IMPROVEMENT OF *IN VITRO* SOMATIC EMBRYOGENESIS IN CASHEW (*Anacardium occidentale* L.)

By

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THESIS

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DECLARATION

I hereby declare that this thesis entitled **"Improvement of *in vitro* somatic embryogenesis in cashew (*Anacardium occidentale* L.)"** is a *bonafide* record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis entitled **"Improvement of *in vitro* somatic embryogenesis in cashew (*Anacardium occidentale* L.)"** is a record of research work done independently by **Miss. Rekha. S. (96.12.04)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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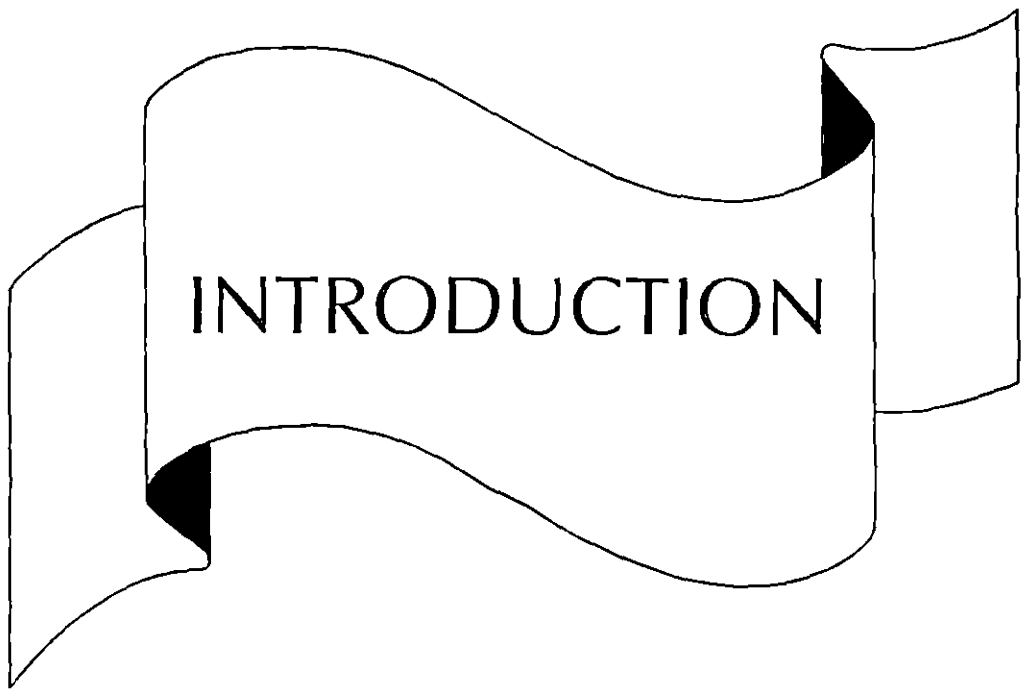
LIST OF ABBREVIATIONS

ABA	-	Abscisic acid
AC	-	Activated charcoal
BA	-	6-benzyl amino purine
CH	-	Casein hydrolysate
CW	-	Coconut water
$CoCl_2$	-	Cobalt chloride
GA_3	-	Gibberellic acid
IAA	-	Indole - 3 - acetic acid
IBA	-	Indole - 3 - butyric acid
MS	-	Murashige and Skoog
NAA	-	Naphthalene acetic acid
PEG	-	Polyethylene glycol
PVP	-	Polyvinyl pyrrolidone
SH	-	Shenk and Hildebrandt
TIBA	-	Tri-iodo benzoic acid
WPM	-	Woody plant medium
2,4-D	-	2,4-dichlorophenoxy acetic acid
2,4-5T	-	2,4-trichlorophenoxy acetic acid


INTRODUCTION

1. INTRODUCTION

India is the largest producer, processor and exporter of cashew kernels in the world. Though it is an important perennial cash crop of Kerala state, the full potential of this crop has not been exploited so far. Being a highly cross-pollinated crop, cashew plantations raised from seeds exhibit wide variation in plant growth, fruiting and productivity. Although, the conventional vegetative propagation methods are quite successful, the rate of multiplication is not sufficient enough to meet the demand for superior planting materials.

