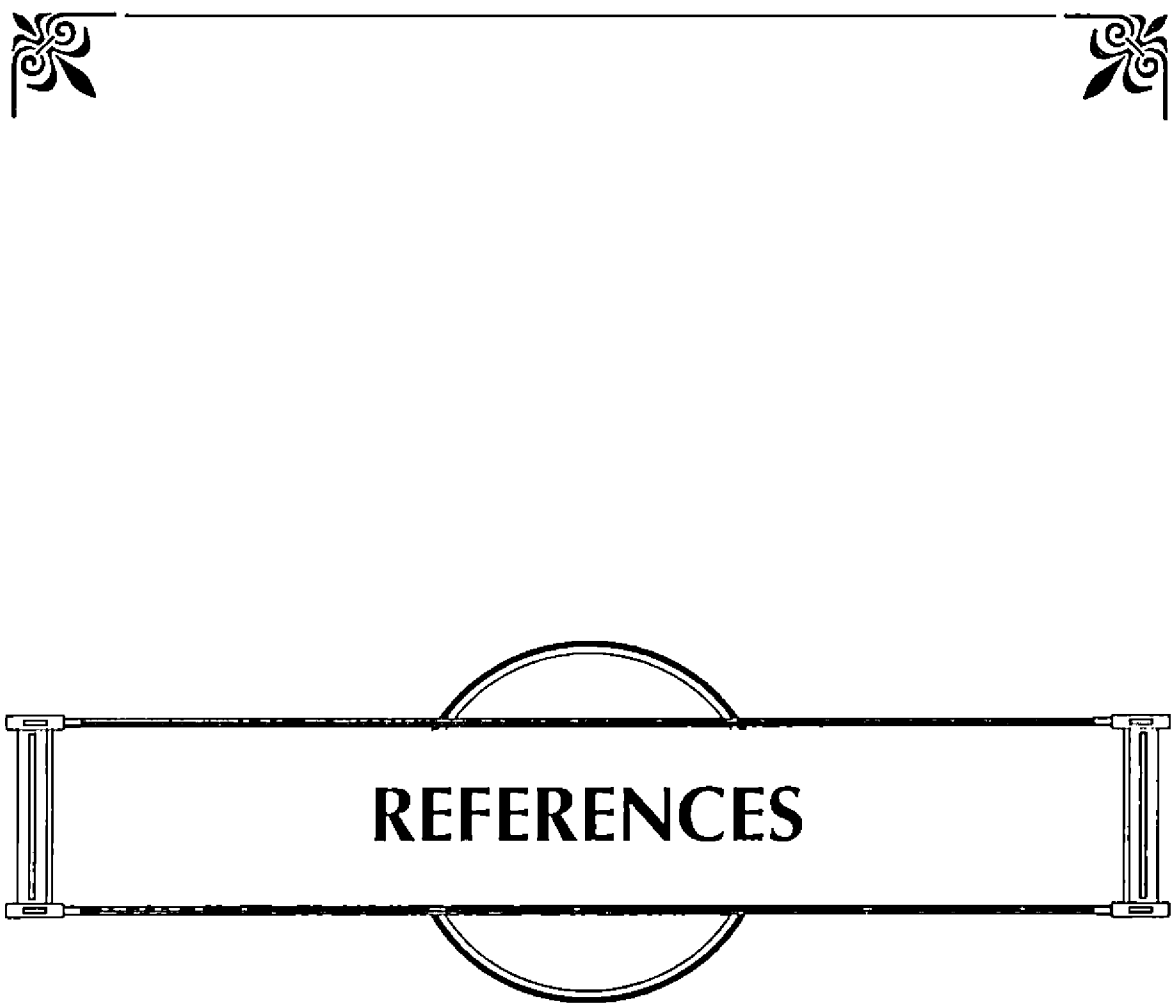
In vitro somatic embryogenesis from nucellus of the mango variety Neelum



Flow Chart - II

In vitro somatic embryogenesis from nucellus of the mango variety Vellari Manga





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* Originals not seen

Standardisation of *in vitro* techniques for the rapid clonal propagation of mango (*Mangifera indica* L.)

By

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ABSTRACT OF A THESIS
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ABSTRACT

Standardisation of techniques for the *in vitro* propagation of mango (*Mangifera indica* L.) varieties was attempted. The studies were carried out at the Plant Tissue Culture Laboratory, Department of Horticulture, College of Agriculture, Vellayani, during 1992-96.

Attempts for the *in vitro* propagation *via* somatic embryogenesis, somatic organogenesis and enhanced release of axillary buds were made. Six monoembryonic and six polyembryonic mango varieties were subjected to the initial response studies. Neelum (monoembryonic) and Vellari Manga (polyembryonic) varieties were selected for further detailed studies. Explants like nucellus, embryo mass, segments of leaf and inflorescence were used.

