EVOLVING TECHNIQUES FOR *IN VITRO* SOMATIC EMBRYOGENESIS IN POLYEMBRYONIC MANGO (*Mangifera indica* L.) VARIETIES

By

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DECLARATION

I hereby declare that this thesis entitled "**Evolving techniques for *in vitro* somatic embryogenesis in polyembryonic mango (*Mangifera indica* L.) varieties**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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CERTIFICATE

Certified that this thesis entitled "**Evolving techniques for *in vitro* somatic embryogenesis in polyembryonic mango (*Mangifera indica* L.) varieties**" is a record of research work done independently by Mr. Ramesh.P. (94-12-20) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
BA	6 - benzyl aminopurine
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
GA_3	Gibberellic acid
IAA	Indole - 3- acetic acid
IBA	Indole - 3- butyric acid
IEDCs	Induced embryogenically determined cells
MS	Murashige and Skoog
NAA	Naphthalene acetic acid
PEDCs	Pre - embryogenically determined cells
PVP	Polyvinyl pyrrolidone
RNA	Ribonucleic acid
SH	Shenk and Hildebrandt
TIBA	Tri - iodo benzoic acid
WPM	Woody plant medium
2, 4 - D	2, 4 - dichlorophenoxy acetic acid

INTRODUCTION

1.INTRODUCTION

Mango (*Mangifera indica* L.) is the most important fruit crop of India. The country has over a thousand of mango varieties. They are either monoembryonic or polyembryonic. Most of the commercial varieties belong to the monoembryonic types. Polyembryonic varieties are mostly confined to the west coast of India, especially Kerala. They are having several desirable qualities such as high productivity, regular bearing habit, good aroma and juice content and resistance to biotic and abiotic stress which can be utilized for breeding purpose. Polyembryonic varieties can be used as root stocks for several commercial varieties. Several polyembryonic varieties like Chandrakaran, Kurukkan, Olour, Salem and Vellari Manga have good culinary and dessert qualities. They are used for making food additives such as pickles and amchoor and for industrial production of dessert products like mango juice and mango jelly.

Seed propagation is the most common method in polyembryonic mango varieties which often gives rise to true to type plants. However, seedlings have long pre-bearing age. Vegetative methods of propagation are useful to ensure true to type plants as well as to reduce the pre-bearing age. But these methods are cumbersome and with low rate of multiplication, insufficient to meet the demand. Polyembryonic mango varieties are extensively used as root stocks. The influence of the root stocks on the performance of the scion has been well documented. The genetic variation in the root stocks used for grafting leads to lack of uniformity in growth, yield and quality of the grafts.

In vitro propagation is useful for large scale clonal propagation in several species. The plants produced will be uniform in field performance. Somatic embryogenesis has been reported to be the most promising route of *in vitro* propagation in several tree crops. This route has higher rate of multiplication compared to the other two routes, *viz.* enhanced release of axillary buds and somatic organogenesis. Plantlets developing from somatic embryoids have good tap root system which aids in proper anchorage of the plants.

Evolving *in vitro* methods of propagation in polyembryonic mango varieties can help to overcome the disadvantages of the conventional vegetative propagation methods. Somatic embryogenesis can provide true to type plants in large numbers. *In vitro* developed plants will have uniform performance. When used as root stocks they will ensure uniformity among the grafts. Many of the polyembryonic varieties have become endangered due to reduced availability of land and popular preference for commercial varieties. *In vitro* somatic embryogenesis can aid in conservation of mango germplasm and prevent several valuable varieties becoming extinct. There are several reports on attempts of somatic embryogenesis in mango (Litz *et al.*, 1982, 1984; Dewald *et al.*, 1989a, b; Litz *et al.*, 1991). The present studies were initiated for evolving techniques for *in vitro* somatic embryogenesis in polyembryonic mango varieties, with respect to the various stages like induction, initiation, maturation and germination of somatic embryogenesis, using nucellus and embryo mass explants.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Mango is one of the most important and widely grown tropical fruit crops. It is commonly propagated through seeds. The phenomenon of polyembryony , characterised by the formation of more than one embryo in the seed, is known to occur in a number of mango varieties. They have seedlings arising from the adventive embryos of nucellar origin, which are highly uniform. In India, polyembryonic varieties are mostly confined to the west coast of southern India, especially Kerala (Singh, 1990). The cultivars from Indochina and Philippines also are polyembryonic (Litz, 1985).

Propagation by vegetative methods is popular in mango. However the availability of quality clonal planting materials at a reasonable price continues to be a major constraint in the cultivation of mango. *In vitro* propagation has proved to be a better alternative to the conventional vegetative propagation methods in many woody fruit crops. Attempts were carried out for the *in vitro* propagation of mango also (Litz *et al.*, 1982, 1984; Litz, 1984b, Dewald *et al.*, 1989 a ,b).

There are three routes of *in vitro* propagation (Murashige, 1974). Among them, somatic embryogenesis seems to have significant superiority over the other two, *viz* enhanced release of

axillary buds and somatic organogenesis, especially in woody plants. Somatic embryos are produced at a higher frequency, hence highly potent for rapid and large scale propagation. They are bipolar structures which do not require a separate rooting phase and provide a tap root system for good anchorage in dicotyledonous trees.

2.1. **Somatic embryogenesis**

Somatic embryogenesis is a non-sexual development process which produces a bipolar embryo like structure from somatic cells (Haccius, 1978). Somatic embryos closely resemble their zygotic counterparts with appropriate root, shoot and cotyledons and do not have vascular connection with mother tissue (Ammirato, 1983). However, they are found to mature incompletely without entering the rest phase unlike zygotic embryos (Gray, 1987). Somatic embryoids are clonal materials compared to zygotic embryos which bring about new recombinations (Kester, 1983).

According to Sharp *et al.* (1980) somatic embryogenesis can be direct or indirect as initiated from pre-embryogenic determined cells (PEDCs) or induced embryogenic determined cells (IEDCs). In PEDCs the embryogenic pathway is predetermined and the cells need only the synthesis of an inducer or removal of an inhibitor to express their potential. The IEDCs on the other hand require an induction treatment to the embryogenic state by exposure to specific auxins.

Once the embryogenic state has been reached, both the cell types proliferate in a similar manner .

Isolated cells have shown the capacity to produce embryos (Backs-Husemann and Reinert, 1970) eventhough determined cells may operate singly or in groups to form somatic embryos (Williams and Maheswaran, 1986). Multicellular aggregates are produced before embryo formation (Reinert, 1973). But the pattern of early segmentation differs from the typical embryogenesis of an egg cell (McWilliam *et al.* 1974). Embryogenesis generally proceeds from the globular to the heart, torpedo, cotyledonary and mature somatic embryo stages of development (Tuleke, 1987). During embryogenesis root and shoot develop simultaneously on the same culture medium (Evans *et al.*, 1984).

Potentially embryoidal cells differ from the other cells as revealed by their differential staining properties, conspicuous size and the presence of large number nucleoli per nucleus (Konar *et al.*, 1972). The embryogenic cell clusters consist of small cells, densely cytoplasmic, rich in ribosomes and containing numerous starch grains (Staritsky, 1970). The developmental activations of somatic embryo involve synthesis of ribosomal RNA and protein (Sussex, 1972). More RNA and proteins are found in embryogenic cells than in non-embryogenic cells (Raghavan, 1983). Verma and Dougall (1978)

observed that the DNA content of the embryogenic cells was increasing exponentially and the nutrient content of the culture medium was the limiting factor for this process.

The initiation and development of embryos from the somatic cells in culture was first recognised by Steward (1958) and Reinert (1958) in carrot explants derived from storage tap root. Tisserat *et al.* (1979) reported somatic embryogenesis in 32 families, 81 genera and 132 species of plants. But according to Rangaswami and Sethi (1991) somatic embryogenesis *sensu stricto* has been reported for only 125 species in 42 families of angiosperms.

Large scale clonal propagation is currently the most important application of somatic embryogenesis (Janick, 1993). Somatic embryos can be encapsulated to produce artificial seeds and dormancy can be induced for long term storage and distant transport (Lutz *et al.*, 1985). Other applications of somatic embryogenesis includes germplasm preservation (Ammirato, 1983), production of secondary metabolites (Al-Abta *et al.*, 1979), molecular and biochemical studies (Raghavan, 1983), genetic transformation (McGranahan *et al.*, 1990) and generating somaclonal variants in tree species (Razdan, 1994).

2.2. **Factors influencing somatic embryogenesis**

The key variables influencing somatic embryogenesis include the explant, culture medium, exogenous plant growth substances, evolved gases, mode of culture, environmental conditions, density

and genetic stability of embryogenic cells and synchrony of development of embryoids(Ammirato, 1983).

2.2.1. **Explant**

Somatic embryogenesis serves as the proof for the manifestation of the totipotency of the plant cells. In carrot, just about any part of the body taken at any time of development has successfully produced somatic embryos (Ammirato, 1983). A complete sexual apparatus is not an essential pre-requisite for embryogeny. Some times individual cell or cells from groups may escape and give rise to either embryoids or nodular embryogenic callus consisting of pro-embryoids which are bipolar structures and can germinate into plantlets under suitable conditions (Williams, 1987).

The underlying ploidy level is not seen to affect somatic embryogensis. Somatic embryos have been reared from haploid microspores (Guha and Maheswari, 1964; Nisch, 1969), diploid cells and triploid cells (Lakshmi Sita *et al.*, 1979). The physiological state of the plant from which the explant is taken is very important, as is the season during which it is removed (Ammirato, 1983). Successful cultures are rarely obtained from senescing tissues (Razdan, 1994).

Because of juvenility, nucellus has proved to be more morphogenic than other tissues from mature trees (Litz, 1986). The first report on the formation of embryoids from nucellus was achieved in citrus by Ohta and Furuzato (1957). Nucellus has been

efficiently utilised for somatic embryogenesis in several crops such as citrus (Kochba *et al.*, 1972), apple (Eichooltz *et al.*, 1979), *Eugenia spp.* (Litz, 1984c), *Myrciaria cauliflora* (Litz, 1984a), rubber (Carron and Enjalric, 1985), grapes (Mullins and Srinivasan,1976) and poly and monoembryonic mango varieties (Litz *et al.*, 1982; Litz, 1984b). Neucellar polyembryony occurs in many woody species representing 16 families (Rangaswami, 1982) including tropical species (Litz, 1985).

Litz and Schaffer (1986) demonstrated direct emergence of somatic embryos from nucellus of mango. The *in vitro* responses of mango nucelli depend on the cultivar or genotype and the type of ovule. Nucelli from polyembryonic mango ovule are more embryogenic (Litz, 1987).

Somatic embryogenesis in mango using explants other than nucellus is reported from cotyledon (Rao *et al.*, 1981) and zygotic embryos (Muralidharan *et al.*, 1994). Explants used in other woody plants for somatic embryogenesis include leaves in coffee (Sondahl and Sharp, 1977), roots and immature inflorescences in oil palm (Smith and Thomas, 1973; Tixeira *et al.*,1994), immature zygotic embryos in *Quercus acutissima* (Kim *et al.*, 1994), mature zygotic embryos in spindle tree (Bonneau *et al.*, 1994)and kernels in *Pistacia vera* (Onay *et al.*, 1995).

2.2.2. **Basal medium**

Somatic embryo development has been reported on a wide range of media from relatively dilute White's medium (White, 1963)

to more concentrated MS medium (Murashige and Skoog, 1962), B-5 medium (Gamborg *et al.* 1968) and SH medium (Shenk and Hildebrandt, 1972). Evans *et al.* (1981) reported that 70 per cent of the explants for somatic embryogenesis were cultured on MS medium or its modifications. Weak salt solutions appear to preserve the potentiality in embryogenic suspensions (Ammirato, 1983).

2.2.2.1. **Induction**

Induction treatment is required for the redetermination of the differentiated cells and the development of embryogenically determined cells (Ammirato, 1987). Induction of somatic embryogenesis depends on the use of an appropriate conditioning medium and providing suitable environment (Tuleke, 1987). The most widely used basal medium for induction of somatic embryogenesis is MS medium (Evans *et al.*, 1981). This medium was used for induction of somatic embryogenesis in apple (Eichooltz *et al.*, 1979) and pistachio (Onay *et al.*, 1995). Modified MS medium with half strength of major salts was used for somatic embryogenesis in coffee (Sondahl and Sharp, 1977).

Rao *et al.* (1981) reported the development of callus from cotyledons of mango on MS medium. Muralidharan *et al.* (1994) used MS medium for induction of somatic embryogenesis in mango. Modified MS medium with half strength of major salts was used for induction of somatic embryogenesis in mango by Litz *et al.* (1982,

1984), Litz (1984b), Litz and Schaffer (1986), Dewald *et al.* (1989a) Mathews and Litz (1990) and Jana *et al.* (1994). Dewald *et al.* (1989a) reported the use of a combination of B-5 major salts and MS minor salts for maintenance of embryogenic tissue while Jaiswal (1990) used this combination for induction of somatic embryogenesis in mango.

2.2.2.2. **Initiation**

For initiation (expression) of somatic embryoids, the cultures are to be transferred from the induction medium to the initiation medium. Initiation of mango somatic embryoids was reported to occur on modified MS medium (Litz *et al.*,1982, 1984; Litz 1984b). Dewald *et al.*, (1989a) reported that modified B-5 medium was significantly more effective than WPM, MS or modified MS for production of somatic embryos. Jaiswal (1990) and Litz *et al.* (1991) used the combination of B-5 major salts and MS minor salts for initiation of somatic embryos.

2.2.2.3. **Maturation**

Proper maturation is required for normal germination of somatic embryoids. Precocious germination is undesirable since it may result in abnormal plants. Transfer of primary explants to maturation medium is a general procedure to assure maturation and prevent abnormal development (Tuleke, 1987). Once the embryo is

transferred to the maturation medium, it is anticipated that the organisational events will proceed as in zygotic embryo maturation (Ammirato, 1987).

Tropical tree species possessing seeds with large embryos are found to have difficulty in controlling somatic embryo development to full maturity (Wang and Janick, 1984). Litz *et al.* (1984) reported that maturation of mango somatic embryos occured on MS medium with half strength major salts. According to Dewald *et al.* (1989b) mango somatic embryos have extremely long maturation periods, exceeding 120 days. They used a combination of B-5 major salts and MS minor salts for maturation. Jana *et al.* (1994) could obtain mature somatic embryos of mango on reduced concentration of MS medium.

2.2.2.4 **Germination**

Germination of somatic embryoid is characterised by rapid elongation of root and shoot poles and greening of the embryoid. Litz *et al.*(1982) reported the use of modified MS medium for germination in polyembryonic mango varieties. MS medium with half strength of major salts was used for germination by Litz *et al.* (1984), but successful regeneration of mango plantlets was not obtained. Dewald *et al.* (1989b) reported that modified B-5 medium with half strength of major salts gave a higher germination percentage in mango. Jaiswal (1990) could obtain germination of

mango somatic embryos on formulation with B-5 major salts and MS minor salts.

2.2.3 **Plant growth substances**

Plant growth substances and their combinations are reported to mediate somatic embryogenesis through various stages of development.

2.2.3.1 **Induction**

Somatic embryogenesis was observed to occur even without the application of exogenous plant growth substances as in the case of *Citrus sinensis* (Vardy *et al.*, 1975) and *Camellia sinensis* (Wachira and Ogada, 1995).

The presence of an auxin is required for induction of somatic embryogenesis, especially in IEDC systems (Razdan, 1994). Some species seem to induce embryogenic callus on a wide range of auxins. Somatic embryogenesis in carrot was induced by 2,4-D (Halperin, 1966), IAA (Sussex and Frei, 1968) and NAA (Ammirato and Steward, 1971). But some species are specific in requirement as in the case of millets in which 2,4-D alone was proved to be useful (Vasil and Vasil, 1981).

Litz *et al* (1982, 1984) and Litz (1984b) reported the use of 2,4-D for induction of somatic embryogenesis in mango. Dewald *et*

al, (1989a) reported the use of kinetin in addition to 2,4-D for maintenance of embryogenic callus. Gibberllic acid was used along with 2,4-D for induction by Jana *et al.* (1994).

Prolonged exposure to 2,4-D may result in suppression of successful regeneration of mango plantlets from mango somatic embryos (Litz,1984b). Litz and Schaffer (1986) demonstrated that somatic embryogenesis from mango nucelli was not dependent on 2,4-D as a stimulus. In the absence of 2,4-D somatic embryogenesis occured at relatively low frequency directly from nucellar explants without an intermediate callus phase.

NAA also has been shown to induce somatic embryogenesis in mango (Litz, 1984b). Auxins other than 2,4-D which were able to induce somatic embryogenesis in woody plants included IBA in *Quercus acutissima* (Kim *et al.*, 1994) and TIBA in coffee (Sreenath *et al.*, 1995). Michaux - Ferriere and Dublin (1988) reported that the cytokinin BA could induce somatic embryogenesis in coffee.

2.2.3.2. **Initiation**

Ammirato (1987) observed that the proembryos grew as embryos when they were transferred from an auxin-free medium. Combinations of plant growth substances are important in initiating and maintaining growth and development of somatic embryos. Cytokinins have been important in a number of plant species

(Ammirato, 1983). Their requirement is often specific. Zeatin was the only cytokinin which could promote somatic embryogenesis in carrot (Fujimura and Komamine, 1975).

In mango, somatic embryogenesis was seen not to proceed beyond the globular stage on medium containing the auxin, 2,4-D (Litz,1984b). Subculture of globular embryoids on to medium without 2,4-D permitted the development of cotyledons and the differentiation of a characteristic bipolar structure (Litz, 1987) However, Jaiswal (1990) and Jana *et al.* (1994) reported the use of 2,4-D for initiation also.

Initiation of somatic embryoids in mango occured in the absence of plant growth substances (Dewald *et al.*, 1989a). But cytokinins were seen to enhance the process. Litz *et al.* (1991) observed that after four weeks of culture, there was only an average of 15.4 cotyledonary somatic embryos per culture in the control while 103.6 and 181.2 cotyledonary somatic embryos per culture in treatments containing kinetin 0.1 mg/l and BA 0.05 mg/l respectively and they developed normally to maturity. Abscisic acid (ABA) also is reported to aid somatic embryo formation (Tsai and Tseng, 1979) and promote its development (Kochba *et al.*, 1978).

2.2.3.3 **Maturation**

Maturation is usually aided by growth inhibitors, mainly the natural growth inhibitor ABA. It permitted embryo maturation to continue, but inhibited abnormal proliferations and repressed precocious germination (Ammirato, 1973; 1974). It can prevent

induction of embryogenic competence and thereby inhibit the formation of secondary embryoids. It provided a high frequency of embryos with two cotyledons and the medium lacking ABA resulted in large number of multiple embryos developing from single proembryos (Ammirato, 1987).