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

Evolving a protocol for the *in vitro* propagation of cashew will help the rapid clonal propagation and early establishment of varieties and

hybrids of known superior traits. Rapid multiplication of scion material and large scale production of clonal root stocks are also possible. Rare and endangered cultivars, especially certain promising local varieties of Kerala, can be multiplied and saved from becoming extinct.

There are three routes of *in vitro* propagation namely *via.* somatic embryogenesis, somatic organogenesis and enhanced release of axillary buds. Among these, somatic embryogenesis is the fastest route of *in vitro* clonal propagation, helping in the production of a large number of bipolar embryos just like that of zygotic embryos. The advantage is that, on germination, the somatic embryos can produce plantlets with well developed tap root system having good anchorage. But on the contrary, plantlets obtained *via.* other routes of *in vitro* propagation as well as from the conventional vegetative propagation method like layering, bear only an adventitious root system, which causes uprooting of cashew trees in coastal belts during heavy rains.

Studies on the *in vitro* propagation of cashew *via* somatic embryogenesis is very limited. Somatic embryogenesis in cashew has been attempted by a few scientists (Hegde, 1988; Jha, 1988; Sy *et al.*, 1991; Kesavachandran, 1991 and 1998; Nair *et al.*, 1993; Lakshmisita, 1994 and Thomas, 1995). But production of normal plantlets was found to be difficult.

Nair *et al.* (1993), under the USDA project on tissue culture in the Department of Horticulture, College of Agriculture, Vellayani during the period from 1988-1993 could make progress in the *in vitro* propagation of cashew *via.* somatic embryogenesis using nucellus as explant. Though the somatic embryoids developed shoots and roots, the shoots formed were compressed and malformed. This can be attributed to many problems such as the imbalance of plant growth substances, accumulation of ethylene or variation in osmotic potential in the media. Hence it was felt for further refinement to get improved response and develop standard protocols.

The present studies were taken up for refining the protocols for the *in vitro* clonal propagation of cashew varieties locally grown in Kerala *via.* somatic embryogenesis. The main objective of the study is to improve the maturation and germination processes of cashew somatic embryoids so as to get normal plantlets. The results may serve as useful guidelines for standardising *in vitro* somatic embryogenesis in improved cashew varieties / hybrids.


REVIEW OF
LITERATURE

2. REVIEW OF LITERATURE

Cashew is an important commercial horticultural crop contributing substantially to the national income through export. It belongs to the family Anacardiaceae.

Cashew is both seed propagated as well as vegetatively multiplied through veener grafting, budding, air layering (Madhava Rao, 1958) and epicotyl grafting (Nagabhushanam, 1984). Cashew is a cross pollinated species and therefore highly heterozygous, exhibiting a wide variation for a number of characters such as growth, yield and quality (Ascenso, 1986). Conventional methods are not adequate to meet the demand for superior planting material. Hence the urgent need had been felt to standardise more rapid and prolific rate of vegetative multiplication, using modern techniques of cell, tissue and organ culture, in order to generate adequate clonal planting materials of high yielding hybrids and selections. Thus tissue culture techniques are expected to supplement the conventional propagation methods in cashew in order to meet the ever increasing demand for superior planting materials (Nambiar and Iyer, 1988).

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 has a tap root system providing good anchorage in dicotyledonous trees.

2.1. Somatic embryogenesis

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

Potentially embryogenic cells are differentiated from cellular aggregates that have differential staining properties, conspicuous size and large number of nucleoli (Konar *et al.*, 1972). Somatic embryos possess a bipolar structure with a vascular system (Haccius, 1978).

Somatic embryogenesis occurs when a single cell or small group of cells initiates the developmental path way normally followed only by the predormant embryo within the seed (Williams, 1987).