The effect of culture medium (basal medium, major and minor nutrients, plant growth substances, casein hydrolysate, sucrose, glutamine, coconut water, activated charcoal, polyvinyl pyrrolidone, sodium butyrate, thidiazuron, polyethylene glycol, sodium chloride, silver nitrate, cobalt chloride, agar), culture conditions (light and temperature) and frequency of subculture on the various stages of somatic embryogenesis were studied.

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Among the various explants tried, somatic embryogenesis could be induced only from nucellus and embryo mass. In Neelum, somatic

embryogenesis could be induced in 66.67 per cent cultures of nucellus and 75.00 per cent cultures of embryo mass. In Vellari Manga 83.33 per cent cultures of nucellus and 66.67 per cent cultures of embryo mass responded.

Somatic embryogenesis from nucellus of Neelum could be best induced on half strength MS basal medium supplemented with GA_3 5.0mg/l, 2,4-D 5.0mg/l, sucrose 60.0g/l, glutamine 400.0mg/l, coconut water 200.0ml/l, activated charcoal 2.5g/l and agar 5.0g/l. The ideal treatment for inducing somatic embryogenesis from nucellus of Vellari Manga was half strength MS basal medium supplemented with 2,4-D 2.0mg/l, GA_3 5.0mg/l, sucrose 60.0g/l, glutamine 600.0 mg/l, coconut water 200.0ml/l, activated charcoal 2.5g/l and agar 6.0g/l. Subculturing in medium of the same composition at an interval of five days increased the percentage induction in Neelum (30.0 per cent) and five to ten days in Vellari Manga (40.0 per cent).

The best treatment identified for the initiation of somatic embryoids from nucellus of Neelum was half strength MS basal medium supplemented with 2,4-D 2.0mg/l, GA_3 5.0mg/l, BA 1.0mg/l, sucrose 60.0g/l, glutamine 400.0mg/l, casein hydrolysate 500.0mg/l, coconut water 200.0ml/l, activated charcoal 2.5g/l and agar 5.0g/l. The ideal treatment for the initiation of somatic embryoids from nucellus of Vellari Manga was half strength MS basal medium supplemented with 2,4-D 0.5mg/l, GA_3 5.0mg/l, BA 1.0mg/l, sucrose 60.0g/l, glutamine 400.0mg/l, casein hydrolysate 600.0mg/l, coconut water 200.0ml/l, activated charcoal 2.5g/l and agar 5.5g/l. Subculturing at an interval of ten days in Neelum and five to ten days in Vellari Manga was

beneficial for the initiation of somatic embryoids. The corresponding percentage of initiation of somatic embryoids was 66.67 in Neelum and 55.56 per cent in Vellari Manga.

A medium containing B_5 major salts and MS minor salts supplemented with abscisic acid 5.0mg/l, sucrose 40.0g/l, casein hydrolysate 100.0mg/l, coconut water 200.0ml/l, polyvinyl pyrrolidone 10.0g/l and agar 4.5g/l was the best for supporting the maturation of the somatic embryoids of Neelum. The best medium for the maturation of the somatic embryoids of Vellari Manga contained B_5 major salts, MS minor salts, abscisic acid 4.22mg/l, sucrose 40.0g/l, casein hydrolysate 100.0mg/l, coconut water 200.0ml/l, polyvinyl pyrrolidone 10.0g/l and agar 5.0g/l. The size of embryoids was the highest (1.0-1.5cm long) when subcultured at an interval of ten days for Neelum and fifteen days (0.5-1.5cm long) for Vellari Manga.

Incubating the cultures in darkness at 26 $\pm$ 2°C favoured the induction, initiation and maturation of somatic embryoids of both the varieties.

Near-normal germination of the somatic embryoids of Neelum was observed when cultured on a medium containing B_5 major salts and MS minor salts, BA 0.1mg/l, sucrose 40.0g/l, sodium chloride 0.5g/l, cobalt chloride 10.0mg/l, polyvinyl pyrrolidone 10.0g/l and agar 5.0g/l. Near-normal germination of the somatic embryoids of Vellari Manga was observed on a medium containing B_5 major salts and MS minor salts, BA 1.0mg/l, sucrose 50.0g/l, sodium chloride 0.5g/l, cobalt chloride 10.0mg/l, polyvinyl

pyrrolidone 10.0g/l and agar 5.5g/l. A few germinated embryoids were planted out. However, they did not survive.

Histological and morphological studies ascertained the status of the somatic embryoids formed. Scanning electron microscope studies depicted the morphological features of the developmental stages of the somatic embryoids.

Attempts to standardise *in vitro* propagation *via* somatic organogenesis and enhanced release of axillary buds were not successful. However, de-differentiation could be induced from leaf segment explants of Neelum and Mulgoa.