Cytokinins are important in fostering somatic embryo maturation (Fujimura and Komamine, 1980), especially cotyledon development (Ammirato and Steward, 1971). Gibberellins are proved to be useful for maturation of somatic embryos in citrus (Kochba *et al* , 1974) and *Santalum album* (Lakshmi Sita *et al*, 1979).

Dewald *et al.* (1989b) reported that the use of ABA was effective for maturation of mango somatic embryos at lower concentrations of sucrose and could control developmental abnormalities. Jaiswal (1990) reported the maturation of mango somatic embryos without growth regulator. Litz *et al.* (1993) observed that ABA could influence the quality of mango somatic embryoids. Jana *et al.* (1994) could obtain mature somatic embryos of mango by employing ABA 1.0 mg/l of. Monsalud *et al.* (1995) observed that ABA could reverse hyperhydricity of secondary somatic embryoids of mango and prevent precocious germination. Somatic embryos grown on ABA were smaller when compared to those grown in the control, but there was no carry over effect of ABA on germination.

2.2.3.4. Germination

Somatic embryos can germinate on agar medium without plant growth substances (Razdan, 1994). Dewald *et al.* (1989b) and Jaiswal (1990) could obtain germination of mango somatic embryos without exogenous application of plant growth substances.

Cytokinins are some times found to be required for development of somatic embryos into plantlets (Kavathekar and Johri, 1978). BA was employed for germination in mango by Litz *et al.* (1984) and Jana *et al.* (1994). Low levels of zeatin (Razdan, 1994) and kinetin (Wachira and Ogada, 1995; Sreenath *et al.*, 1995) were also found useful for germination of somatic embryoids.

According to Button and Bornman (1971) addition of GA_3 enhanced root development in fully developed embryos but suppressed shoot and plantlets needed transfer to another medium lacking GA_3 to avoid development of weak, spindly plants. Gibberellins can be used for breaking dormancy in somatic embryoids (Kavathekar and Johri,1978). It was used for root and shoot development of somatic embryos in citrus (Kochba *et al.*, 1974) and *Santalum album* (Lakshmi Sita *et al.*, 1979).

2.2.4. Other supplements

2.2.4.1 Coconut water

The earliest success in somatic embryogenesis was achieved in media supplemented with coconut water (Steward, 1958). It continued to be extremely useful both in embryo induction and maturation (Pense *et al.*, 1979; Litz and Conover, 1980) though it is

not a prerequisite for induction of embryogenesis (Homes, 1967)

The most efficient somatic embryo production in mango was obtained on medium supplemented with 20 per cent (v/v) coconut water and it was essential for normal plantlet development (Litz *et al.*, 1982). It was also found to delay necrosis of mango somatic embryos on maturation medium (Litz, 1984b). It could enhance somatic embryo production in mango by 18 per cent (Dewald *et al.*, 1989a), stimulate production of greater frequency of somatic embryo and was able to mediate their normal maturation, along with ABA (Dewald *et al.*, 1989b).

2.2.4.2. **Carbon source**

Sucrose has been reported to be the most useful reduced carbon source for somatic embryogenesis (Ammirato, 1983). It also act as an osmoticum that can stimulate and regulate morphogenesis (Wethrell, 1984). In embryogenic systems, sucrose was reported to be useful at a higher concentration of upto 12 per cent (Lu and Ozias-Akins, 1982). Higher concentration supressed precocious germination (Ammirato and Steward, 1971). The requirement of sucrose for maturation could be reduced by the addition of inositol (Steward *et al.*, 1975). Other carbohydrate such as glucose (Homes, 1967), galactose (Kochba *et al.*, 1978) and soluble starch (Kao and Michayluk, 1980) were also reported to be useful for somatic embyogenesis.

Somatic embryo production in mango was reported to be clearly sucrose dependent (Dewald *et al.*, 1989a). Relatively higher concentration of 6.0 per cent (w/v) was optimum for somatic embryo production in mango with more normal appearance while it did not occur at 2.0 per cent level. However sucrose at higher concentration was found to mask the effect of ABA in maturation medium (Dewald *et al.*, 1989b). Germination of mango somatic embryos occured at 4.0 per cent (w/v) of sucrose (Jana *et al.*, 1994).

2.2.4.3. **Reduced nitrogen**

The benefits of reduced nitrogen in addition to nitrate for somatic embryo initiation and maturation have been well established (Ammirato, 1983). A key element of MS medium is the presence of high levels of reduced nitrogen as ammonium nitrate. The nitrogenous compounds are utilised for protein synthesis, but how exactly they effect a change in gene expression deflecting a somatic cell from its normal course to the pathway of an embryo is not known (Narayanaswamy, 1992).

The supply of reduced nitrogen can be in the form of complex organic addenda such as coconut water (Steward and Schantz, 1956), a mixture of amino acids (Kato and Takeyuchi, 1966), casein hydrolysate (Ammirato and Steward, 1971), a single amino acid such as L-glutamine or L-alanine (Wethrell and Dougall, 1976), or in the form of inorganic ammonium alone (Dougall and Verma, 1978).

Glutamine seems to be more important to promote somatic embryogenesis when added singly (Kamada and Harada, 1979). It enhanced somatic embryogenesis in medium which already contained reduced nitrogen (Litz and Conover, 1980).

Glutamine has been extensively used in embryogenic cultures of mango. Dewald *et al.* (1989a,b) reported the use of glutamine at all stages of somatic embryo development in mango. Litz *et al.*(1982, 1984), Litz (1984b) and Mathews and Litz (1990) reported the use of glutamine for somatic embryo production in mango.

Addition of casein hydorlysate was reported to be useful in somatic embryogenesis of citrus (Rangaswami, 1961). But it was reported to inhibit somatic embryo production in mango (Dewald *et al.*, 1989a) and had no effect on maturation (Dewald *et al.*, 1989b). It was reported to be ineffective to prevent necrosis of mango somatic embryos in maturation medium (Litz, 1984b). Jana *et al.*, 1994 reported the use of casein hydrolysate at the rate of 100 mg per litre in maturation medium.

2.2.4.4. **Agents for reducing phenolic exudation**

Addition of activated charcoal has been useful for somatic embryo development in many woody species such as date palm (Tisserat and De Mason, 1980) and oil palm (Tixeira *et al.*, 1994). It substantially reduced the level of phenyl acetic acid and parahydroxy

benzoic acid which inhibited somatic embryogenesis (Fridborg *et al.*, 1978). It absorbed 5-hydroxymethyl furfural, an inhibitor formed by the degradation of sucrose while autoclaving, but found to absorb auxins and cytokinins also(Weatherhead *et al.*, 1978). It did not appear to absorb iron EDTA (Heberle-Bors, 1980).

Muralidharan *et al.*, (1994) reported the use of 1.0 per cent activated charcoal for somatic embryogenesis in mango. Activated charcoal was reported to have deleterious effects in somatic embryogenesis in mango, as most of the somatic embryos resulted from treatments using activated charcoal died during the late heart-shaped stage of development (Litz *et al.*, 1984).

2.2.5. **Mode of culture**

Somatic embryogenesis in mango occurred most efficiently in liquid medium (Litz *et al.*, 1982). The entire callus induced in liquid medium appeared to be embryogenic (Litz *et al.*, 1984). The growth of callus was more rapid in liquid medium though it could be maintained on solid medium also(Litz, 1986). Only fewer somatic embryoids developed on solid medium, but they were unable to advance to maturity (Litz *et al.*, 1991). Liquid shaker culture could help in overcoming phenolic interference in mango (Raghuvanshi and Srivastava, 1995).

Eventhough high rate of growth of embryogenic cultures occurred in liquid medium, subculture on to solid medium was necessary for high frequency production of normal somatic embryos (Dewald *et al.*, 1989a). Mango somatic embryos grown on solid

maturation medium developed more normally as liquid medium gave larger somatic embryo, but with greater developmental abnormality such as polycotyledony (Dewald *et al.*, 1989b), fasciation and loss of bipolarity (Litz *et al.*, 1993) and vitrification or hyperhydricity which was particularly severe in cultures that were highly embryogenic (Monsalud *et al.*, 1995).

Gelrite was reported to be more efficient when compared to agar for somatic embryogenesis in mango (Dewald *et al.*, 1989a). Jaiswal (1990) reported that induction of somatic embryogenesis occured on medium with agar as solidifying agent while initiation of embryoids occured on the same medium when gelrite was used in place of agar. Mango somatic embryos developed more vigorously with apparently more normal morphology on maturation medium with gelrite than with Difco-Bacto-agar (Dewald *et al.*, 1989b). Increased Gel-Gro content of the medium resulted in a higher frequency of reversion of hyperhydricity (Monsalud *et al.*, 1995). But gelrite alone could not reverse vitrification (Litz *et al.*, 1991).

2.2.6. **Culture conditions**

Somatic embryo development is extremely plastic and is subject to cultural and environmental variables (Ammirato, 1983). Certain physical and environmental conditions may be critical for somatic embryogenesis which include quality, intensity and duration of light, period of interruption of darkness, temperature, rate of gas exchange

and volume of culture medium (Tuleke, 1987). The often observed poor ability of regeneration in tree cultures is probably due to improper culture conditions (Simola, 1987).

2.2.6.1. **Light**

The requirement of light for somatic embryogenesis varies according to the crop and stage of development. Prolonged culture in dark was required for coffee (Sondahl and Sharp, 1977) while light was better for cocoa (Kong and Rao, 1981). High light intensity was required for tobacco (Haccius and Lakshmanan, 1965) while far red illumination enhanced somatic embryogenesis in carrot (Newcomb and Wethrell, 1970).

In mango, induction of callus is reported to occur in complete darkness (Rao *et al.*, 1981). Initiation of somatic embryos of mango occurred in suspension cultures kept in darkness (Dewald *et al.*, 1989a). They were maintained in darkness until they acquired physiological maturity and provided with a photoperiod of 16 hours using cool, white fluorescent tubes for germination (Dewald *et al.*, 1989b).

2.2.6.2. **Temperature**

Somatic embryogenesis has been reported to occur at relatively higher temperature. Embryogenic potential was reduced in citrus culture when temperature was lowered from 27^0C to 12^0C (Ammirato, 1983). A temperature of 27^0C was seen ideal for coffee (Michaux - Ferriere and Dublin,1988) and eucalyptus (Muralidharan *et al.*, 1994) also.

Induction of somatic embryogenesis in mango was reported to occur when incubated at 25^{0}C (Litz *et al.*,1991). Suspension culture initiated embryoids when cultured at 24^{0}C to 27^{0}C (Dewald *et al.*, 1989a). Somatic embryoids in maturation medium were incubated at 24^{0}C to 26^{0}C and germination occurred at 25^{0}C (Dewald *et al.*, 1989b).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

Investigations were carried out at the Plant Tissue Culture Laboratory, Department of Horticulture, College of Agriculture, Vellayani with the objective of evolving techniques for *in vitro* somatic embryogenesis in polyembryonic mango varieties during January 1995 to August 1996. The materials and methods tried for induction, initiation, maturation and germination of somatic embryoids are described in this chapter.

3.1. **Varieties**

Six polyembryonic mango varieties namely Pulichi, Vellari Manga, Kalluvarikka, Olour, Kurukkan and Thalimanga were tried for the study.

3.2. **Explants**

Nucellus and embryo mass were used as explants. They were obtained from the ovules of tender mango fruits of about 30 - 45 days after fertilization.

3.2.1. **Nucellus**

Nucellus is a paranchymatous tissue that makes the body of the ovule It is seen surrounded by two integuments of the megasporangium and the embryo is usually seen embedded in it. The major function of this tissue is providing nourishment to the developing embryo. It is a part of the mother plant having the same ploidy level of other somatic cells. Hence it can be used for clonal propagation.

3.2.2. **Embryo mass**

Embryo is usually developing from the fertilized egg or zygote which follows a pre-determined pathway of development. In general an embryo possesses an embryonic root or radicle, an embryonic shoot or plumule and two cotyledons which arise at two sides of the hypocotyl. In polyembryonic mango varieties, several embryos are found to develop from the nucellus in addition to the zygotic embryo and they often suppress the development of the zygotic embryo. They are generally referred to as embryo mass.

3.3. **Collection and preparation**

Tender mango fruits of 30 to 45 days age were collected from different parts of Thiruvananthapuram and Kanyakumari. The collection was made based on the size of fruits, containing nucellus in the right stage, varied from 2 to 4 cm. depending on the variety. The correct size in each variety was identified by cutting the fruit longitudinally and observing the proper stage of development of the nucellus or embryo mass within it. Premature fruits contained watery nucellus while embryo fill up utilizing the nucellus in mature fruits. The pedicel of the fruits were removed, they were washed thoroughly in tap water with a few drops of 'Labolein' as wetting agent and then in double glass distilled water. They were kept in open containers with proper aeration until surface sterilization and inoculation were carried out.

3.4. **Surface sterilization**

Surface sterilization of the fruits was carried out inside a laminar air flow chamber just before inoculation. The fruits were transferred to a sterile beaker and treated with 70 per cent ethyl alcohol for 10 minutes. The alcohol

3.6 Media preparation

The basal media used for the study were MS (Murashige and Skoog, 1962) and B-5 (Gamborg *et al.*, 1968). Analytical grade chemicals from British Drug House (Bombay), Sisco Research Laboratory (Bombay) and E Merck (Bombay) were used for the preparation of the media.

Standard (Thorpe, 1980) procedures were followed for the preparation of MS and B-5 media. Stock solutions of major and minor nutrients, organics and plant growth substances were prepared by dissolving the required quantities of chemicals in specific volume of double glass distilled water. They were stored under refrigerated condition (+4^{0}C).

The culture vessels used were 'Borosil' brand test tubes (25 x 150 mm) and Erlenmeyer flasks (100 ml). They were washed with diluted 'Labolene' and tap water, rinsed with double glass distilled water and kept overnight in a hot air oven (160^{0}C) for drying and pre-sterilization.

All items of glassware used for the preparation of culture media were washed thoroughly in diluted 'Labolene' and tap water and rinsed with double glass distilled water. Specific quantities of stock solutions were pipetted out into a 1000 ml beaker. Coconut water, when used in the medium, was collected from freshly harvested tender coconut, filtered and measured quantities were added. Myo-inositol, sucrose and all other solids, except agar and activated charcoal (when used) were added directly in the medium. The

total volume was made up to 980 ml using double glass distilled water. The pH of the medium was adjusted between 5.6 and 5.8 using 0.1 N NaOH or 0.1 N HCl with the aid of an electronic pH meter (Philips make, model PP 9046). Agar (in the case of solid medium) was added to the medium and final volume made up to 1000 ml.

The medium was heated by placing the beaker on a heating mantle with constant stirring using a glass rod till the agar melted. Activated charcoal when used in the medium was added at this stage and stirred well for uniform distribution. The medium was then poured into the pre-sterilized culture vessels at the rate of 15 ml in test tube and 30 ml in Erlenmeyer flasks. The mouth of the culture vessels were plugged tightly with non-absorbent cotton covered with aluminium foil or paper, labelled and autoclaved at 121 °C temperature and 1.06 kg per cm^2 pressure for 20 minutes.

3.7 **Somatic embryogenesis**

3.7.1 **Using nucellus as explant**

3.7.1.1 **Induction**

Explants were subjected to different treatments for induction of somatic embryogenesis.

The treatments involved different levels and combinations of the basal media (MS and B-5), plant growth substances (2,4-D, BA and GA_3), different concentrations of glutamine, sucrose, coconut water and activated charcoal

The MS medium with half strength of major saltss was compared with MS medium having the minor saltss, substituted with B-5 major saltss in order to assess the effect of basal medium on the induction of somatic embryogenesis Sixteen treatments were tried for optimising the level of plant growth substances. Four treatments each were tried for glutamine (Table 1), sucrose (Table 2), coconut water (Table 3) and activated charcoal (Table 4).

The effect of light on induction was assessed by keeping the cultures either in darkness or in light. Darkness was provided by keeping the cultures in a temperature controlled (26 ± 2 ^{0}C) dark room. Light (3000 lux, 16 hours photoperiod) was provided using cool white fluorescent tubes.

Six to twenty four replications were provided for treatments. Observations were recorded on the number of cultures producing embryogenic callus / initiating direct embryoids.

3.7.1.2 **Initiation (Expression)**

The cultures that were showing response in the induction medium were transferred to the initiation (expression) medium.

Murashige and Skoog medium containing half strength of major salts was used as the basal medium. Eighteen treatments were tried to assess the effect of plant growth substances (2,4- D, BA and GA_3; Table 5) eight for casein hydrolysate (Table 6) and five each for coconut water and glutamine

Plate 1. Nucellus inoculated on the induction medium.

Plate 2. Somatic embryoes initiating from nucellar explant.