Embryogenesis generally proceeds from the globular to the heart, torpedo, cotyledonary and mature somatic embryo stages of development

(Tulecke, 1987). During embryogenesis root and shoot develop simultaneously on the same culture medium (Evans *et al.*, 1984). Somatic embryogenesis consists of all the stages of embryo development, from the onset of embryogenesis to the production of a fully mature developmentally arrested embryo (Redenbough *et al.*, 1991).

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

According to Sharp *et al.* (1982) 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 path way is predetermined and the cells need only the synthesis of an inducer to express their potential. In 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 the same manner.

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

Other uses include crop improvement, metabolite production, disease elimination (Al-Abta *et al.*, 1979), germ plasm preservation (Ammirato, 1983) and generating somaclonal variants in the species (Razdan, 1993).

2.2. Factors influencing somatic embryogenesis

The key factors which influence somatic embryogenesis are pretreatments, explants, culture media, exogenous plant growth substances, environmental conditions, mode of culture, density and genetic stability of embryogenic cells and synchronous development of embryoids (Ammirato, 1983).

2.2.1. Explant

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

The physiological state of the plant from which the explant is taken is very important, and also the season during which it is removed (Ammirato, 1983).

Because of juvenility, nucellus has proved to be more morphogenetic than other tissues from mature trees (Litz, 1986a). The first response on the formation of embryoids from nucellus was reported in citrus by Ohta and Furuzato (1957).

Nucellus has been efficiently utilised for somatic embryogenesis in several crops such as apple (Eichooltz *et al.*, 1979), *Eugenia* spp. (Litz 1984 c), *Myrciaria cauliflora* (Litz, 1984 a), rubber (Carron and Enjalric, 1985), grapes (Mullins and Srinivasan, 1976) and poly and mono embryonic mango varieties (Litz *et al.*, 1982; Litz, 1984 b). Stevenson (1956), Rangan *et al.* (1968), Kochba *et al.* (1972), Mitra and Chaturvedi (1972), Ben-Hayyim and Neumenn (1983) and Navarro *et al.* (1985) used nucellus as explant for inducing somatic embryogenesis in citrus.

Litz and Schaffer (1986) demonstrated direct emergence of somatic embryos from nucellus of mango. Although monoembryonic and polyembryonic cultivars appeared to respond equally well, somatic embryogenesis was observed to be cultivar dependent (Litz *et al.* 1991). Nucelli from polyembryonic mango ovule are more embryogenic (Litz, 1987). Jaiswal (1990), Jana *et al.* (1994), Sulekha (1996) and Ramesh (1998) could induce *in vitro* somatic embryogenesis in mango from nucellar tissue.

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) and cocoa (Litz, 1986b) and immature inflorescence 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 *camelia japonica* (Vieitez and Barciela, 1990), kernels in *Pistachio vera* (Onay *et al.*, 1995), stem in *santalum album* (Lakshmi sita *et al.*, 1979), root and stem in *Actinidia chinensis* (Harada, 1975).

Nair *et al.* (1993) could induce somatic embryogenesis from cashew nucellus.

Hegde (1988), Jha (1988), Sy *et al.* (1991), Nair *et al.* (1993) Lakshmisita (1994) and Thomas (1995) used immature cotyledons as explant for inducing somatic embryogenesis in cashew.

Keshavachandran (1991, 1998) reported that the immature cotyledon bits, stem and petiole bits were responsive to callus induction and higher percentage of callus was induced from immature cotyledon bits.

2.2.2. Basal medium

2.2.2.1. Induction

According to Christianson (1985), an event must initially occur that involves a change in the determination of fate of certain cells. This change in the commitment of a cell is referred to as an inductive event. 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 (Tulecke, 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), *Citrus sinensis* (Wachira and Ogada, 1995), *Ginkgo biloba* (Laurain *et al.*, 1996) *Azadirachta indica* (Su *et al.*, 1997). Modified MS medium with half strength of major salts was used for somatic embryogenesis in coffee (Sondahl and Sharp, 1977). Kim *et al.*(1994) used modified MS medium for culturing embryos of *Quercus accutissima*. Somatic embryogenesis and subsequential regeneration of European spindle tree were obtained on MS semi solid basal medium. (Bonneau *et al.*, 1994)

Rao *et al.* (1981) reported the induction of callus from cotyledons of jack 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 (1984 b), Litz and Schaffer (1986), Dewald *et al.* (1989 a), Mathews and Litz (1990) and Jana *et al.* (1994). Dewald *et al.* (1989a) reported the use of a combination B5 major salts and MS minor salts for maintenance of embryogenic tissue while Jaiswal (1990) used this combination for

induction of somatic embryogenesis in mango. Litz *et al.* (1991) obtained somatic embryos when nucellus of polyembryonic and monoembryonic mango varieties were cultured on medium containing modified B5 major salts and MS minor salts and organics.

Hegde (1988) reported the development of globular structures from immature cotyledon sections of cashew on LS medium. Jha (1988) obtained callus when embryos excised from immature seeds were cultured on MS medium with modifications. SH medium with supplements was used for induction of somatic embryogenesis in cashew cotyledons by Sy *et al.* (1991). Nair *et al.* (1993) observed callusing from immature cotyledons of cashew on MS medium. Lakshmisita (1994) cultured cotyledonary pieces on MS medium and obtained callus. Thomas (1995) recorded high callusing from cotyledon bits when cultured on MS medium. Maximum callusing with 30 per cent cultures showing somatic embryoid was obtained in this medium. By using different explants, Kesavachandran (1998) obtained callus induction from Y3, LS and MS media.

2.2.2.2. Initiation

When tissues are transferred from an auxin containing medium to an auxin free medium having nitrogenous compounds like aminoacids, embryogenesis will be triggered (Reinert, 1958).

Initiation of mango somatic embryoids was reported to occur on modified MS medium (Litz *et al.*, 1982, Litz 1984 b). Dewald *et al.*

(1989 a) reported that modified B5 medium was significantly more effective than WPM, MS or modified MS for production of mango somatic embryos.

Jaiswal (1990) and Litz *et al.* (1991) used the combination of B5 major salts and MS minor salts for initiation of mango 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 (Tulecke, 1987).

Once the embryo is transferred to maturation medium it is anticipated that the organisational events will proceed as in zygotic embryo maturation (Ammirato, 1987). According to Litz *et al.* (1991) control of somatic embryo maturation was the most critical and difficult process in mango regeneration.

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

Jana *et al.* (1994) could obtain mature somatic embryos of mango on half strength MS basal medium. Modified basal medium consisting B5 major salts and MS minor salts was identified as the best for the maturation of somatic embryoids by Sulekha (1996).

2.2.2.4. Germination

If somatic embryos are not physically mature, they cannot germinate normally and survive. Poor germination is typical in many embryogenic culture systems. Razdan (1993) reported that germination of somatic embryos can occur only when it was mature enough to have functional root and shoot apices capable of meristematic growth. According to Capuana and Debergh (1997) to obtain normal plants from the somatic embryoids are difficult due to the asynchronous maturation of the embryogenic tissues and low germination and conversion rates.

Litz *et al.* (1982) reported the use of modified MS medium for germination in polyembryonic mango varieties. MS medium with half strength major salts was used for germination of mango somatic embryos by Litz *et al.* (1984), but successful regeneration of plantlets was not obtained. Dewald *et al.* (1989 b) reported that modified B5 medium with half strength of major salts gave a higher germination percentage in mango.

Jaiswal (1990), Sulekha (1996) and Ramesh (1998) could obtain germination of mango somatic embryoids on formulation with B5 major salts and MS minor salts.

Plantlet regeneration from the germinated somatic embryos of *Azadiracta indica* was achieved in a half strength MS agar basal medium with 1.0 per cent sucrose. (Su *et al.*, 1997)

2.2.3. Plant growth substances

2.2.3.1. Induction

The presence of auxin in the medium is generally essential for embryo initiation. The type and concentration of auxin also had some specific effects on the process of somatic embryogenesis, both in terms of efficiency and frequency.