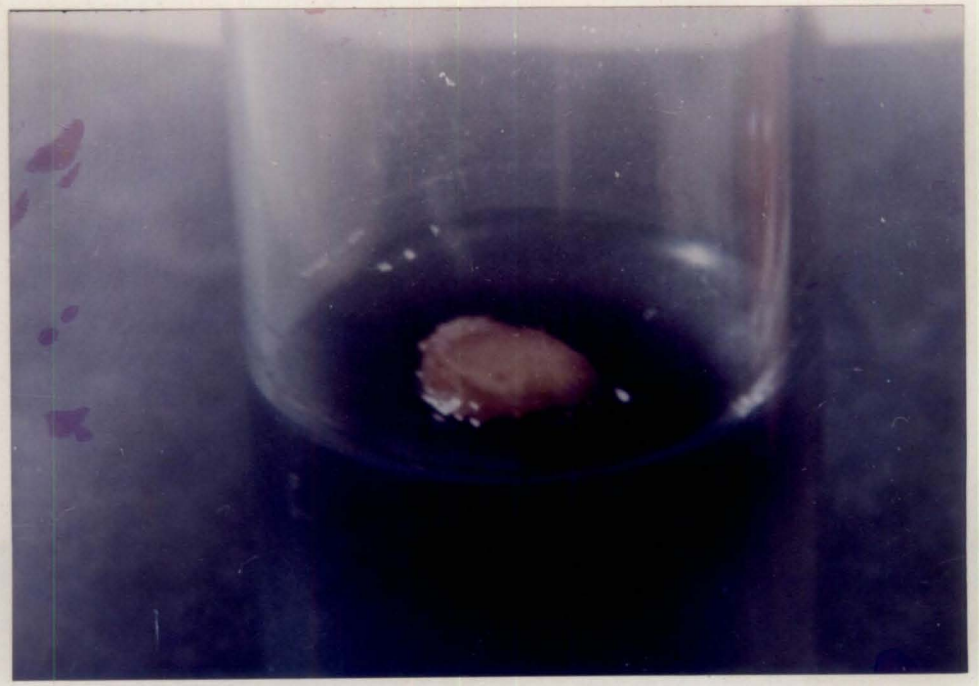


Table 1. **Treatments tried to assess the effect of glutamine on the induction of somatic embryogenesis from nucellus of polyembryonic mango varieties**

Medium : 1/2 MS + inositol 150 mg / l + GA_3 5.0 mg / l + 2, 4-D 5.0 mg / l + sucrose 60 g / l + coconut water 200 ml / l + agar 6.0 g /l + activated charcoal 2.5g /l

Treatment No.	Glutamine (mg/l)
IG_1	0
IG_2	200
IG_3	400
IG_4	600

Table 2 **Treatments tried to assess the effect of sucrose on the induction of somatic embryogenesis from nucellus of polyembryonic mango varieties**

Medium : 1/2 MS + inositol 150 mg / l + GA_3 5.0 mg / l + 2, 4-D 5.0 mg / l + glutamine 400 mg/l + coconut water 200 ml / l + agar 6.0 g /l + activated charcoal 2.5 g /l

Treatment No.	Sucrose (g/l)
IS_1	30
IS_2	40
IS_3	50
IS_4	60

Table 3 **Treatments tried to assess the effect of coconut water on the induction of somatic embryogenesis from nucellus of polyembryonic mango varieties**

Medium : 1/2 MS + inositol 150 mg / l + GA_3 5.0 mg / l + 2, 4-D 5.0 mg / l + glutamine 400 mg/l + sucrose 60 g/l + agar 6.0 g /l + activated charcoal 2.5 g /l

Treatment No.	Coconut water (ml/l)
ICW_1	0
ICW_2	150
ICW_3	200
ICW_4	250

Table 4 **Treatments tried to assess the effect of activated charcoal on the induction of somatic embryogenesis from nucellus of polyembryonic mango varieties**

Medium : 1/2 MS + inositol 150 mg / l + GA_3 5.0 mg / l + 2, 4-D 5.0 mg / l + glutamine 400 mg/l + sucrose 60 g/l + agar 6.0 g /l + coconut water 200 ml/l

Treatment No.	Activated charcoal (g/l)
IAC_1	0
IAC_2	1.25
IAC_3	2.50
IAC_4	3.75

Table 5 **Treatments tried to assess the effect of plant growth substances on the initiation (expression) of somatic embryoids from nucellus of polyembryonic mango varieties**

Medium : 1/2 MS + inositol 150 mg / l + glutamine 400 mg/l + casein hydrolysate 500 mg/l + sucrose 60 g/l + coconut water 200 ml /l + agar 5.5 g /l + activated charcoal 2.5 g/l

Treatment No.	Plant growth substances (mg/l)
E_1	GA_3 0 + BA 0 + 2,4-D 0
E_2	GA_3 0 + BA 0 + 2,4-D 2
E_3	GA_3 0 + BA 0 + 2,4-D 4
E_4	GA_3 0 + BA 0.5 + 2,4-D 0
E_5	GA_3 0 + BA 0.5 + 2,4-D 2
E_6	GA_3 0 + BA 0.5 + 2,4-D 4
E_7	GA_3 0 + BA 1.0 + 2,4-D 0
E_8	GA_3 0 + BA 1.0 + 2,4-D 2
E_9	GA_3 0 + BA 1.0 + 2,4-D 4
E_{10}	GA_3 5.0 + BA 0 + 2,4-D 0
E_{11}	GA_3 5.0 + BA 0 + 2,4-D 2
E_{12}	GA_3 5.0 + BA 0 + 2,4-D 4
E_{13}	GA_3 5.0 + BA 0.5 + 2,4-D 0
E_{14}	GA_3 5.0 + BA 0.5 + 2,4-D 2
E_{15}	GA_3 5.0 + BA 0.5 + 2,4-D 4
E_{16}	GA_3 5.0 + BA 1.0 + 2,4-D 0
E_{17}	GA_3 5.0 + BA 1.0 + 2,4-D 2
E_{18}	GA_3 5.0 + BA 1.0 + 2,4-D 4

(Table 8). The effect of chelated iron was assessed with five treatments by providing different levels of ferrous sulphate heptahydrate and sodium salts of EDTA in the basal medium (Table 7).

Four to six replications were provided for the treatments. The cultures were kept in darkness (26 ± 2 ^{0}C). Observations were recorded on the number of cultures initiating embryoids, number of embryoids initiated per culture, size of embryoids and other visual characters such as colour and abnormalities.

3.7.1.3 **Maturation**

The somatic embryoids initiated were subjected to various maturation treatments in order to have normal germination.

3.7.1.3.1 **Culture medium**

The basal medium having B-5 major salts and MS minor saltss, was used for maturation treatments. Ten treatments were tried to assess the effect of the plant growth substances - ABA and BA (Table 9), four each for sucrose (Table 10) and coconut water (Table 11). Nine treatments were tried for the effect of cobaltous chloride, sodium butyrate and sodium chloride (Table 12).

3.7.1.3.2 **Mode of culture**

Liquid medium as well as solid medium were tried in order to assess the effect of mode of culture on maturation of somatic embryoids.

Table 6 **Treatments tried to assess the effect of casein hydrolysate on the initiation (expression) of somatic embryoids from nucellus of polyembryonic mango varieties**

Medium : 1/2 MS + inositol 150 mg / l + 2, 4-D 2.0 mg/l + BA 1.0 mg/l + GA_3 5.0 mg/l + glutamine 400 mg/l + sucrose 60 g/l + coconut water 200 ml /l + agar 5.5 g /l + activated charcoal 2.5 g/l

Treatment No.	Casein hydrolysate (mg/l)
ECH_1	0
ECH_2	100
ECH_3	200
ECH_4	300
ECH_5	400
ECH_6	500
ECH_7	600
ECH_8	700

Table 7 **Treatments tried to assess the effect of chelated iron on the initiation (expression) of somatic embryoids from nucellus of polyembryonic mango varieties**

Medium : Modified MS without ferrous sulphate and EDTA (disodiam salts) + inositol 150 mg / l + 2, 4-D 2.0 mg/l + BA 1.0 mg/l + GA_3 5.0 mg/l + glutamine 400 mg/l + casein hydrolysate 500 mg/l + sucrose 60 g/l + coconut water 200 ml /l + agar 5.5 g /l + activated charcoal 2.5 g/l

Treatment No.	Chelated iron (mg/l)
ECI_1	Ferrous sulphate 0 + EDTA (Na salts) 0
ECI_2	Ferrous sulphate 6.95 + EDTA (Na salts) 9.33
ECI_3	Ferrous sulphate 13.90 + EDTA (Na salts) 18.65
ECI_4	Ferrous sulphate 27.80 + EDTA (Na salts) 37.30
ECI_5	Ferrous sulphate 55.60 + EDTA (Na salts) 74.60

Table 8 **Treatments tried to assess the effect of coconut water on the initiation (expression) of somatic embryoids from nucellus of polyembryonic mango varieties**

Medium : 1/2 MS + inositol 150 mg / l + 2, 4-D 2.0 mg/l + BA 1.0 mg/l + GA_3 5.0 mg/l + glutamine 400 mg/l + casein hydrolysate 500 mg/l + sucrose 60 g/l + agar 5.5 g /l + activated charcoal 2.5 g/l

Treatment No.	Coconut water (ml/l)
ECW_1	0
ECW_2	100
ECW_3	150
ECW_4	200
ECW_5	250

Table 9 **Treatments tried to assess the effect of plant growth substances on the maturation of somatic embryoids derived from nucellus of polyembryonic mango varieties**

Medium : B-5 major salts + MS minor salts + inositol 150 ml/l + casein hydrolysate 100 ml/l + sucrose 40 g/l + PVP 10 g/l + coconut water 200 ml/l + agar 5.0 g/l

Treatment No.	Plant growth substances (mg/l)
M_1	BA 0 + ABA 1.0
M_2	BA 0 + ABA 2.0
M_3	BA 0 + ABA 3.0
M_4	BA 0 + ABA 4.0
M_5	BA 0 + ABA 5.0
M_6	BA 0.1 + ABA 1.0
M_7	BA 0.1 + ABA 2.0
M_8	BA 0.1 + ABA 3.0
M_9	BA 0.1 + ABA 4.0
M_{10}	BA 0.1 + ABA 5.0

Table 10 **Treatments tried to assess the effect of sucrose on the maturation of somatic embryoids derived from nucellus of polyembryonic mango varieties**

Medium : B-5 major salts + MS minor salts + inositol 150 ml/l + ABA 3.0 mg/l + casein hydrolysate 100 ml/l + PVP 10 g/l + coconut water 200 ml/l + agar 5.0 g/l

Treatment No.	Sucrose (g/l)
MS_1	30
MS_2	40
MS_3	50
MS_4	60

Table 11 **Treatments tried to assess the effect of coconut water on the maturation of somatic embryoids derived from nucellus of polyembryonic mango varieties**

Medium : B-5 major salts + MS minor salts + inositol 150 ml/l + ABA 3.0 mg/l + casein hydrolysate 100 ml/l + PVP 10 g/l + sucrose 40 g/l + agar 5.0 g/l

Treatment No.	Coconut water (ml/l)
MCW_1	0
MCW_2	100
MCW_3	150
MCW_4	200

Table 12 **Treatments tried to assess the effect of cobaltous chloride, sodium butyrate and sodium chloride on the maturation of somatic embryoids derived from nucellus of polyembryonic mango varieties**

Medium : B-5 major salts + MS minor salts + inositol 150 ml/l + ABA 3.0 mg/l + casein hydrolysate 100 ml/l + PVP 10 g/l + sucrose 40 g/l + coconut water 200 ml/l + agar 5.0 g/l

Treatment No.	Media Supplement
MI_1	Control
MI_2	Cobaltous chloride 5.0 mg/l
MI_3	Cobaltous chloride 10.0 mg/l
MI_4	Cobaltous chloride 15.0 mg/l
MI_5	Sodium butyrate 1.0 mM/l
MI_6	Sodium butyrate 2.0 mM/l
MI_7	Sodium chloride 0.5 g/l
MI_8	Sodium chloride 1.0 g/l
MI_9	Sodium chloride 2.0 g/l

3.7.1.3.3 **Culture conditions**

The performance of somatic embryoids was assessed in the presence and absence of light. Light (3000 lux, 16 hours photoperiod) was provided using cool white fluorescent tubes. Darkness was provided by keeping the culture in a temperature controlled dark room (26 ± 2 ^{0}C).

Three to six replication were provided for the treatments. Observations were recorded on the number, size, colour and other visual characteristics such as root emergence and developmental abnormalities of somatic embryoids (if any).

3.7.1.4 **Germination**

The mature somatic embryoids were subjected to various germination treatments involving culture media, mode of culture and culture conditions.

3.7.1.4.1 **Culture medium**

The basal medium used for germination treatments was a combination of B-5 major saltss and MS minor saltss. Fifteen treatments involving plant growth substances (BA, kinetin and GA_3 ; Table 13) six treatments for sucrose (Table 14) and seven for cobaltous chloride and sodium butyrate (Table 15) were tried for germination of somatic embryoids.

Table 13 **Treatments tried to assess the effect of plant growth substances on the germination of embryoids of polyembryonic mango varieties**

Medium : B-5 major salts + MS minor salts + inositol 150 ml/l + sucrose 50 g/l + PVP 10 g/l + agar 5.0 g/l

Treatment No.	Plant growth substanses (mg/l)
G_1	Control
G_2	BA 0.05
G_3	BA 0.1
G_4	BA 0.2
G_5	BA 0.4
G_6	BA 0.8
G_7	BA 1.6
G_8	Kinetin 0.05
G_9	Kinetin 0.1
G_{10}	Kinetin 0.2
G_{11}	Kinetin 0.4
G_{12}	Kinetin 0.8
G_{13}	Kinetin 1.6
G_{14}	GA_3 1.0
G_{15}	GA_3 1.0 + BA 0.1

Table 14 **Treatments tried to assess the effect of sucrose on the germination of embryoids derived from nucellus of polyembryonic mango varieties**

Medium : B-5 major salts + MS minor salts + inositol 150 ml/l + BA 0.1 mg/l + PVP 10 g/l + agar 5.0 g/l

Treatment No.	Sucrose (g/l)
GS_1	10
GS_2	20
GS_3	30
GS_4	40
GS_5	50
GS_6	60

Table 15 **Treatments tried to assess the effect of cobaltous chloride / sodium butyrate on the germination of embryoids derived from nucellus of polyembryonic mango varieties**

Medium : B-5 major salts + MS minor salts + inositol 150 ml/l + BA 0.1 mg/l + sucrose 50 g/l + PVP 10 g/l + agar 5.0 g/l

Treatment No.	Media supplement
GI_1	Control
GI_2	Cobaltous chloride 5.0 mg/l
GI_3	Cobaltous chloride 10.0 mg/l
GI_4	Cobaltous chloride 15.0 mg/l
GI_5	Sodium butyrate 1.0 mM/l
GI_6	Sodium butyrate 2.0 mM/l
GI_7	Sodium butyrate 4.0 mM/l

3.7.1.4.2 **Mode of culture**

Liquid medium as well as solid medium were tried in order to assess the effect of mode of culture on germination of somatic embryoids.

3.7.1.4.3 **Culture conditions**

The cultures were kept in light (3000 lux, 16 hours photoperiod) provided by cool white fluorescent tubes or in darkness in order to assess the effect of light on somatic embryoids.

Observations were recorded on the number of cultures germinating, showing root / shoot emergence and developmental abnormalities of embryoids (if any).

3.7.2 **Using embryo mass as explant**

3.7.2.1 **Induction**

The embryo mass scooped out from the ovules of tender mango fruits were inoculated on induction media. Fifteen treatments were tried for the induction of somatic embryogenesis from embryo mass. The treatments involved different levels and combinations of basal media (MS and B-5), plant growth substances *viz.* NAA, 2, 4-D, BA, kinetin and GA_3 (Table 16) sucrose and coconut water. An average of twelve replications were provided. The cultures were kept under darkness at 25 ± 2^{0}C. Observations were recorded on the number of cultures initiating direct embryoids, multiple embryoids, number of embryoids per culture, size of embryoids and other visual characteristics such as colour and developmental abnormalities of embryoids (if any).

Table 16 **Treatments tried for the induction of somatic embryogenesis from embryo mass of polyembryonic mango varieties**

Medium : 1/2 MS + inositol 150 ml/l + G_{A3} 5.0 mg/l + glutamine 400 mg/l + sucrose 60 g/l + coconut water 200 ml/l + agar 6.0 g/l + activated charcoal 2.5 g/l

Treatment No.	Plant growth substances (mg/l)
IE_1	BA 0.5
IE_2	BA 1.0
IE_3	Kinetin 0.5
IE_4	Kinetin 2.0
IE_5	Kinetin 5.0
IE_6	Kinetin 64.0
IE_7	2, 4-D 5.0
IE_8	NAA 1.0
IE_9	2, 4-D 0.1 + NAA 1.0 l

3.7.2.2 **Germination**

Somatic embryoids of three to four cm. size were obtained directly form the induction treatments using embryo mass as explants. Hence the initiation and maturation stages could be avoided. The embryoids obtained from the embryo mass in the induction medium were transferred directly on to the germination treatments (Table 13). Six replications were provided for each treatments. Observations were recorded on the number of cultures showing normal germination, having root / shoot development and other visual characteristics such as colour and developmental abnormalities.

RESULTS

4. RESULTS

The results of the investigations carried out for evolving techniques for *in vitro* somatic embryogenesis in polyembryonic mango varieties are presented in this chapter.

4.1 Varieties

The response of the nucellar tissue of six polyembryonic mango varieties to the induction treatments for somatic embryogenesis was documented (Table 17, Fig. 1). The variety Kalluvarikka showed the highest level of response (85.0 %) of cultures initiating embryogenic callus. This was followed by Thalimanga (81.9 %). Vellari Manga had the lowest response level (only 68.85 %) of cultures producing embryogenic callus.

The highest callus index also was recorded by Kalluvarikka (170.00) which was followed by Thalimanga (163.80). Vellari Manga had the lowest callus index (120.31).

Three varieties *viz.*, Kalluvarikka, Olour and Kurukkan showed direct emergence of somatic embryoids. Kalluvarikka recorded the highest response (10 per cent cultures initiating somatic embryoids) followed by Olour and Kurukkan (5.0 per cent each).

4.2 Somatic embryogenesis using nucellus as explant

4.2.1 Induction

4.2.1.1 Basal media

Out of the two treatments tried to compare the effect of basal media on induction of somatic embryogenesis from nucellus, MS medium having half strength of major salts recorded better performance with 65.7 per cent of cultures showing

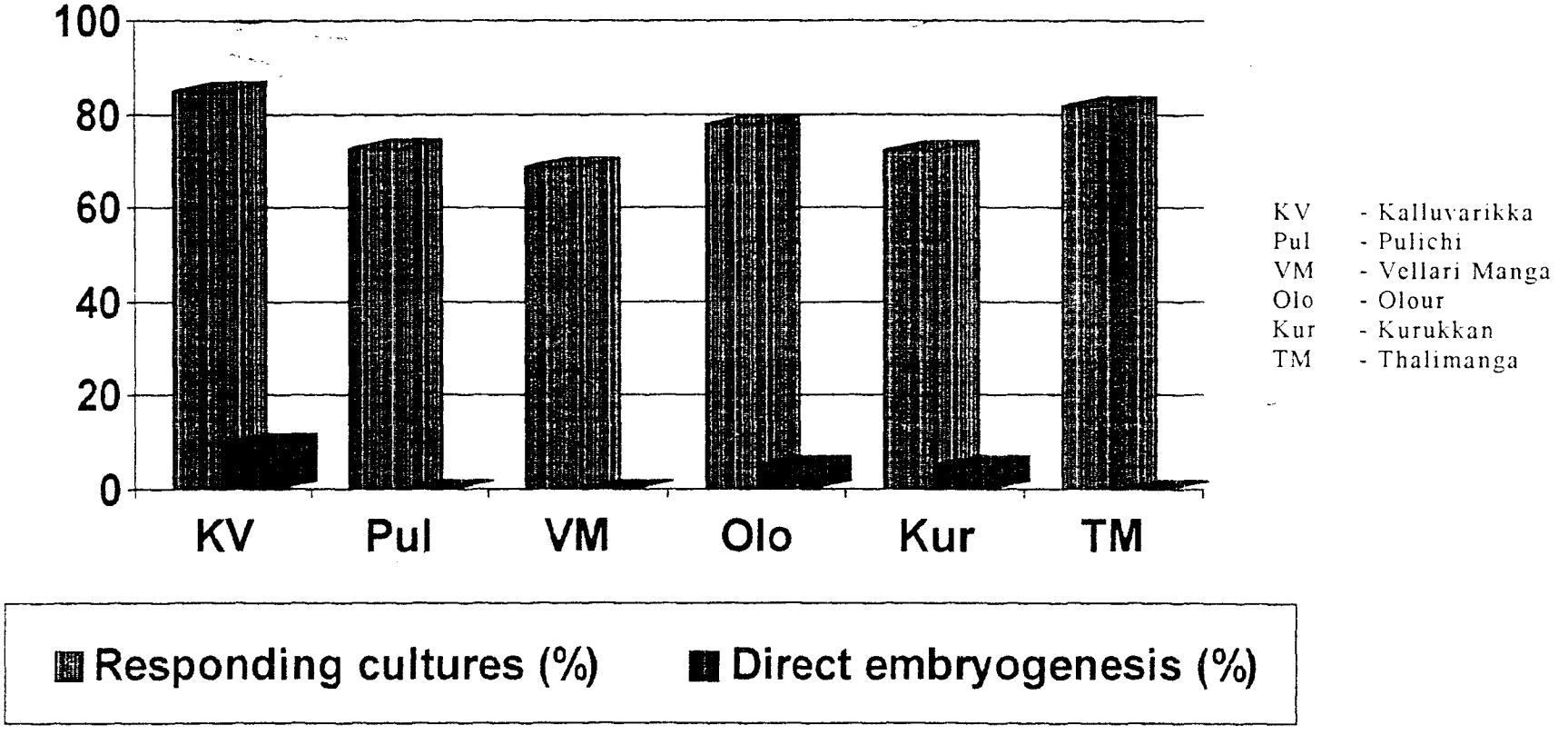
Table 17 **Response of different polyembryonic mango varieties to the induction treatments for somatic embryogenesis from the nucellus**

Medium : 1/2 MS + inositol 150 mg/l + 2, 4-D 5.0 mg/l + GA_3 5.0 mg/l + glutamine 400 mg/l + sucrose 60 g/l + coconut water 200ml/l + agar 6.0g/l + activated charcoal 2.5g/l

Variety	Cultures showing response (%)	Growth score	Callus index	Cultures showing direct embryogenesis (%)
Kalluvarikka	85.00	2.00	170.0	10
Pulichi	72.70	2.00	145.4	0
Vellari Manga	68.75	1.75	120.3	0
Olour	77.80	2.00	155.6	5
Kurukkan	72.20	2.00	144.4	5
Thalimanga	81.90	2.00	153.8	0

* The data represent average of 20 replications

Fig. 1 Response of nucellar explant of polyembryonic mango varieties to induction treatments for somatic embryogenesis



response with a callus index of 115.0. When MS major salts were substituted with B-5 major salts, only 63.6 per cent of cultures showed response and recorded a callus index of 111.3. The medium having half strength MS major salts produced direct embryoids at a higher rate of 6.0 per cent cultures. The medium with B-5 major salts produced direct embryoids in only 5.0 per cent cultures.

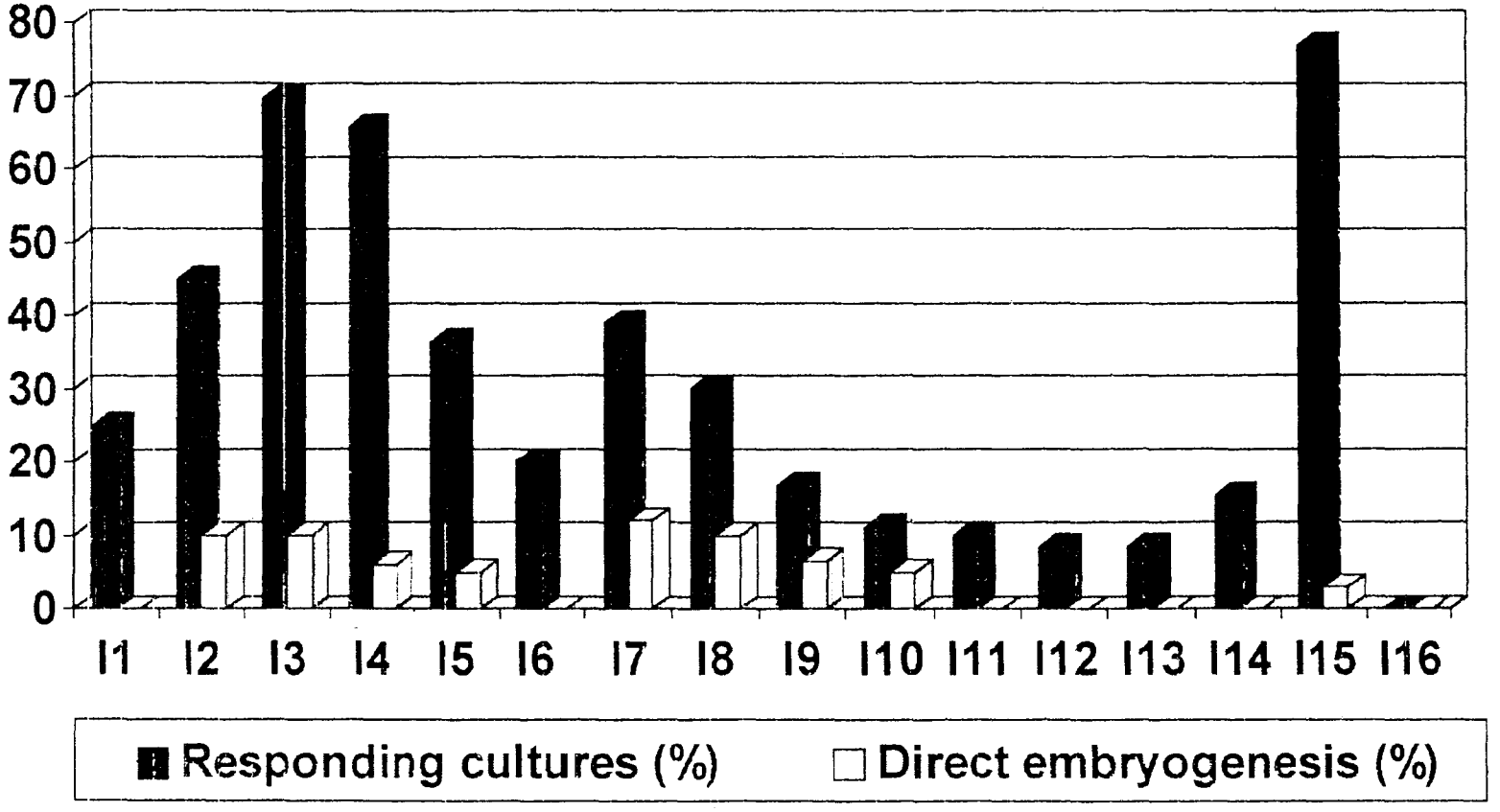
4.2.1.2 Plant growth substances

Sixteen treatments were tried to assess the effect of plant growth substances on induction of somatic embryogenesis from nucellus (Fig. 2). The treatment having 2,4-D 5.0 mg/l recorded the highest per cent of cultures having response (76.9) followed by BA 16 mg/l (69.7) and BA 32 mg/l (65.7). The highest callus index was recorded for the treatment with BA 16 mg/l (156.8) followed by 2,4-D 5.0 mg/l (153.8) and BA 32 mg/l (115.0). The highest percentage of cultures with direct embryogenesis was recorded by the treatment with kinetin 8.0 mg/l (12.0). Emergence of direct embryoids at the rate of 10.0 per cent was recorded by the treatments with BA 5.0 mg/l, BA 8.0 mg/l and kinetin 16 mg/l while 2,4-D 5.0 mg/l produced only 3.0 per cent cultures showing direct embryogenesis. Combinations of BA and kinetin at lower concentrations showed very poor response while combination of 2,4-D and NAA showed no response at all.

4.2.1.3 Glutamine

The effect of glutamine on induction of somatic embryogenesis is presented in Table 18. Glutamine at the rate of 400 mg/l recorded the highest response (81.8 %) of cultures showing induction of somatic embryogenesis. But the highest callus index

Fig. 2. Effect of plant growth substances on the induction of somatic embryogenesis from the nucellus of polyembryonic mango varieties



(163.6) as well as the percentage of cultures with direct embryoids (20.0) was obtained in the absence of glutamine.

4.2.1.4 Sucrose

The effect of sucrose on induction of somatic embryogenesis from nucellus is presented in Table 19. The highest level of response was obtained at an increased level of sucrose (60 g/l) at which 83.3 per cent of cultures showed response with a callus index of 250.0. At this level 20 per cent of cultures showed direct somatic embryogenesis. A gradual reduction in the level of response was shown by the decreasing levels of sucrose both in the percentage of cultures showing response and callus index. Eventhough there was no change in the response of cultures (83.3) while the level of sucrose was reduced from 60 g/l to 50 g/l, the callus index decreased to 166.6. Also the cultures did not show direct emergence of somatic embryoids. The percentage of cultures showing response was found reduced to 66.6 and 50.0 and the callus indices to 100.0 and 66.5 when the sucrose level was reduced to 40 g/l and 30 g/l, respectively.

4.2.1.5 Coconut water

The effect of coconut water on induction of somatic embryogenesis from nucellus is presented in Table 20. In the absence of coconut water, only 16.7 per cent of cultures showed response. The corresponding callus index also was 16.7. Induction of somatic embryogenesis was improved when the coconut water was used at higher concentrations. Coconut water at 200 ml/l in the medium showed the highest response with 83.3 per cent of cultures producing embryogenic callus, having a callus index of 200.0. In this case 25.5 per cent of cultures showed direct emergence of somatic embryoids.

Table 18 Effect of glutamine on induction of somatic embryogenesis from the nucellus of polyembryonic mango varieties

Treatments	Cultures having response (%)	Growth score	Callus index	Cultures initiating direct embryoids (%)
IG_1	72.72	2.25	163.62	20.0
IG_2	54.55	1.50	81.83	12.0
IG_3	45.45	1.25	56.81	4.0
IG_4	81.81	1.75	143.17	16.0

* The data represent average value of 11 replications

** Treatment details given in Table 1

Table 19 Effect of sucrose on induction of somatic embryogenesis from the nucellus of polyembryonic mango varieties

Treatments	Cultures having response (%)	Growth score	Callus index	Cultures initiating direct embryoids (%)
IS_1	50.00	1.33	66.50	8.5
IS_2	66.66	1.50	100.00	17.0
IS_3	83.33	2.00	166.66	0.0
IS_4	83.33	3.00	250.00	20.0

* The data represent average value of 6 replications

** Treatment details given in Table 2

4.2.1.6 Activated charcoal

The effect of activated charcoal on induction of somatic embryogenesis from nucellus is presented in Table 21. The cultures failed to respond in the absence of activated charcoal while 80.0 per cent of cultures showed the induction of somatic embryogenesis at all concentrations of activated charcoal. A higher callus index of 120.0 could be obtained when the level of activated charcoal was increased to 3.75 g/l. Direct emergence of somatic embryoids was observed in 20.0 per cent of cultures at this level.

4.2.1.7 Culture conditions

Darkness was found to be ideal for induction of somatic embryogenesis from nucellus. The cultures failed to respond in presence of light (3000 lux, 16 hours photoperiod). In the absence of light 76.9 per cent of cultures showed response with a callus index of 153.8 and 3.0 per cent direct embryogenesis.

4.2.2 Initiation

4.2.2.1 Plant growth substances

Eighteen treatments involving different combinations of 2,4-D, BA and GA_3 were tried to assess the effect of plant growth substances on initiation of somatic embryoids from cultures that showed response to the induction treatments. The results are presented in Table 22 (Fig. 3). Among the treatments, the treatment involving BA alone at 1.0 mg/l (E_7) showed the best results by providing cent per cent response with fifteen to twenty five easily separable somatic embryoids per culture which were having 0.5 to 1.0 cm. size and normal visual characters of colour and shape. Treatment

Table 20 Effect of coconut water on induction of somatic embryogenesis from the nucellus of polyembryonic mango varieties

Treatments	Cultures having response (%)	Growth score	Callus index	Cultures initiating direct embryoids (%)
ICW_1	16.67	1.0	15.67	8.5
ICW_2	33.33	1.0	33.33	0.0
ICW_3	83.33	2.4	200.00	25.5
ICW_4	83.33	2.2	183.33	17.5

* The data represent average value of 6 replications

** Treatment details given in Table 3

Table 21 Effect of activated charcoal on induction of somatic embryogenesis from the nucellus of polyembryonic mango varieties

Treatments	Cultures having response (%)	Growth score	Callus index	Cultures initiating direct embryoids (%)
IAC_1	0.0	0.00	0.0	0.0
IAC_2	80.0	1.25	100.0	10.0
IAC_3	80.0	1.25	100.0	10.0
IAC_4	80.0	1.50	120.0	20.0

* The data represent average value of 5 replications

** Treatment details given in Table 4

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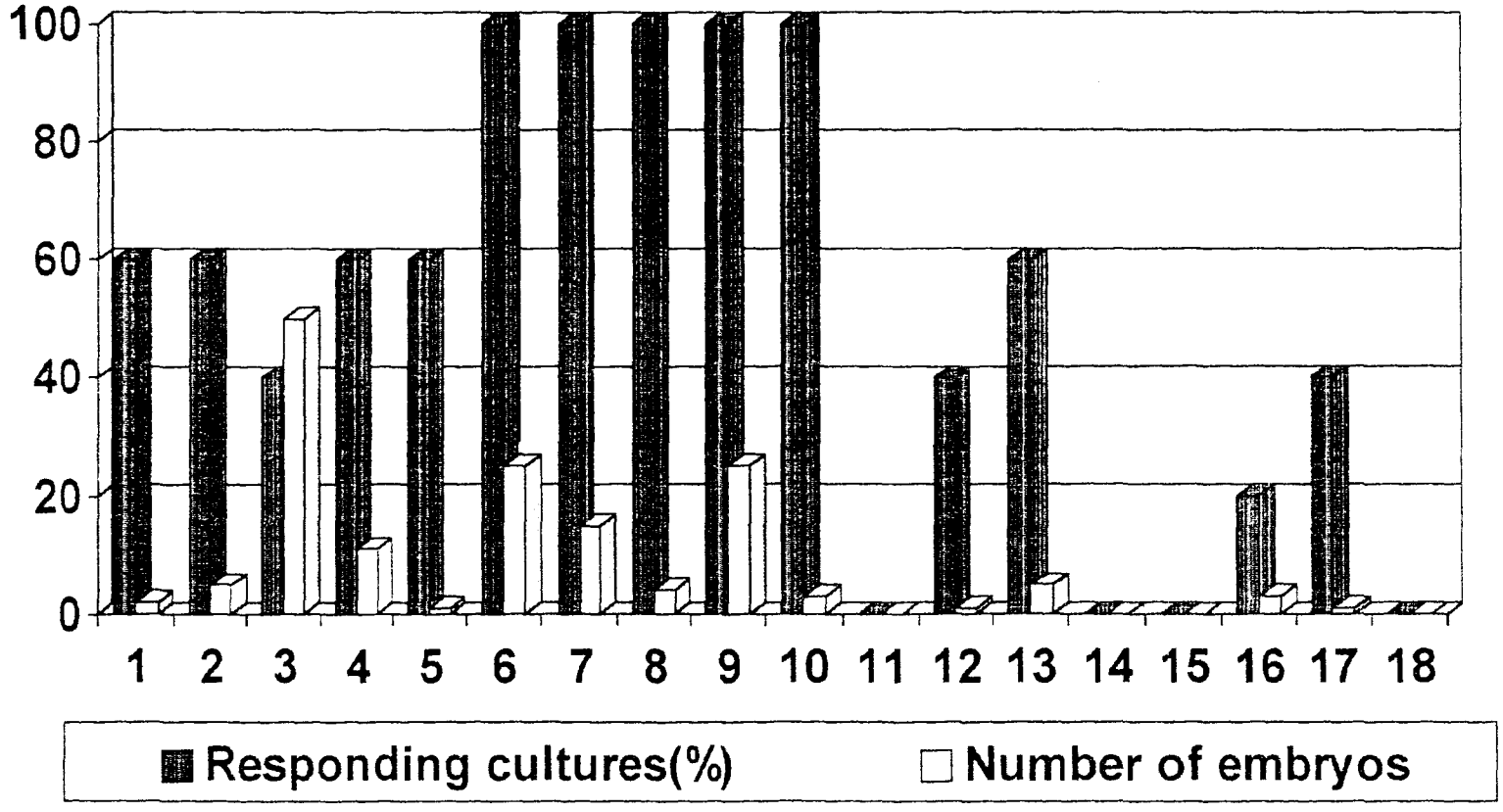
Table 22 **Effect of plant growth substances on the initiation of somatic embryoids from the nucellus of polyembryonic mango varities**

Treatments**	Cultures having response (%)	Average number of embryoids per culture	Average size of embryoids (cm)	Description of embryoids
E_1	60	2	1.25	Greenish, malformed
E_2	60	5	<0.5	Creamy, secondary embryoids
E_3	40	>50	very small	Creamy, secondary embryoids
E_4	60	11	0.5-1.0	Creamy, normal shape
E_5	60	1	1.5	Creamy, malformed with multiple cotyledons
E_6	100	25-50	very small	Creamy, fused secondary embryoids
E_7	100	15-25	0.5-1.0	White, easily separable embryoids with normal shape
E_8	100	4	0.5-1.0	Creamy, easily separable embryoids with normal shape
E_9	100	25-50	very small	Creamy, vitrified secondary embryoids
E_{10}	100	3	0.5-1.5	Greenish, malformed
E_{11}	0	0	-	-
E_{12}	40	1	0.5-3	Creamy, malformed
E_{13}	60	5-10	0.5-1.0	Creamy, normal shape
E_{14}	0	0	-	-
E_{15}	0	0	-	-
E_{16}	20	3	0.75	Creamy, malformed
E_{17}	40	1	1.0-2.0	Cream, twisted and malformed
E_{18}	0	0	-	-

* The data represent five replications

** Treatment details given in Table 5

Fig. 3. Effect of plant growth substances on the initiation of somatic embryos from the nucellus of polyembryonic mango varieties



having BA 1.0 mg/l and 2,4-D 2.0 mg/l (E_8) also initiated embryoids having good size and shape. However the embryoids were fewer in number. Proliferation of secondary embryoids and higher rates of malformation were observed in all the treatments having 2,4-D at higher concentrations (4.0 mg/l). Vitrification and aggregation of somatic embryoids were found in several treatments when the level of 2,4-D was increased. Addition of GA_3 5.0 mg/l was found to be deleterious for initiation of somatic embryoids. Four treatments involving GA_3 5.0 mg/l (E_{11}, E_{14}, E_{15} and E_{18}) failed to evoke any response. The rate of response in other treatments having GA_3 were also lower. Very few and malformed embryoids were produced in the treatments devoid of plant growth substances (E_1).