Basal medium supplemented with auxins alone induced somatic embryos from embryonal explants of leguminous crops such as soyabean (Lazzeri *et al.*, 1987), Pea (Kysely and Jacobsen, 1990) and peanut (Ozias-Akins *et al.*, 1992); while cytokinins were required for somatic embryogenesis in *Trifolium* (Maheswaran and Williams, 1986) and *Phaseolus* species (Malik and Saxena, 1992).

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)

Mostly 2,4-D has been used to induce somatic embryogenesis (Razdan, 1993). 2,4-D is the most commonly used auxin for the induction of primary and secondary somatic embryogenesis in cassava (Sofiari *et al.*, 1997). 2,4-D was most effective in producing the highest frequency of responding cultures in peanut (Eapen and George, 1993), Papaya (Jordan and Velozo, 1996) and sesame (JeyaMary and Jayabalan 1997).

Litz *et al.* (1982, 1984) and Litz (1984 b) reported the use of 2,4-D for induction of somatic embryogenesis in mango. Somatic embryogenesis could be induced using MS medium supplemented with 2,4-D 5.0 mg/l, GA3 5.0 mg/l (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 occurred 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, 1984 b and cocoa (Adu Ampomah *et al.*, 1988). Auxins other than 2,4-D which are 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).

Litz *et al.* (1982) obtained somatic embryogenesis from mango nucellus when cultured on media supplemented with either coconut water or BA. Michaux Ferriere and Dublin (1988) reported that the cytokinin BA could induce somatic embryogenesis in coffee. Direct somatic embryogenesis from *Ginkgo biloba* was observed when BA 10 Mm was used as the sole plant growth regulator in the induction media (Laurain *et al.*, 1996).

Callus and somatic embryos were induced using a MS semisolid media supplemented with several combinations of auxins and cytokinins in spindle tree (Bonneau *et al.*, 1994). According to Laurain *et al.* (1996) the addition of different concentrations of NAA and BA to the induction medium led to an indirect embryogenesis in *Ginkgo biloba*. Embryogenic callus from cotyledons of *Azadirachta indica* were initiated when the medium is supplemented with 0.5 mg/l NAA and 1.0 mg/l BA (Su *et al.*, 1997).

Jha (1988) observed compact mass of calli from cotyledons of cashew in a medium containing 2,4-D and NAA. Nair *et al.* (1993) obtained callusing from immature cotyledons of cashew when 2,4-D, NAA and BA were incorporated into the medium. They also observed somatic embryogenesis in MS basal medium supplemented with IBA and BA combination. Thomas (1995) reported maximum callusing with somatic embryoids from a medium containing 2,4-D, NAA and BA combination. BA and GA produced direct somatic embryoids from cotyledonary bits of cashew. According to Kesavachandran (1998), 2,4-D induced

comparatively higher percentage of callusing followed by 2,4-5 T and NAA. Profuse callusing with a higher callus index was induced with a combination of 2,4-D and NAA 4.0 mg/l each with kinetin at 2.0 mg/l.

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

2.2.3.2. Initiation

The embryogenic clump that have been induced in the induction medium is then transferred to a medium free of auxin or with very low levels of auxin to initiate the development of embryos.

Litz *et al.* (1984) reported that efficient somatic embryogenesis occurred when nucellar callus was transferred to liquid media without 2,4-D. Jaiswal (1990) and Jana *et al.* (1994) reported the use of 2,4-D for initiation.

Cytokinins have been important in a number of plant species (Ammirato, 1983). The requirement is often specific. Zeatin was the only cytokinin which could promote somatic embryogenesis in carrot (Fujimura and Komamine, 1975). Litz *et al.* (1982) could initiate mango somatic embryoids on medium containing coconut water, BA or no growth regulators. Litz *et al.* (1991) used kinetin and BA in medium and could promote somatic embryogenesis in mango.

ABA also is reported to aid somatic embryo formation (Tsai and Tseng, 1979) and promote its development (Kochba *et al.*, 1978).

Initiation of somatic embryoids in mango occurred in the absence of plant growth regulators (Dewald *et al.*, 1989a).