4.2.2.2 Casein hydrolysate

The effect of casein hydrolysate on initiation of somatic embryoids from nucellus is presented in Table 23. Casein hydrolysate at 300 mg/l (ECH_4) was found to be highly beneficial to induce response in all the cultures, producing on an average of six somatic embryoids per culture having good size and other visual characters such as shape and colour. Casein hydrolysate 400 mg/l and 500 mg/l (ECH_5 and ECH_6) also provided normal embryoids. Eventhough the treatment having casein hydrolysate 200 mg/l (ECH_3) provided 100.0 per cent response, it caused the proliferation secondary embryoids. Cultures failed to respond in the absence of casein hydrolysate (ECH_1) as well as at higher levels (600 mg/l and 700 mg/l) tried.

4.2.2.3 Chelated iron

The effect of chealated iron (Table 24) at its normal concentration in the MS medium (ECI_4) was found to be ideal for the initiation of somatic embryoids. Cultures

Table 23 **Effect of casein hydrolysate on the initiation of somatic embryoids from the nucellus of polyembryonic mango varieties**

Treatment**	Cultures showing response (%)	Average No. of emobryoids	Average size of embryoids (cm)	Description of embryoids
ECH_1	0.0	-	-	-
ECH_2	33.3	2.0	05-1.0	Cream, thin, elongated
ECH_3	100.0	4.0	very small	Cream, secondary embryoids
ECH_4	100.0	6.0	0.5-1.0	White, normal shape
ECH_5	66.7	4.0	0.5	Cream, normal shape
ECH_6	66.7	5.0	0.5	Cream, normal shape
ECH_7	0.0	0.0	-	-
ECH_8	0.0	0.0	-	-

** The data represent six replications
** Treatment details given in Table 6

Table 24 **Effect of chelated iron on the initiation of somatic embryoids from the nucellus of polyembryonic mango varieties**

Treatment**	Cultures showing response (%)	Average No. of emobryoids	Average size of embryoids (cm)	Description of embryoids
ECI_1	0	-	-	-
ECI_2	0	-	-	-
ECI_3	50	2.0	0.5-1.0	Cream, normal shape
ECI_4	75	4.0	0.5-1.0	Cream, normal shape
ECI_5	0	-	-	-

* The data represent four replications
** Treatment details given in Table 7

failed to respond in the absence of chelated iron (ECI_1), at 1/4th level (ECI_2) and at double concentration (ECI_5).

4.2.2.4 Glutamine

The effect of glutamine on initiation of somatic embryoids from nucellus is presented in Table 25. Glutamine at all the levels were of not much use in initiating somatic embryoids. All the treatments, except glutamine 200 mg/l (EG_4), induced response in only 25.0 per cent cultures. However the embryoids had about 0.75 cm size and near normal appearance. Glutamine 200 mg/l could increase the percentage of responded cultures to 50.0. The number of embryoids per cultures also was increased. However the average size of embryoids was reduced.

4.2.2.5 Coconut water

The effect of coconut water on initiation of somatic embryoids from nucellus is presented in Table 26. The cultures failed to respond in the absence of coconut water as well as at 50 ml/l level. The response was gradually increasing with increasing concentrations of coconut water and was the highest at 250 ml/l. At this concentration the response was observed in 75.0 per cent cultures. The treatment also recorded the maximum number of embryoids per culture with good size, shape and colour.

4.2.3 Maturation

4.2.3.1 Plant growth substances

The effect of plant growth substances on maturation of somatic embryoids derived from nucellus is presented in Table 27 (Fig. 4). Abscisic acid 3.0 mg/l (M_3) was found to be highly beneficial for maturation of somatic embryoids. At this level all

Plate 3. Mature somatic embryoes developed from nucellus.

Plate 4. Germinating somatic embryo developed from nucellus

Table 25 **Effect of glutamine on the initiation of somatic embryoids from the nucellus of polyembryonic mango varieties**

Treatment	Cultures having response (%)	Average No. of emobryoids per culture	Average size of embryoids (cm)	Description of embryoids
EG_1	25.0	3.0	0.75	White, near normal
EG_2	25.0	4.0	0.75	White, near normal
EG_3	25.0	3.0	0.75	White, near normal
EG_4	50.0	5.0	0.50	White, near normal
EG_5	25.0	3.0	0.75	Cream, near normal

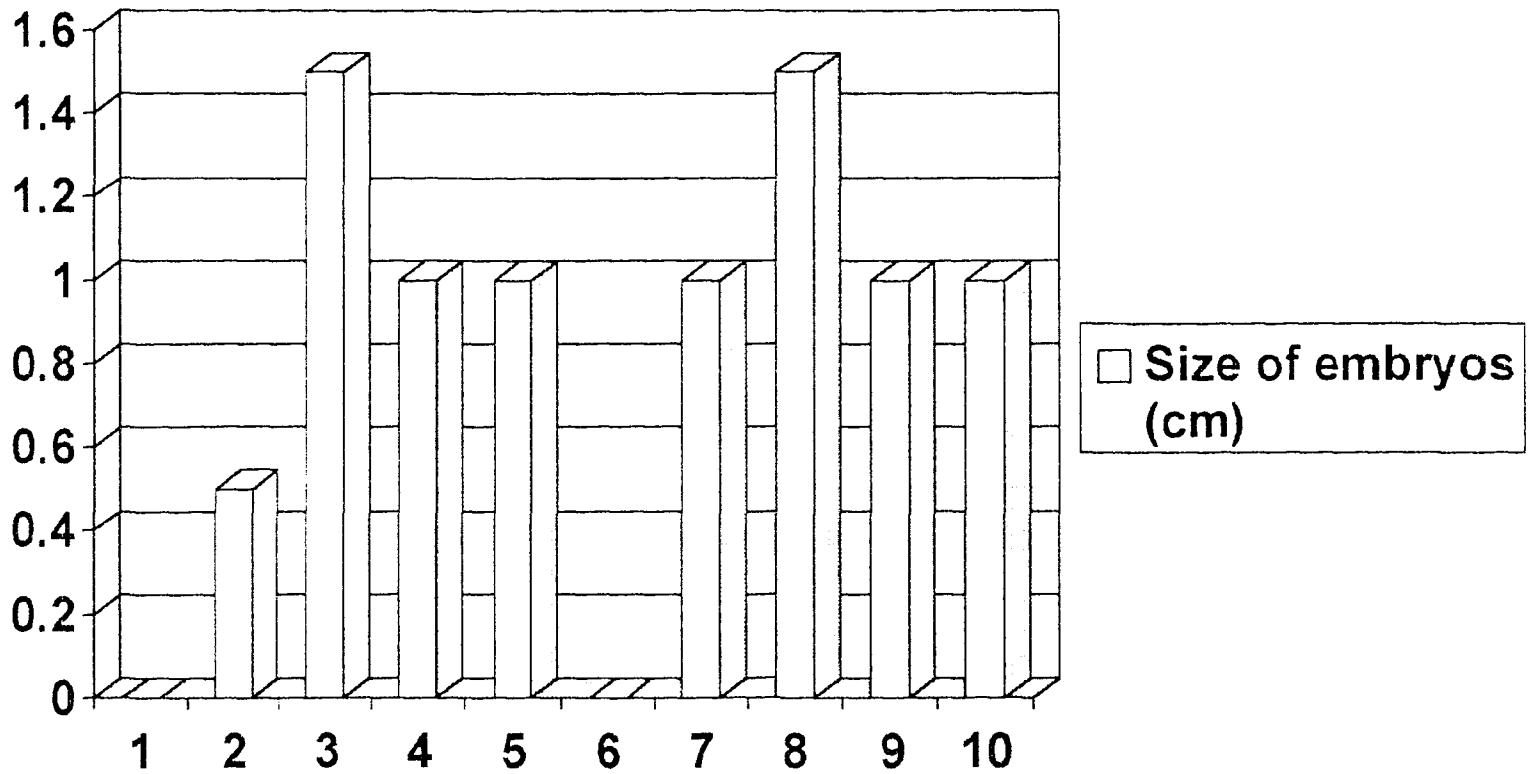
* The data represent four replications

Table 26 **Effect of coconut water on the initiation of somatic embryoids from the nucellus of polyembryonic mango varieties**

Treatment**	Cultures showing response (%)	Average No. of emobryoids per culture	Average size of embryoids (cm)	Description of embryoids
ECW_1	0.0	-	-	-
ECW_2	0.0	-	-	-
ECW_3	50.0	3	0.5-1.0	Greenish, elongated
ECW_4	75.0	5	0.5-1.0	Creamy, normal shape, slightly vitrified
ECW_5	75.0	6	0.5-1.0	Creamy, normal shape

* The data represent four replications
** Treatment details given in Table 18

Fig. 4. Effect of plant growth substances on the maturation of somatic embryos



the cultures showed response. The embryoids showed increase in size and were normal in shape, except for rooting in some. The response was good for treatment with BA 0.1 mg/l along with ABA 3.0 mg/l (M_8) which also provided 100.0 per cent response as well as normal embryoids with good increase in size. Cultures failed to respond when the concentration of ABA was reduced to 1.0 mg/l (M_1 and M_6). The response in most of the other treatments were not good. Higher rates of abnormal embryoids were observed.

4.2.3.2 Sucrose

The effect of sucrose on maturation of somatic embryoids derived from nucellus is presented in Table 28. Sucrose at 50 g/l (MS_3) was found to be ideal for maturation. All the cultures showed response to this treatment and the embryoids attained the maximum size of 2.5 cm and were devoid of abnormality. Sucrose 60 g/l (MS_4) also showed better results compared to other treatments with sucrose 30 g/l (MS_1) and 40 g/l (MS_2). At lower levels secondary embryogenesis and abnormalities were observed.

4.2.3.3 Coconut water

Increasing the concentration of coconut water was found to be beneficial for maturation of somatic embryoids. Coconut water at its highest level (200 ml/l) tried provided the highest per cent (83.3) cultures responding. The embryoids had the maximum size and good visual characters. Only 33.3 per cent of cultures showed response in the absence of coconut water. This response was increased to 66.7 when 100 ml/l of coconut water was incorporated. But the embryoids were very small and proliferation of secondary embryoids occurred.

Table 27 **Effect of plant growth substances on the maturation of somatic embryoids from the nucellus of polyembryonic mango varieties**

Treatment**	Cultures surviving (%)	Average size of embryoids (cm)	Description of embryoids
M_1	0.0	-	Secondary embryoids occured before drying up
M_2	33.3	0.5	White, embryoids dried up partly
M_3	100.0	1.5	White, increase in size and rooting observed, embryoids normal in shape
M_4	100.0	1.0	White, malformation
M_5	66.7	1.0	White, rooting of embryoids observed
M_6	0.0	-	-
M_7	33.3	1.0	Cream, normal shape of embryoids
M_8	100.0	1.5	White, normal shape of embryoids
M_9	100.0	1.0	White, normal shape of embryoids
M_{10}	66.7	1.0	White, embryoids dried up partly

* The data represent six replication
** Treatment details given in Table 9

Table 28 **Effect of sucrose on the maturation of somatic embryoids from the nucellus of polyembryonic mango varieties**

Treatment**	Cultures surviving (%)	Average size of embryoids (cm)	Description of embryoids
MS_1	66.7	0.5	White, rooting of embryoids observed
MS_2	66.7	1.0	White, secondary embryogenesis observed
MS_3	100.0	2.5	White, normal shape, good increase in size observed
MS_4	100.0	2.0	White, normal shape

* The data represent six replications
** Treatment details given in Table 10

4.2.3.4 Other supplements

The effect of cobaltous chloride, sodium butyrate and sodium chloride on maturation of somatic embryoids derived from nucellus is presented in Table 29. Cobaltous chloride did not record any significant improvement in maturation. Treatments using sodium butyrate 2.0 mM/l (MI_6) and sodium chloride 0.5 g/l (MI_7) had given normal embryoids, but the size of these were not satisfactory.

4.2.3.5 Mode of culture

Solid medium was found to be better for maturation of somatic embryoids. The embryoids undergoing maturation in liquid medium initially showed increase in size. But they became vitrified within a month and dried up later. Media solidified using agar 5.5 g/l produced embryoids with significant increase in size and normal visual characters of colour and shape.

4.2.3.6 Culture conditions

Darkness was found to be ideal for maturation of somatic embryoids derived from nucellus. The embryoids kept in light showed greening, but no significant increase in size was noticed. Several cultures showed rooting and abnormal shape. Cultures kept in darkness showed increase in size without greening and lesser abnormalities.

4.2.4 Germination

4.2.4.1 Plant growth substances

The effect of plant growth substances on germination of somatic embryoids from nucellus is presented in Table 30 (Fig. 5). Among them BA alone was effective

Table 29 **Effect of cobaltous chloride / sodium butyrate / sodium chloride on the maturation of somatic embryoids derived from the nucellus of polyembryonic mango varieties**

Treatment**	Cultures surviving (%)	Average size of embryoids (cm)	Description of embryoids
MI_1	33.3	0.5	White, partly dry up
MI_2	66.7	0.5	White, malformed
MI_3	66.7	0.5	White, malformed
MI_4	33.3	0.5	White, partly dry up
MI_5	66.7	0.25	White, secondary embryoids
MI_6	66.7	0.5	Creamy, normal shape
MI_7	66.7	1.0	White, normal shape
MI_8	66.7	1.0	Creamy, malformed
MI_9	66.7	1.0	White, malformed

* The data represent three replications

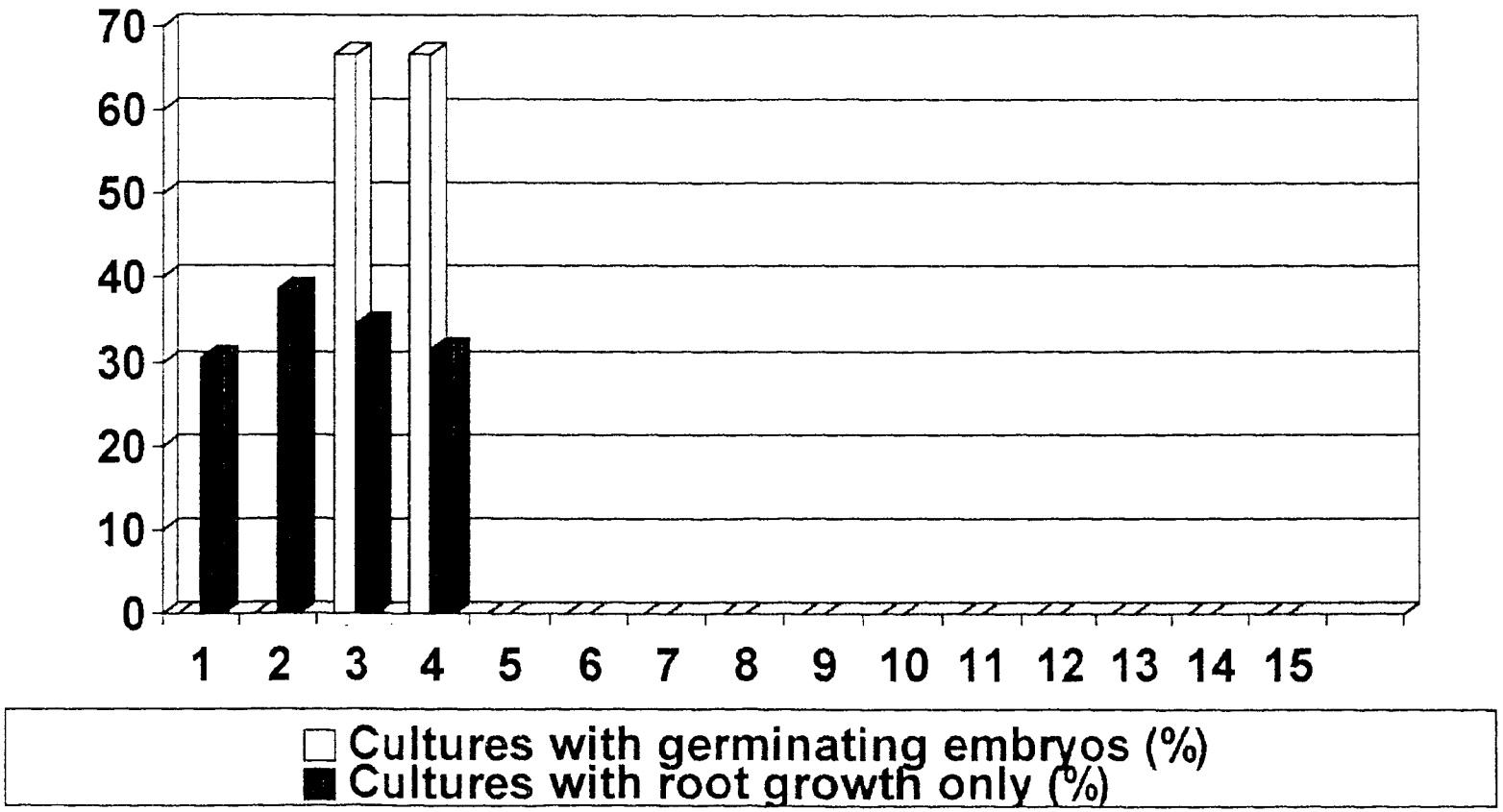
** Treatment details given in Table 12

Table 30 **Effect of plant growth substances on the germination of somatic embryoids derived from the nucellus of polyembryonic mango varieties**

Treatment**	Surviving cultures (%)	Cultures with simultaneous shoot and root growth (%)	Cultures with shoot growth only (%)	Cultures with root growth only (%)	Description of embryoids
G_1	0.0	-	-	-	-
G_2	0.0	-	-	-	-
G_3	66.7	66.67	-	-	Near normal
G_4	66.7	66.67	-	-	Near normal
G_5	0.0	-	-	-	-
G_6	0.0	-	-	-	-
G_7	33.3	-	-	33.3	Malformed
G_8	66.7	-	33.3	33.3	Malformed
G_9	0.0	-	-	-	-
G_{10}	0.0	-	-	-	-
G_{11}	33.3	-	-	33.3	Malformed
G_{12}	0.0	-	-	-	-
G_{13}	33.3	-	-	33.3	Multiple roots observed
G_{14}	0.0	-	-	-	-
G_{15}	33.3	-	-	33.3	Malformed

* The data represent six replications
** Treatment details given in Table 13

Fig. 5. Effect of plant growth substances on the germination of somatic embryoids



in promoting normal germination. BA 0.1 mg/l (G_3) and 0.2 mg/l (G_4) supported 66.7 per cent survival of cultures. The embryoids showed simultaneous shoot and root growth and near normal germination. Treatments without plant growth substances as well as the other treatments of BA failed to support the survival of the cultures. Treatments involving kinetin and GA_3 either failed to make any response or caused malformations in the cultures survived.

4.2.4.2 Sucrose

The effect of sucrose on germination of somatic embryoids derived from nucellus is shown in Table 31. Sucrose at concentrations of 30 g/l (GS_3), 40 g/l (GS_4) and 50 g/l (GS_5) supported the survival of all the cultures. But sucrose 40 g/l alone had near normal germination. The cultures failed to survive at lower concentrations of sucrose.

4.2.4.3 Other supplements

The effect of supplements in the culture media *viz.*, cobaltous chloride and sodium butyrate on germination of somatic embryoids derived from nucellus is presented in Table 32. Cobaltous chloride 10 mg/l (GI_3) supported near normal germination in 33.3 per cent cultures. All the other treatments of both cobaltous chloride and sodium butyrate either failed to make the cultures respond or caused malformations.

4.2.4.4 Mode of culture

Liquid medium was tried against solid medium with agar in order to assess the effect of mode of culture. Liquid medium did not support normal germination. The

Table 31 **Effect of sucrose on the germination of somatic embryoids derived from the nucellus of polyembryonic mango varieties**

Treatment**	Surviving cultures (%)	Cultures with simultaneous shoot and root growth (%)	Cultures with shoot growth only (%)	Cultures with root growth only (%)	Description of embryoids
GS_1	0.0	-	-	-	-
GS_2	0.0	-	-	-	-
GS_3	100.0	33.3	33.3	33.3	Malformed
GS_4	100.0	100.0	-	-	Near normal
GS_5	100.0	-	33.3	66.7	Malformed
GS_6	66.7	-	-	66.7	Root only formed

* The data represent six replications
** Treatment details given in Table 14

Table 32 **Effect of cobaltous chloride / sodium butyrate on the germination of somatic embryoids derived from the nucellus of polyembryonic mango varieties**

Treatment**	Surviving cultures (%)	Cultures with shoot and root growth (%)	Cultures with shoot growth only (%)	Cultures with root growth only (%)	Growth of embryoids
GI_1	100.0	0.0	33.3	66.7	Malformed
GI_2	33.3	0.0	0.0	33.3	Malformed
GI_3	33.3	33.3	0.0	0.0	Near normal
GI_4	0.0	-	-	-	-
GI_5	0.0	-	-	-	-
GI_6	66.7	0.0	33.3	33.3	Malformed
GI_7	33.3	0.0	33.3	0.0	Malformed

* The data represent six replications
** Treatment details given in Table 15

cultures showed vitrification and dried up. Solid medium supported near normal germination of several embryoids.

4.2.4.5 Culture conditions

Light was found to be essential for normal germination of somatic embryoids derived from nucellus. In presence of light the embryoids showed rapid greening, shoot emergence and elongation. The cultures kept under darkness remained white in colour and did not show shoot emergence eventhough root emergence was observed in a few instances.

4.3 Somatic embryogenesis using embryo mass as explant

4.3.1 Induction

The effect of treatments tried to induce somatic embryogenesis from embryo mass is presented in Table 33. None of the treatments showed induction of callus from explants while several treatments caused direct embryogenesis. Among the plant growth substances, 2,4-D 5.0 mg/l (IE_7) was found to give high response. It caused direct embryogenesis in all the cultures, with an average of eight embryoids per culture. However abnormalities were observed in several embryoids. Combination of 2,4-D 0.1 mg/l and NAA 1.0 mg/l (IE_4) also recorded 100 per cent response with an average of 5.0 embryoids. These treatments also caused some abnormalities. Several treatments such as BA 0.5 mg/l (IE_1), kinetin 0.5 mg/l (IE_3), kinetin 2.0 mg/l (IE_4) and kinetin 64 mg/l (IE_6) failed to produce any response, except for slight swelling of the explants.

Table 33 **Effect of treatments tried for the induction of somatic embryogenesis from the embryo mass of polyembryonic mango varieties**

Treatment **	Cultures surviving (%)	Cultures showing direct embryogenesis (%)	Average No. of direct embryoids per culture	Description of embryoids
IE_1	100.0	0.0	0.0	Swelling of explants
IE_2	33.3	33.3	1.0	Single normal embryoid
IE_3	66.7	0.0	0.0	-
IE_4	66.7	0.0	0.0	-
IE_5	66.7	66.7	1.0	Single normal embyoid
IE_6	33.3	0.0	0.0	-
IE_7	100.0	100.0	8.0	Normal and abnormal embryoids observed
IE_8	100.0	100.0	1.0	Abnormal embryoids
IE9	100.0	100.0	5.0	Aggregated embryoids

*The data represent avg. 12 replications

** Treatment details given in Table 16

Lower levels of sucrose (IE_{11} and IE_{12}) caused the proliferation of secondary embryoids from embryo mass. Higher levels of sucrose (IE_7 and IE_{13}) supported the production of direct embryoids with normal shape.

Coconut water was found to be essential to induce response from embryo mass explants. In the absence of coconut water (IE_{14}) the embryoids were not produced. Increasing response was exhibited while the level of coconut water was increased to 150 ml/l (IE_{15}) and 200 ml/l (IE_7).

4.3.2 Germination

The effect of treatments tried for germination of embryoids derived from embryo mass is presented in Table 34. Among the plant growth substances BA was found to promote normal germination of embryoids very efficiently. Treatment using BA 0.1 mg/l (G_3) caused near normal germination in all the cultures. Treatments using BA 0.2 mg/l (G_4) also caused higher percentage of cultures with near normal germination, except for occurrence of multiple shoots. Kinetin 0.2 mg/l (G_{10}) also supported near normal germination, eventhough at a lower percentage. Root development was not found to be a problem as most of the treatments effected satisfactory root development. However many treatments failed to control abnormalities of embryoids and effect shoot emergence.

Plate 5. Germinating embryo developed from embryo mass explant.

Plate 6. Vitrified somatic embryo

Table 34 **Effect of treatments tried for the germination of somatic embryoids from the embryo mass of polyembryonic mango varieties**

Treatment	Surviving cultures (%)	Cultures with root and shoot development (%)	Cultures with shoot development alone (%)	Cultures with root development alone (%)	Description of embryoids
G_1	33.3	-	-	33.3	Rooting & greening of embryoids observed
G_2	66.7	-	-	66.7	Multiple root emergence
G_3	100.0	100.0	-	-	Near normal embryoids
G_4	100.0	66.7	33.3	-	Multiple shoots formed from embryoids
G_5	66.7	33.3	-	33.3	Embryoids with long root
G_6	-	-	-	-	-
G_7	33.3	-	-	33.3	Secondary embryoids observed
G_8	-	-	-	-	-
G_9	33.3	-	-	33.3	Necrosis observed
G_{10}	33.3	33.3	-	-	Near normal
G_{11}	100.0	-	-	100.0	Secondary embyoids occur
G_{12}	33.3	-	-	33.3	-
G_{13}	-	-	-	-	-
G_{14}	33.3	33.3	-	-	Embryoids remain white in colour
G_{15}	66.7	33.3	-	33.3	Axillary buds developed

* The data represent six replications

DISCUSSION

5. DISCUSSION

Mango is generally propagated through seeds. Vegetative methods also are gaining popularity. However, the rate of multiplication is not sufficient enough to meet the demand for high quality planting materials. *In vitro* method has been proposed as a useful alternative for the rapid vegitative propagation in many woody crops. Evolving techniques for *in vitro* propagation of mango can help rapid clonal propagation of desirable varieties and elite plants. Large scale *in vitro* production of polyembryonic seedlings can provide large number of uniform root stocks for other commercial varieties, which in turn will ensure uniform performance of the grafts. Many of the polyembryonic varieties have become endangered as commercial varieties gained popularity due to fragmentation of cultivated land. *In vitro* propagation can help in preventing them from becoming extinct.

Somatic embryogenesis has been observed to be highly efficient route of *in vitro* propagation in many woody species. It is a process by which somatic cells develop into plants through a series of stages characteristic of zygotic embryo development. It represents the most striking confirmation of totipotency. With its immense rate of multiplication, it is able to provide large number of plants having uniform size within a short time. The present study was initiated for evolving techniques for *in vitro* somatic embryogenesis in polyembryonic mango varieties. The outcome of the investigations are discussed in this chapter.

The response of six polyembryonic mango varieties *viz* Kalluvarikka, Pulichi, Vellari Manga, Olour, Kurukkan and Thalimanga, to the induction of somatic embryogenesis using nucellus explant was studied. All the six varieties showed satisfactory response, eventhough at varying levels. Kalluvarikka showed the highest degree of response (85.0) followed by Thalimanga (81.9). Vellari Manga recorded the lowest level (68.85). According to Litz *et al.* (1982) the degree of polyembryony directly influenced the level of response and only five out of the nine varieties used were observed to have somatic embryogenesis. Mathews and Litz (1990) reported that certain cultivars like Red Itamaraca did not respond to the treatments for inducing somatic embryogenesis. Three varieties *viz* Kalluvarikka, Olour and Kurukkan exhibited direct emergence of somatic embryoids from the nucellus. This direct permissive pattern of somatic embryogenesis may be due to the presence of pre-embryogenically determined cells (PEDCs) in the explant as proposed by Sharp *et al.* (1980). Such direct emergence of somatic embryos from nucellus was reported in mango by Litz *et al.* (1982) and Mathews and Litz (1990). Direct embryogenesis was reported to occur in citrus (Rangan *et al.*, 1968) and apple (Eichooltz *et al.*, 1979) also.

Selection of basal medium was found to influence the induction of somatic embryogenesis from nucellar explants of polyembryonic

mango varieties. Evans *et al.* (1981) observed that 70.0 per cent of the explants of various crops for somatic embryogenesis were cultured on MS medium or on its modifications. Jaiswal (1990) reported the medium having a combination of B-5 major salts and MS minor salts to be beneficial for induction of somatic embryogenesis in mango. In the present study the performance of MS basal medium with half strength major salts was compared with the medium having a combination of B-5 major salts and MS minor salts. The MS medium with half strength major salts was found to be superior for induction of somatic embryogenesis from nucellar explants of polyembryonic mango varieties. Several workers like Litz *et al.* (1982, 1984), Litz (1984b), Litz and Schaffer (1986), Dewald *et al.* (1989a), Mathews and Litz (1990) and Jana *et al.* (1994) also report the superiority of the same medium for the induction of somatic embryogenesis from nucellus of a number of mango varieties.

Plant growth substances have substantial influence on the developmental stages of somatic embryogenesis, the requirement of which varies according to the plant species and varieties (Razdan, 1994). The presence of an auxin is required for induction of somatic embryogenesis especially in induced embryogenically determined cell (IEDC) systems. In the present instance 2,4-D 5.0 mg/l recorded the highest in the percentage of cultures responding (76.9). According to Litz *et al.* (1982, 1984) and Litz (1984b) induction of somatic

embryogenesis in mango occurred in presence of 2,4-D. However, the highest callus index (156.8) was recorded for the treatment involving BA 16mg/l. In the highest percentage of cultures producing direct embryoids (12.0) was recorded for treatment with kinetin 8.0mg/l. Somatic embryogenesis might have occurred in the absence of 2,4-D due to the pre-embryogenically determined state of the cells of the nucellus in polyembryonic mango varieties. Morphogenesis in polyembryonic mangoes confirm with the pattern of direct somatic embryogenesis from predetermined embryogenic callus (Sharp *et al.* 1980). Michaux-Ferriere and Dublin (1988) reported the use of BA for induction of somatic embryogenesis in coffee.

The optimal form and concentration of nitrogen appears to be critical for somatic embryogenesis (Sharp *et al.*, 1980). The benefits of reduced nitrogen in addition to the nitrate nitrogen is well established (Evans *et al.*, 1981). Among the sources of reduced nitrogen, glutamine was found to be more important to promote somatic embryogenesis (Kamada and Harada, 1979). In the present instance the effect of glutamine on induction of somatic embryogenesis in polyembryonic mango varieties was assessed. The highest percentage (81.8) of cultures having response was recorded for the treatment with glutamine 400mg/l. However, the highest callus index (163.6) as well as the percentage of cultures having direct embryoids (20.0) was recorded in the absence of glutamine.

Glutamine has been extensively used for somatic embryo production in mango (Litz *et al.*, 1982, 1984; Litz,1984b ; Mathews and Litz, 1990).

Sucrose has been reported to be the most useful carbon source for somatic embryogenesis (Ammirato, 1983). In the present study sucrose at 60g/l has provided response in 83.3 per cent of cultures with callus index of 250.0 and 20.0 per cent of cultures produced direct embryoids. When the concentration of sucrose was reduced to 30g/l, the percentage of cultures with response and callus index was reduced to 50.0 and 66.5, respectively. According to Dewald *et al.* (1989a) induction of somatic embryogenesis and early stages of somatic embryo development require moderate to high concentration of sucrose. Five to six per cent of sucrose was required to maximise somatic embryo production in mango varieties James Saigon and Parris.

The benefits of coconut water has been reported even in the earliest works in somatic embryogenesis (Steward, 1958). It was found to be highly beneficial for somatic embryogenesis in mango also (Litz *et al.*,1982). In the present study, coconut water 200ml/l in the induction medium induced response in 83.3 per cent of cultures with a callus index of 200.0. Direct emergence of embryoids was recorded in 25.5 per cent of cultures. In the absence of coconut

water, only 16.7 per cent cultures showed response. The results were similar to the findings of Litz *et al.* (1982) who reported that the most efficient somatic embryo production in mango was obtained on medium supplemented with 20 per cent (v/v) coconut water.

Activated charcoal has been found useful for somatic embryo production in many woody species such as date palm (Tisserat and De Mason, 1980) and oil palm (Tixeira *et al.*, 1994). It has been found to absorb several inhibitors such as phenyl acetic acid and parahydroxy benzoic acid which inhibited somatic embryogenesis (Fridborg *et al.*, 1978). In the present study, the cultures failed to induce somatic embryogenesis in the absence of activated charcoal while 80.0 per cent of cultures showed response at all other levels. The highest callus index (120.0) was recorded at the highest level (3.75g/l) of activated charcoal. Muralidharan *et al.* (1994) also reported the beneficial effects of activated charcoal for somatic embryogenesis in mango. However according to Litz *et al.* (1984) activated charcoal had deleterious effect in somatic embryogenesis of mango as most of the somatic embryoids resulted from treatments using activated charcoal died during the late heart shaped stage of development.

Culture conditions are important for the induction of somatic embryogenesis. Darkness was found to be essential for somatic

embryogenesis in mango in the presents instance. In darkness, 76.9 per cent of cultures showed induction of embryogenic callus with a callus index of 153.8. On the other hand cultures failed to respond in presence of light. Rao *et al.* (1981) reported that the induction and growth of callus in mango was better in darkness than in light with a normal photo period. Litz *et al.* (1982) maintained nucellar cultures in the induction medium in darkness at 25°C. Jana *et al.* (1994) also observed that darkness was better for induction of somatic embryogenesis in mango.

The embryogenic calli obtained on the induction medium were transferred to the initiation (expression) medium for furthering somatic embryogenesis. The use of 2,4-D exhibited deleterious effects. At higher concentrations of 2,4-D, proliferation of secondary embryoids and higher rates of malformations were found to occur. Litz (1984b) reported that the development of mango somatic embryos did not proceed beyond the globular stage in presence of 2,4-D. However Jaiswal (1990) reported the use of 2,4-D for initiation of somatic embryoids in mango. Cytokinins were found to promote initiation of somatic embryoids from embryogenic callus of polyembryonic mango varieties. The treatment using BA 1.0mg/l showed the best results. It provided response in cent per cent of the cultures with 15-25 easily separable somatic embryoids in each cultures. The embryoids had good size and visual characters of

colour and shape. Litz *et al.* (1991) observed nearly twelve times increase in the initiation of somatic embryoids (as compared to the control) by incorporating BA 0.05mg/l in the medium. The presence of GA_3 in the initiation medium was found to affect the rate of embryo development (Nitsch, 1969). In the present study it was observed that the incorporation of GA_3 suppressed the initiation of somatic embryoids in polyembryonic mango varieties. In the absence of plant growth substance production of only very few, malformed embryoids was observed.

Casein hydrolysate is a non-specific organic nitrogen source and serves as an amino acid supplement (Skoog and Miller, 1957). In the present study casein hydrolysate 300mg/l was found to be beneficial for initiation of somatic embryoids in polyembryonic mango varieties. However at higher concentrations it caused the cultures to dry up. Dewald *et al.* (1989a) observed that casein hydrolysate inhibited somatic embryo production in mango.

Chelated iron at its normal concentration in the MS medium was found to be ideal for somatic embryo production in polyembryonic mango varieties in the present study. Cultures did not show response in the absence of chelated iron as well as when the concentration was doubled (Table 24).

The amino acid, glutamine was reported to supplement the existing ammonium to nitrate ratio in the medium, there by influencing the initiation of somatic embryogenesis and morphogenesis (Litz and Gray, 1992). Glutamine was found to be of not much significance for initiation of somatic embryoids in polyembryonic mango varieties. The different concentrations of glutamine was found not to create much variation in somatic embryo production. However there are several reports such as Litz *et al.* (1982, 1984), Litz (1984b), Dewald *et al.* (1989a) and Mathews and Litz (1990) who indicate the use of glutamine for somatic embryo production in mango.

Coconut water was found to promote somatic embryo production from the nucellar explants of polyembryonic mango varieties. Coconut water 250ml/l recorded the highest response (75.0 per cent) with an average of six embryoids per culture. Initiation of somatic embryos did not occur in the medium with coconut water. Dewald *et al.* (1989a) also observed that coconut water could enhance somatic embryo production in mango by 18.0 per cent.

Somatic embryoids obtained in the initiation medium were transferred to the maturation medium. According to Litz *et al.* (1991) control of somatic embryo maturation was the most critical and difficult process in mango regeneration. The natural growth

inhibitor, ABA is used to aid maturation of somatic embryos. It inhibited abnormal proliferations and repressed precocious germinaiton (Ammirato, 1973; 1974). In the present work, ABA 3.0mg/l was found to be highly beneficial for somatic embryo maturation in polyembryonic mango varieties. The embryoids showed much increase in size, maintaining normal shape in the maturation medium supplemented with ABA. Cultures dried up when the concentration of ABA was reduced to 1.0mg/l. Effective use of ABA for maturation of mango somatic embryoids and control of abnormalities was reported by several workers (Dewald *et al.*, 1989b; Litz *et al.*, 1993; Jana *et al.*,1994; Monsalud *et al.*,1995).