Removal of 2,4-D enhanced the process of somatic embryogenesis in cashew (Jha, 1988). According to him the morphogenetic pattern increased when 2,4-D was omitted from the medium and concentration of NAA and kinetin was reduced to 0.5-0.005 mg/l and 0.5 mg/l respectively. Initiation of cashew somatic embryoids was reported to occur on MS medium with combination of 2,4-D, NAA and BA (Nair *et al.*, 1993). Embryo differentiation of cashew was obtained from callus on media supplemented with 1.0 mg/l NAA and 0.5 mg/l kinetin or BA (Lakshmisita, 1994).

2.2.3.3. Maturation

Litz *et al.* (1993), Jaiswal (1990), Litz and Gray (1992) and Razdan (1993) reported that maturation of mango somatic embryos occurred on medium without growth regulators.

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 embryogenesis in citrus (Kochba *et al.*, 1974) and *Santalum album* (Lakshmisita *et al.*, 1979). Razdan (1993) observed that

high auxin levels inhibited the development and growth of shoot meristem of embryoids.

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).

The endogenous concentration of ABA in embryogenic cultures showed a rapid increase during somatic embryo development and reached a peak at maturation (Rajasekharan *et al.*, 1982). Pence (1991) demonstrated that the endogenous level of ABA in developing *Theobroma cacao* L. recalcitrant zygotic embryos reaches maximum early in maturation phase of development, resembling the pattern in orthodox embryos.

ABA 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).

In experiments on Norway Spruce, Von Arnold and Hakman (1988) showed that addition of cytokinin to ABA containing medium reduced the yield of somatic embryos. Bozhkov *et al.* (1992) obtained a sharp increase of recovery of somatic embryos after one or two subcultures on medium supplemented with ABA and BA.

Dewald *et al.* (1989b) reported that the use of ABA was effective for maturation of mango somatic embryos at lower concentration of sucrose and could control developmental abnormalities. 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.

Monsalud *et al.* (1995) observed that ABA could reverse hyper hydricity of secondary somatic embryoids of mango and prevent precocious germination. Somatic embryos grown on ABA were smaller when compared to those grown in control, but there was no carry over effect of ABA on germination.

Development of cotyledonary stage of nucellar embryos of mango was arrested *in vitro* by exposure to 750 - 1750 µM ABA (Alfaro *et al.*, 1996). The enlargement and germination of nucellar embryos was inhibited for as long as four weeks after subculture from ABA containing medium.

According to Sulekha (1996) maturation of somatic embryoids of mango variety Neelum was attained when ABA 5.0 mg/l was supplemented in the medium.

An increase in viability, shoot elongation and conversion was observed for the chestnut embryos previously cultured on medium enriched with ABA (80 µM) alone or plus PEG (5.0 g/l) (Capuana and Debergh, 1997)..

Sofiari *et al.* (1997) observed that maturation period for secondary somatic embryos was shorter in NAA medium than in 2,4-D.

According to Ramesh (1998) ABA 3.0 mg/l was highly beneficial for somatic embryo maturation in polyembryonic mango varieties.

2.2.3.4. Germination

Somatic embryos can germinate on agar medium without plant growth substances (Razdan, 1993). Dewald *et al.* (1989b) and Jaiswal (1990) could obtain germination of mango somatic embryos without exogenous application of plant growth substances.

Gibberillin application is found to be beneficial in some cases. Gibberillins can be used for breaking dormancy in somatic embryoids (Kavathekar and Johri, 1978). Exogenously supplied GA_3 caused germination of somatic embryos in *Citrus sinensis* (Rangaswami, 1961) and *Santalum album* (Lakshmisita *et al.*, 1979).

Kavathekar and Johri (1978) reported that cytokinins are sometimes required for the growth of embryos into plantlets. BA was required for the germination in *Quercus accutissima* (Kim *et al.*, 1994). BA was employed for germination in mango by Litz *et al.* (1984) and Jana *et al.* (1994).

Low levels of zeatin (Razdan, 1993) and kinetin (Wachira and Ogada, 1995; Sreenath *et al.*, 1995) were also found useful for

germination of somatic embryoids. BA stimulated both tap root formation and shoot formation (Sofiari *et al.*, 1997). According to him the desiccated secondary somatic embryos required a medium supplemented with BA for high frequency germination.