The concentration of sucrose was found to be critical for maturation of nucellus derived embryoids of polyembryonic mango varieties in the present instance. A higher concentration of sucrose 50g/l was found to be the best for maturation at which the embryoids attained a size of 2.5cm without showing abnormalities. Lower levels of sucrose resulted in drying up of the cultures. Litz *et al.* (1993) 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. However, higher concentration of sucrose has been reported to mask the effect of ABA in the maturation medium (Dewald *et al.*, 1989b).

Coconut water was found to promote somatic embryo maturation in polyembryonic mango varieties (Table 32). Lower levels of coconut water resulted in abnormalities and proliferation of secondary embryoids. The abnormalities were decreasing with increasing concentrations of coconut water and at 200ml/l level it aided the maturation of 83.3 per cent of cultures with normal shape. Coconut water was reported to prevent necrosis in mango somatic embryos in the maturation medium (Litz , 1984b) and to mediate normal maturation along with ABA (Dewald *et al.*, 1989b).

Endogenous ethylene level above critical concentration can cause inhibitory effects on maturation process of somatic embryoids. In order to check this, ethylene inhibitor *viz* cobaltous chloride was tried in the medium. However, these treatements failed to aid maturation of embryoids without causing malformations.

Sodium butyrate is known to influence the histone deacetylation and the expression of genes that are switched off in the developmental sequence (Perry and Chalkey, 1981). In the present instance sodium butyrate could not prove to be successful as the embryoids did not increase in size eventhough they maintained normal shape.

Osmotic potential was found to influence somatic embryogenesis. Treatments using sodium chloride was tried to assess the effect of osmotic potential on maturation of somatic embryoids of

mango. However, this did not show any positive results as the embryoids remained small in size.

Solid medium was found to be ideal for maturation of nucellus derived embryoids of polyembryonic mango varieties. Only those embryoids subjected for maturation in solid medium increased in size and were normal in shape. The embryoids in liquid medium showed an initial increase in size. But they became vitrified within a month and dried up later. Dewald *et al.* (1989b) also reported more abnormalities, such as polycotyledony in the liquid medium. There are other reports also on fasciation and loss of bipolarity (Litz *et al.*, 1993) and vitrification, especially in highly embryogenic cultures (Monsalud *et al.*, 1995) of mango somatic embryoids in liquid maturation medium.

The requirement of light for somatic embryo maturation varies for different crops. In the present study darkness was found to be ideal for maturation of somatic embryoids without greening and abnormalities. In presence of light greening of somatic embryoids occurred and they did not increase in size. Dewald *et al.* (1989b) also reported similar response in mango.

Germination of mango somatic embryoids is very critical, at which several developmental abnormalities can occur (Litz *et al.*, 1982). In the present study the embryoids were transferred to the

germination medium after attaining a size of 1.0 to 2.0cm. Polyembryonic mango varieties did not germinate without the aid of plant growth substances. The cytokinin, BA at concentrations 0.1mg/l and 0.2mg/l was found to be very effective to promote normal germination in the present instance. Litz *et al.* (1984b) and Jana *et al.* (1994) reported the use of BA for normal germination of mango somatic embryoids. However, Dewald *et al.* (1989b) and Jaiswal (1990) could obtain germination of mango somatic embryoids without exogenous application of plant growth substances. According to Button and Bornman (1971), the addition of GA_3 enhanced root development in fully developed somatic embryos, but suppressed shoot development. In the present instance GA_3 was found to produce malformations when incorporated in the germination medium.

Sucrose at a concentration of 40g/l was found to be ideal for normal germination of somatic embryoids of polyembryonic mango varieties. Lower concentrations were unable to support the cultures and they dried up. Jana *et al.* (1994) were also able to attain normal germination of mango somatic embryoids at 4.0 per cent (w/v) concentration of sucrose.

Internal ethylene level can influence the germination of somatic embryoids. Among the treatments involving the ethylene inhibitor - cobaltous chloride 10mg/l alone was able to mediate normal germination and that too in only 33.3 per cent of cultures.

Sodium butyrate, which is known to influence the histone deacetylation process, was not found to aid effective germination.

Solid medium alone was able to give normal germination of nucellus derived embryoids. Vitirfication and drying up was observed in the embryoids subjected for germination in the liquid medium. Dewald *et al.* (1989b) and Jana *et al.* (1994) also reported the positive effects of solid medium for normal germination of mango somatic embryoids.

Light was found to be essential for normal germination of somatic embryoids of mango. In presence of light rapid greening, shoot emergence and elongation were observed, characteristics of normal germination. Dewald *et al.* (1989b) reported normal germinaiton of mango somatic embryos in the light of cool, white fluorescent tubes with a photo period of sixteen hours.

Production of somatic embryoids in mango from explants other than nucellus is reported from cotyledonous explants (Rao *et al.*, 1981) and zygotic embryos (Muralidharan *et al.*, 1994). In the present investigations somatic embryoids were found to occur directly from the embryo mass explants. None of the cultures showed the induction of embryogenic callus. Among the plant growth substance, 2,4-D 5.0mg/l was found to be highly efficient for initiation of somatic embryos directly from embryo mass. But these embryos showed abnormalities to a greater extent. Combination of 2.4-D 0.1mg/l and NAA 1.0mg/l also was able to produce satisfactory level of response, eventhough these embryoids also

exhibited several abnormalities. Other plant growth substances were not efficient to produce good response.

Lower level of sucrose resulted in secondary embryogenesis in the embryoids derived from embryo mass explants. At higher levels (60g/l) sucrose was able to give good response and production of normal embryoids. Dewald *et al.* (1989a) reported that induction of somatic embryogenesis and early stages of somatic embryo development require moderate to high concentration of sucrose.

Coconut water was found to be essential for somatic embryo production from embryo mass. In its absence embryos were not found to occur. Coconut water at 200ml/l was found to be ideal for the production of embryoids from embryo mass of polyembryonic mango varieties which is same as in the case of nucellas when used as explants. Litz *et al.* (1982), Litz (1984b) and Dewald *et al.* (1989a) also indicate success in induction of somatic embryogenesis in mango in media supplemented with coconut water.

The somatic embryoids obtained from embryo mass were having sufficient size so that they could be transferred directly on to the germination media. As in the case of nucellus derived embryoids, BA was found to be suitable for germination of embryoids from embryo mass also. This plant growth substance at concentrations 0.1mg/l

and 0.2mg/l recorded satisfactory germination. Kinetin 0.2mg/l also favoured normal germination. Several other treatments failed to give germination without abnormalities. Kavathekar and Johri (1978) reported the requirement of cytokinins for the development somatic embryos into plantlets.

The initial two stages *viz* induction and initiation in the somatic embryogenesis of polyembryonic mango varieties was found comparatively easy. Maturation of somatic embryoids was the most critical stage. Proper maturation of somatic embryoids without abnormalities was found difficult to control. This stage of development require further refinement. Eventhough germination occured in several cultures, these plantlets failed *ex vitro* establishment. Hence this aspect also find more attention in the future line of work.

SUMMARY

6. SUMMARY

Attempts were made in the Plant Tissue Culture Laboratory of the Department of Horticulture, College of Agriculture, Vellayani during 1994-1996 for evolving techniques for *in vitro* somatic embryogenesis in polyembryonic mango varieties. Standardisation of basal media, culture medium components and culture conditions during various stages of somatic embryogenesis, namely, induction, initiation, maturation and germination was attempted using nucellus and embryo mass as explants.

The salient findings of the studies are summarised below.

1. Varietal difference was observed in induction of somatic embryogenesis from nucellus. The highest percentage of response (85.0) and callus index (170.0) were observed for the variety Kalluvarikka. The lowest were for the variety Vellari Manga (68.9 and 120.3 respectively).
2. Direct somatic embryogenesis was observed in Kalluvarikka (10.0%), Olour and Kurukkan (5.0 % each).
3. The MS medium with half strength of major nutrient salts was found better for induction of somatic embryogenesis from nucellus, compared to the medium having a combination of B-5 major nutrient salts and MS minor nutrient salts.
4. Among the plant growth substances, 2,4-D at 5.0 mg/l induced the highest percentage of response (76.0 %). The highest callus index was recorded by BA 16 mg/l (156.8) and the highest percentage of direct embryogenesis by kinetin 8.0 mg/l (12.0).

5. The highest percentage of induction of somatic embryogenesis (81.8 %) was effected by glutamine 400 mg/l, but the highest callus index (163.62) and direct embryogenesis (20.0 %) were recorded in the absence of glutamine.
6. Sucrose 60 g/l was ideal for induction of somatic embryogenesis from nucellus which effected highest response (83.3 %), callus index (250.0) and direct embryogenesis (20.0 %)
7. Coconut water 200 ml/l was found good for induction of somatic embryogenesis from nucellus (83.3 %).
8. Activated charcoal was found essential for induction of somatic embryogenesis from nucellus. At 3.75 g/l it recorded the highest level of response (80 %).
9. Darkness was found essential for induction of somatic embryogenesis from nucellus.
10. Among the plant growth substances, BA 1.0 mg/l was good for initiation of somatic embryogenesis from nucellus. Addition of 2,4-D and GA_3 was deleterious due to either reduced level of response or malformations in cultures.
11. Addition of casein hydrolysate 300 mg/l was beneficial for initiation of somatic embryogenesis from nucellar cultures.
12. Chelated iron at its normal concentration in the basal medium (ferrous sulphate 27.8 mg/l + EDTA - Na salt 37.3 mg/l) was found to be ideal for initiation of somatic embryogenesis.
13. The level of glutamine was found insignificant for initiation of somatic

embryoids from nucellus.

14. Coconut water 250 ml/l recorded the maximum response in initiation of somatic embryoids from nucellus.

15. Abscisic acid 3.0 mg/l was highly beneficial for maturation of nucellus derived embryoids.

16. Maturation of nucellar somatic embryoids was promoted by sucrose 50 g/l at which the embryoids attained a maximum size of 2.5 cm without abnormalities.

17. Coconut water 200 ml/l supported the maturation of somatic embryoids from nucellar cultures.

18. Media supplements such as cobaltous chloride, sodium butyrate and sodium chloride were not beneficial for the maturation of somatic embryoids derived from nucellus.

19. Solid medium alone supported maturation of nucellus derived somatic embryoids. The embryoids in liquid medium became vitrified and dried up.

20. Darkness was found to be ideal for maturation of somatic embryoids.

21. Among the plant growth substances normal germination of nucellar somatic embryoids was aided by BA (0.1 mg/l and 0.2 mg/l) alone.

22. Sucrose 40 g/l aided near normal germination of nucellus derived somatic embryoids.

23. Cobaltous chloride and sodium butyrate were not useful for the germination of nucellar embryoids.

24. Solid medium was good for germination somatic embryoids from nucellus. Liquid medium caused vitrification and drying up of the embryoids.

25. Light was essential for germination of somatic embryoids.
26. Direct somatic embryogenesis was observed when embryo mass was used as explant.
27. The auxin 2,4-D (5.0 mg/l) effected the induction of somatic embryogenesis from embryo mass.
28. Sucrose 60 g/l was ideal for induction of somatic embryogenesis from embryo mass.
29. Coconut water (150-200 ml/l) was found to be essential for induction of somatic embryogenesis from embryo mass.
30. Treatments using, BA 0.1 mg/l caused near normal germination of somatic embryoids, derived from embryo mass.

REFERENCES

REFERENCES

Al-Abta, S., Galpin, I. J. and Collin, H. A. 1979. Flavour compounds in tissue cultures of celery. *Plant Sci. Lett.*, **16** : 129-134.

Ammirato, P. V. 1973. Some effects of abscisic acid on the development of embryos from caraway cells in suspension culture. *Am. J. Bot.*, **60** : 22-23.

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

Ammirato, P. V. 1983. Embryogenesis. In : *Handbook of Plant Cell Culture*. Vol. I. *Technique for Propagation and Breeding* (Evans, D. A., Sharp, W. R., Ammirato, P. V. and Yamada, Y. eds.) Macmillan Publishing Company, New York, pp. 83-123.

Ammirato, P. V. 1987. Organisational events during somatic embryogenesis. In : *Plant Tissue and Cell Culture* (Green, C. E., Somres, D. A., Hackett, W. P. and Biesboer, D. D., eds.). Alan R. Liss, Inc., New york, pp. 57-81.

Ammirato, P. V. and Steward, F.C. 1971. Some effects of the environment on the development of embryos from cultured free cells. *Bot. Gaz.*, **132** : 149-158.

* Backs-Husemann, D. and Reinert, J. 1970. Embryobildung durch isolierte Einzelzellen aus Guwebekulturen von *Daucus carota*. *Protoplasma*, **70** : 49-60.

Bonneau, L., Beranger-Novart, L. and Monin, J. 1994. Somatic embryogenesis and plant regeneration in a woody species: the European Spindle Tree. (*Euonymus europaeus* L.). *Plant Cell Rep.*, **13** : 135-138.

Button, J. and Bornman, C.H. 1971. Development of nucellar plants from unfertilized ovules of Washington Navel Orange *in vitro*. *J. South African Botany*, **37** : 127-134.

Carron, M.P. and Enjalric, F. 1985. Somatic embryogenesis from inner integument of the seed of *Hevea brasiliensis* (Kunth., Mull. Arg.). *Comp. Rend. Ser. III.*, **300** : 653-658.

Dewald, S.G., Litz, R.E. and Moore, G.A. 1989a. Optimising somatic embryo production in mango *J. Amer. Soc. HortSci.*, **114** (4) : 712-716.

Dewald, S.G., Litz, R.E. and Moore, G.A. 1989b. Maturation and germination of mango somatic embryos. *J. Amer. Soc. HortSci.*, **114** (5) : 837-841.

Dougall, D. K. and Verma, D. C. 1978. Growth and embryo formation in wild-carrot suspension cultures with ammonium ion as sole nitrogen source. *In Vitro*, **14** : 180-182.

Eichooltz, D. A., Robitaille, H. A. and Hazegawa, P. M. 1979. Adventive embryony in apple. *HortSci.*, **14** : 699-700.

Evans, D. A., Sharp, W. R. and Bravo, J. E. 1984. Cell culture methods for crop improvement. In : *Handbook of Plant Cell Culture.*, Vol. *2. Crop Species.* (Sharp, W. R., Evans, D.A., Ammirato, P. V. and Yamada, Y., eds.) Macmillan Publishing Co. New York, pp. 47-68.

Evans, D. A., Sharp, W. R. and Flick, C. E. 1981. Growth and behaviour of cell cultures : Embryogenesis and organogenesis. In : *Plant Tissue Culture Methods and Applications in Agriculture.* (Thorpe, T. A. ed.) Academic Press, New York, pp. 45-113.

Fridborg, G., Pederson, M., Landstron, L-E. and Erikson, T. 1978. The effect of activated charcoal on tissue cultures: Adsorption of metabolites inhibiting morphogenesis. *Physiol. Plant.*, **43** : 104-106.

Fujimura, T. and Komamine, A. 1975. Effects of various growth regulators on embryogenesis in a carrot cell suspension culture. *Plant Sci. Lett.*, **5** : 359-364.

*Fujimura, T. and Komamine, A. 1980. Mode of action of 2,4-D and zeatin on somatic embryogenesis in a carrot cell suspension culture. *Z. Pflanzenphysiol.* **99** : 1-8

Gamborg, O. L., Miller, R. A. and Ojima, K. 1968. Plant cell cultures I. Nutrient requirement of suspension cultures of soyabean root cells. *Exp. Cell Res.*, **50** : 151-158.

Gray, D. J. 1987. Quiscence in monocotyledenous and dicotyledenous somatic embryos induced by dehydration. *HortSci.*, **22** : 810-814.

Guha, S. and Maheswari, S. C. 1964. *In vitro* production of embryos from anthers of *Datura*. *Nature*, **204** : 497.

Haccius, B. 1978. Question of unicellular origin of non-zygotic embryos in callus cultures. *Phytomorphology*, **28** : 74-81.

*Haccius, B. and Lakshmanan, K. K. 1965. Adventiv-embryonen aus *Nicotiana* Kallus, der bei hohen Lichtintensitaten kultiviert wurde. *Planta*, **65** : 102-104.

Halperin, W. 1966. Alternative morphogenetic events in cell suspensions. *Am. J. Bot.*, **53** : 443-453.

*Heberle-Bors, E. 1980. Interaction of activated charcoal and iron chelates in anther cultures of *Nicotiana* and *Atropa belladonna* Z. *pflanzenphysiol.*, **99** : 339-347.

*Homes, J. L.A.1967. Influence de la concentration en glucose sur la development *et al* differenciation d'embryons formes dans des tissue de carrotte cultives *in vitro*. In : *Les Cultures de Tissus de Plantes. Coll. Nat. CNRS*, Paris pp. 49-60.

Jaiswal, V. S. 1990. Somatic embryogeny in nucellus of some Indian cultivars of mango. *Abstracts of the International Seminar on New Frontiers in Horticulture*, Bangalore pp. 33.

Jana, M.M., Nadgauda, R. S., Rajmohan, K. and Mascarenhas, A. F. 1994. Rapid somatic embryogenesis from the nucelli of monoembryonic varieties. *In vitro Plant*, **30** (1) : 55-57.

Janick, J. 1993. Agricultural uses of somatic embryos. *Acta. Hort.*, **336** : 207-215.

*Kamada, H. and Harada, H. 1979. Studies on the organogenesis in carrot tissue cultures. II. Effects of amino acids and inorgainc nitrogenous compounds on somatic embryogenesis. *Z. Pflanzenphysiol.*, **91** : 453-463.

*Kao, K. N. and Michayluk, M. R. 1980. Plant regeneration from mesophyll protoplasts of alfalfa. *Z. Pflanzenphysiol.*, **96** : 135-141.

Kato, H. and Takeuchi, M. 1966. Embryogenesis from the epidermal cells of carrot hypocotyl. *Sci. Pap. College Gen. Educ. Univ.* Tokyo, **16** : 245-254.

Kavathekar, A. K. and Johri, B. M. 1978. *In vitro* responses of embryoids of *Eschscholzia californica*. *Biol. Plant*, **20** : 98-106.

Kester, D. H. 1983. The clone in horticulture. *HortSci.*, **18** : 831-837.

Kim, Y. W., Lee, B. C., Lee, S. K. and Jing, S. S. 1994. Somatic embryogenesis and plant regeneration in *Quercus acutissima*. *Plant Cell Rep.*, **13** : 315-318.

Kochba, J., Button, J., Spiegel-Roy, P., Bornman, C. H. and Kochba, M. 1974. Stimulation of rooting of *Citrus* embryoids by giberellic acid and adenine sulphate. *Ann. Bot.*, **38** : 795-802.

*Kochba, J., Spiegel-Roy, P., Newmann, H. and Saad, S. 1978. Stimulation of embryogenesis in *Citrus* ovular callus by ABA, Ethephon, CCC and Alar and its suppression by GA. *Z. Pflanzenphysiol*, **89** : 427-432.

*Kochba, J., Spiegel-Roy, P. and Safran, H. 1972. Adventive plants from ovules and nucelli in *Citrus*. *Planta*, **106** : 237-245.

Konar, R. N., Thomas, E. and Street, H. E. 1972. The diversity of morphogenesis in suspension cultures of *Atropa belladona* L. *Ann. Bot.*, **36** : 249-258.

Kong, L. S. and Rao, A. N. 1981. Induction of callus and organogenesis in cocoa tissues. In : *Tissue Culture of Economically Important Plants* (Rao, A. N. ed.) COSTED and ANBS, Singapore, pp. 107-112.

Lakshmi Sita. G., Raghava Ram, N. V. and Vaidyanathan, C. S. 1979. Differentiation of embryoids and plantlets from shoot callus of sandal wood. *Plant Sci. Lett.*, **15** : 265-270.

Litz, R. E. 1984a. *In vitro* somatic embryogenesis from callus of jaboticaba (*Myrciaria cauliflora*). *HortSci.*, **19** : 62-64.

Litz, R. E. 1984b. *In vitro* somatic embryogenesis from nucellar callus of monoembryonic mango. *HortSci.*, **19** : 715-717.

Litz, R. E. 1984c. *In vitro* responses of adventitious embryos of two polyembryonic *Eugenia* species. *HortSci.*, **19** : 720-722.

Litz. R. E. 1985. Somatic embryogenesis in tropical fruit trees. In : *Tissue Culture in Forestry and Agriculture* (Henke, R. R., Hughes, K. W., Constantin, M. P., Hollaender, A. and Wilson, C. M., eds.) Plenum Publishing Corp., New York, pp. 179-193.

Litz, R. E. 1986. Mango In : *Handbook of Plant Cell Culture* Vol. 4. *Techniques and Applications.* (Evans, D. A., Sharp, W. R. and Ammirato, P.V., eds.) Macmillan Publishing Co., New York, pp. 612-615.

Litz, R. E. 1987. Application of tissue culture to tropical fruits. In : *Plant Tissue and Cell Culture* (Green, C. E., Somers, D. A., Hackett, W. P. and Biesboer, D. D., eds.) Alan R. Liss Inc., New York, pp. 407-418.

Litz, R. E. and Conover, R. A. 1980. Somatic embryogenesis in cell cultures of *Carica stipulata*. *Hortic. Sci.*, **15** : 733-744.

Litz, R.E. and Gray, D.J. 1992. Organogenesis and somatic embryogenesis. In : *Biotechnology of Perennial Fruit Crops*. (F.A. Hammerschlag and R.E. Litz, eds.) pp. 3 - 34. CAB International, U.K.

Litz, R. E., Knight, R. J. and Gazit, S. 1982. Somatic embryos from cultured ovules of polyembryonic *Mangifera indica* L. *Plant Cell Reports*, **1** : 264-266

Litz, R. E., Knight, R. J. and Gazit. S. 1984. *In vitro* somatic embryogenesis from *Mangifera indica* L. callus. *Scientia Hort.*, **22** : 233-240.

Litz, R. E., Mathews, V. H., Hendrix, R. C. and Yurgalevitch, C. 1991. Mango somatic cell genetics. *Acta Hort.*, **291** : 133-140.

Litz, R. E., Mathews, V. H., Moore, G. A., Pleigo Alfaro, F., Yurgalevitch, C. and Dewald, S. G. 1993. Somatic embryos of mango (*Mangifera indica* L.) In : *SYNSEEDS: Application of Synthetic Seeds to Crop Improvement* (Redenbough, K., ed.) CRC Press, New York, pp. 409-425.

Litz, R. E. and Schaffer, B. 1986. Polyamines in adventitious and somatic embryogenesis in mango (*Mangifera indica* L.). *J. Plant Physiol.*, **128** : 251-258.

Lu, C. and Ozias-Akins, P. 1982. Somatic embryogenesis in *Zea mays* L. *Theor. Appl. Genet.*, **62** : 109-112.

Lutz, J. D., Wong, J. R., Rowe, J., Tricoli, D. M. and Lawrence, R. H. Jr. 1985. Somatic embryogenesis for mass cloning of crop plants. In : *Tissue Culture in Forestry and Agriculture.* (Henke, R. R., Hughes, K. W., Constantin, M. P., Hollaender, A. and Wilson, C. M., eds.) Plenum Publishing Corp., New York, pp. 105-116.

Mathews, H. and Litz, R. E. 1990. Kanamycin sensitivity of mango somatic embryos. *HortSci.*, **25** : 965-966.

McGranahan, G. H., Leslie, C. A., Uratsu, S. L. and Dandekar, A. M. 1990. Improved efficiency of the walnut somatic embryo gene transfer system. *Plant Cell Rep.*, **8** : 512-516.

McWilliam, A. A., Smith, S. M. and Street, H. E. 1974. The origin and development of embryoids in suspension cultures of carrot (*Daccus carota*). *Ann. Bot.*, **38** : 243-250.

*Michaux-Ferriere, N. and Dublin, P. 1988. Somatic embryogenesis in *Coffea arabica* : induction and development of embryogenic cells. *Twelfth International Scientific Colloquium on Coffee, Paris.*, pp. 418-425.

Monsalud, M. J., Mathews, H., Litz, R. E. and Gray, D. J. 1995. Control of hyperhydricity of mango somatic embryos. *Plant Cell, Tissue and Organ Culture*, **42** : 195-206.

Mullins, M. G. and Srinivasan, C. 1976. Somatic embryos and plantlets from an ancient clone of the grapevine (W Cabernet-Sauvignon) by apomixis *in vitro J. Exp. Bot.*, **27** (100) : 1022-1030.

Muralidharan, E. M., Nadgauda, R. S., Jana, M. M., Parashurami, V. A. and Mascarenhas, A. F. 1994. Somatic embryogenesis in some woody perennials. In : *Advances in Plant Tissue Culture in India*. (Pramod Tandan., ed.) Pragathi Prakashan, Meerut, pp. 1-7.

Murashige, T. 1974. Plant regeneration through tissue cultures. *Ann. Rev. Plant Physiol.*, **25** : 135-136.

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

Narayanaswamy, S. 1992. Regeneration of plants from tissue cultures. In : *Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture* (Reinert, J. and Bajaj, Y. P. S., eds.) Narosa Publishing House, New Delhi, pp. 179-248.

Newcomb, W. and Wethrell, D. F. 1970. The effects of 2,4,6-trichlorophenoxy acetic acid on embryogenesis in wild carrot tissue culture. *Bot. Gaz.*, **131** : 242-245.

Nitsch, J. P. 1969. Experimental androgenesis in *Nicotiana*. *Phytomorphology*, **19** : 389-404.

*Ohta, Y. and Furuzato, K. 1957. Embryo culture in *Citrus*. *Seiken Ziho*, **8** : 49-54.

Onay, A., Jeffree, C. F. and Yeomann, M. M. 1995. Somatic embryogenesis in cultured immature kernals of pistachio (*Pistacia vera* L.). *Plant Cell Rep.*, **15** : 192-195.

Perry, M. and Chalkey, L. 1981. The effect of histone hyperacetylation on the nuclear sensitivity and solubility of chromatin. *J. Biol. Chem.*, **258** : 3313 - 3318.

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

Raghavan, V. 1983. Biochemistry of somatic embryogenesis. In : *Handbook of Plant Cell Culture.* Vol. I. *Techniques for Propagation and Breeding* (Evans, D. A., Sharp W. R., Amirato, P. V. and Yamada, Y. eds.) Macmillan Publishing Co., New York, pp. 655-671.

Raghuvansi, S. S. and Srivasthava, A. 1995. Plant regeneration of *Mangifera indica* using liquid shaker culture to reduce phenolic exudation. *Plant Cell, Tissue and Organ Culture*, **41** : 83-85.

Rangan, T.S., Murashige, T. and Bitters, W.P. 1968. *In vitro* initiation of nucellar embyos in monoembryonic citrus. *HortSci.*, **3** : 226-227.

Rangaswami, N. S. 1961. Experimental studies on female reproductive structures of *Citrus microcarpa* Binge. *Phytomorphology*, **11** : 109-127.

Rangaswami, N. S. 1982. Nucellus as an experimental system in basic and applied tissue culture research. In : *Tissue Culture of Economically Important Plants.* (Rao, A. N., ed.) COSTED, Singapore, pp. 269-286.

Rangaswami, N. S. and Sethi, M. 1991. Polyembryoni and embryos from somatic cells, pollen grains and microspores. In : *Reproduction in Flowering Plants* (Rashid, A., ed.) CRC Press Inc., Florida.

Rao, A. N., Sin, Y. M., Kathagoda, N. and Hut.Chinson, J. F. 1981. Cotyledon tissue culture of some tropical fruits. In : *Tissue Culture of Economically Important Plants.* (Rao, A. N., ed.) COSTED, Singapore, pp. 124-137.

Razdan, M. K. 1994. *An Introduction to Plant Tissue Culture.* Oxford and IBH Publishing Co. Pvt.Ltd., New Delhi, pp. 87-102.

*Reinert, J. 1958. Morphogenese und itre kontrolle an Gewebekulturen aus Carotten. *Naturwissenschaften*, **45** : 344-345.

Reinert, J. 1973. Aspects of organisation-Organogenesis and embryogenesis. In : *Plant Tissue and Cell Culture* (Street, H. E., ed.) University Press, Oxford pp. 338-355.

Sharp, W. R., Sondahl, M. R., Caldas, L. S. and Maraffa, S. B. 1980. Physiology of *in vitro* asexual embryogenesis. *Hortic. Rev.*, **2** : 268-310.

Shenk, R. U. and Hildebrandt, A. C. 1972. Medium and techniques for induction of growth of monocotyledonous and dicotyledonous plant cell cultures. *Can. J. Bot.*, **50** : 199-204.

Skoog, F. and Miller, C.O. 1957. Chemical regulation of growth and organ formation in plant tissue cultivated *in vitro*. In : *Biological Action, Action of growth substances. Symp. Soc. Exp. Biol.*, **11** : 118 - 131.

Simola, L. K. 1987. Structure of cell organelles and cell wall in tissue culture of trees. In : *Cell and Tissue Culture in Forestry. Vol. I. General Principles and Biotechnology* (Bonga, J. M. and Durzan, Don, J., eds.) Martinus Nijhoff Publishers, Dordrecht, Netherlands, pp. 389-418.

Singh. R.N. 1990. *Mango.* Indian Council of Agricultural Research, New Delhi.

*Smith, W. K. and Thomas. J. A. 1973. The isolation and *in vitro* cultivation of cells of *Elaeis guineensis*. *Oleagineaux*, **28** : 123-127.

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

Sreenath, H. L., Shantha, H. M., Harinath Babu, K. and Naidu, M. M. 1995. Somatic embryogenesis from integument (perisperm) cultures of coffee. *Plant Cell Rep.*, **14** : 670-673.

*Tsai, H. S. and Tseng, M. T. 1979. Embryoid formation and plantlet regeneration from anther callus of sweet potato. *Bot. Bull. Acad. Sin.*, **20** : 117-112.

Tuleke, W. 1987. Somatic embryogenesis in woody perennials. In : *Cell and Tissue Culture in Forestry* Vol. 2. *Specific Principles and Methods : Growth and Developments* (Bonga, J. M. and Durzan, Dan, J., eds.) Martinus Nijhoff Publishers, Dordrecht, Netherlands pp. 61-91.

Vardy, A., Spiegel-Roy, P. and Galun, E. 1975. *Citrus* cell culture : Isolation of protoplasts, planting densities, effect of mutagens and generation of embryos. *Plant Sci. Lett,*. **4** : 231-236.

Vasil, V. and Vasil, I. K. 1981. Somatic embryogenesis and plant regeneration from tissue cultures of *Pennisetum americanum* and *P. americanum* x *P. purpureum* hybrid. *Am. J. Bot.*, **68** : 864-872.

Verma, D.C. and Dougall, D. K. 1978. DNA, RNA and protein content of tissues during growth and embryogenesis in wild carrot suspension cultures. *In Vitro*. **14** : 183-191.

Wachira, F. and Ogada, J. 1995. *In vitro* regeneration of *Camellia sinensis* (L.) O. Kuntze. by somatic embryogenesis. *Plant Cell Rep.* **14** : 463-466.

Wang, Y. C. and Janik, J. 1984. Inducing precocious germination in asexual embryos of cacao. *HortSci.* **19** : 839-841.

*Weatherhead, M. A., Burdon, J. and Henshaw, G. G. 1978. Some effects of activated charcoal as an additive to plant tissue culture media. *Z. Pflanzenphysiol.* **89** : 141-147.

Wethrell, D. F. 1984. Enhanced adventive embryogenesis resulting from plasmolysis of cultured wild carrot cells. *Plant Cell, Tissue and Organ Culture* **3** : 221-227.

Wetherell, D. F. and Dougall, D. K. 1976. Sources of nitrogen supporting growth and embryogenesis in cultured wild carrot tissue. *Physiol. Plant* **37** : 97-103.

Staritsky, G. 1970. Embryoid formation in callus of coffee . *Acta Bot. Neerl.*, **19** : 509-514.

Steward, F. C. 1958. Growth and development of cultivated cells III. Interpretations of the growth from free cell to carrot plant. *Am. J. Bot.*, **45**. 709-713.

*Steward, F. C., Israel, H. W., Mott, R. L., Wilson, H. J. and Krikorian, A. D. 1975. Observations on growth and morphogenesis in cultured cells of carrot (*Daucus carota* L.) *Philos. Trans. R. Soc. London Ser. B.*, **273** : 33-53.

Steward, F. C. and Shantz, E. M. 1956. The chemical induction of growth in plant tissue cultures. In : *The Chemistry and Mode of Action of Plant Growth Substances*. (Wain, R. L. and Whitman, F. eds.) Academic Press, New York, pp. 165-187.

Sussex, I. M. 1972. Somatic embryos in long term carrot tissue culture: histology, cytology and development. *Phytomorphology*, **22** : 50-58.

Sussex, I. M. and Frei. K. A. 1968. Embryoid development in long term tissue cultures of carrot. *Phytomorphology*. **18** : 339-349.

Thorpe, T.A. 1980. Organogenesis *in vitro* : structural, physiological and biochemical aspects. *Int. Rev. Cytol. Sup.*, **11 A** : 71 - 111.

Tisserat, B. and De Mason, D. A. 1980. A histological study of development of adventive embryos in organ cultures of *Phoenix dactylifera* L. *Ann. Bot.* **46** : 465-472.

Tisserat, B., Esan, E. B. and Murashige, T. 1979. Somatic embryogenesis in angiosperms. In : *Horticultural Reviews* (Janick, J., ed.) Vol. I, pp. 1-78, Pardue University, AVI Pub., Westport, Connecticut.

Tixeira, J. B., Sondahl, M. R. and Kirby, E. G. 1994. Somatic embryogenesis from immature inflorescences of oil palm. *Plant Cell Rep.*, **13** : 247-250.

White, P. R. 1963. *A Handbook of Plant and Animal Tissue Culture*. Jaques Cattel Press, Lancaster, Pennsylvania.

Williams, E. G. 1987. Somatic embryogenesis as a tool in plant improvement. In : *Biotechnology in Agriculture*. (Natesh, S. ed.) Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi, pp. 179-184.

Williams, E. G. and Maheswaran, G. 1986. Somatic embryogenesis: Factors influencing co-ordianted behaviour of cells as an embryogenic group. *Ann. Bot.*, **57** : 443-462.

EVOLVING TECHNIQUES FOR *IN VITRO* SOMATIC EMBRYOGENESIS IN POLYEMBRYONIC MANGO (*Mangifera indica* L.) VARIETIES

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ABSTRACT

Studies were conducted for evolving techniques for *in vitro* somatic embryogenesis in polyembryonic mango varieties during 1994 - 1996 in the Plant Tissue Culture Laboratory of the Department of Horticulture, College of Agriculture, Vellayani. Attempts were made to standardise the various stages of somatic embryogenesis, namely, induction, initiation, maturation and germination using nucellus and embryo mass as explants.

Among the six varieties attempted for induction of somatic embryogenesis from nucellus, Kalluvarikka recorded the highest percentage of response (85.0) and Vellari Manga the least (68.9).

Induction of somatic embryogenesis from nucellus was found to occur at its maximum on MS medium having half strength major salts, supplemented with 2,4 - D 5.0 mg/l, GA_3 5.0 mg/l, glutamine 400 mg/l, sucrose 60 g/l, coconut water 200 ml/l, agar 6.0 g/l and activated charcoal 2.5 g/l, in darkness. The highest callus index was recorded when 2,4 - D 5.0 mg/l was substituted with BA 1.0 mg/l in the same medium.

Initation of somatic embryogenesis from nucellus occurred at its best in darkness on MS medium having half strength major salts, supplemented with BA 1.0 mg/l, glutamine 400 mg/l, casein hydrolysate 500 mg/l, sucrose 60 g/l, coconut water 200 ml/l agar 5.5 g/l and activated charcoal 2.5 g/l.

Maturation of nucellus derived somatic embryoids was found at its best on a combination of basal media with B-5 major salts and MS minor salts supplemented with ABA 3.0 mg/l, casein hydrolysate 100 mg/l, sucrose 40 g/l, PVP 10 g/l, coconut water 200 ml/l and agar 5.0 g/l, in darkness.

Germination of somatic embryoids derived from nucellus occurred only in presence of light, on a combination of basal media with B-5 major salts, MS minor salts, supplemented with BA 0.1 mg/l, sucrose 50 g/l, PVP 10 g/l and agar 5.0 g/l.

Somatic embryos were produced directly from embryo mass explants in darkness on solid MS medium having half strength of major salts supplemented with 2,4 - D 5.0 mg/l, GA_3 5.0 mg/l, glutamine 400 mg/l, sucrose 60 g/l, coconut water 200 ml/l, agar 6.0 g/l and activated charcoal 2.5 g/l. The embryoids having sufficient size resulted from this treatment were able to attain near normal germination in presence of light on medium having a combination of B - 5 major salts and MS minor salts, supplemented with BA 0.1 mg/l or 0.2 mg/l, sucrose 50 g/l, PVP 10 g/l and agar 5.0 g/l.

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