Hegde (1988) reported that with reduced levels of auxin bipolar differentiation of shoots and roots were observed from cashew embryoids, but there was no further growth into plantlet. The embryoids of cashew produced well developed root system and rudimentary shoots when germinated in medium supplemented with 0.2 mg/l NAA (Lakshmisita, 1994).

2.2.4. Other supplements

2.2.4.1. Coconut water

Coconut water acts as a source of reduced nitrogen in the medium (Tulecke *et al.*, 1961). A substantial amount of reduced form of nitrogen is required for embryo initiation and maturation. Razdan (1993) reported that coconut water or casein hydrolysate could be used as the source of reduced nitrogen.

Homes (1967) reported that coconut water was not a pre requisite for inducing embryogenesis.

In mango, somatic embryogenesis was induced on medium supplemented with 20 per cent coconut water, and it was essential for

normal plantlet development (Litz *et al.*, 1982). According to Dewald *et al.* (1989b) maturation and germination of somatic embryos of mango were achieved by sequential transfer of somatic embryos onto medium containing 20 per cent coconut water. Litz *et al.* (1993) routinely added filter sterilized coconut water to the maturation medium in mango.

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 upto 12 per cent (Lu and Ozias - Akins, 1982). Higher concentration suppressed precocious germination (Ammirato and Steward, 1971). For maturation of somatic embryos the optimum concentration of sucrose was six per cent (Dewald *et al.*, 1989b). Litz *et al.* (1993) reported that sucrose concentration should be maintained at moderately high levels in order to prevent germination of somatic embryoids. Germination of mango somatic embryos occurred at 4.0 per cent of sucrose (Jana *et al.*, 1994). Other carbohydrate such as glucose (Homes, 1967), galactose (Kochba *et al.*, 1978) and soluble starch (Kao and Michayluk, 1981) were also reported to be useful for somatic embryogenesis.

2.2.4.3. Agents for reducing phenolic oxidation

Fridborg *et al.* (1978) showed that activated charcoal could adsorb some phenols commonly produced by wounded tissues. It substantially reduced the level of phenyl acetic acid and para hydroxy benzoic acid which inhibited somatic embryogenesis. It adsorbed 5-hydroxy methyl furfural, an inhibitor formed by the degradation of sucrose while autoclaving, but found to adsorb auxins and cytokinins also (Weatherhead *et al.*, 1978). According to Eriksson (1978) and Henshaw (1978) activated charcoal, along with adsorbing inhibitory growth substances, can also bind useful hormones and other metabolites. In date palm activated charcoal effectively prevented browning of explants from mature trees (Tisserat, 1982).

Activated charcoal hastened the differentiation of somatic embryos in mango (Litz *et al.*, 1984, Litz 1986a). However, the mortality of embryoids was high. Jana *et al.* (1994) obtained matured somatic embryoids of mango when activated charcoal 2.5 g/l was used in the medium. Muralidharan *et al.* (1994) reported the use of 1.0 per cent activated charcoal for somatic embryogenesis in mango. Bindu (1995) and Sulekha (1996) found that charcoal was essential in mango somatic embryogenesis. Browning of tissue and media were severe when cultured in media without activated charcoal.

Jaiswal (1990), Sulekha (1996) reported that polyvinyl pyrrolidone (PVP) was more effective in removing phenolic substances than activated charcoal in mango somatic embryogenesis.

In the case of teak somatic embryogenesis, Gupta *et al.* (1980) observed PVP to be more effective. Hu and Wang (1983) found that treating the explants with PVP, washing them with sterile water and inclusion of activated charcoal in the medium reduced the oxidation of polyphenols because of the adsorption of the oxidation products by these chemicals.

2.2.4.4. Ethylene inhibitors

Ethylene can influence the process of maturation and germination of somatic embryoids. Endogenous ethylene level above a critical concentration can be inhibitory. According to Tisserat and Murashige (1977) ethylene suppressed embryo germination in citrus and carrot cultures. According to them the availability, uptake, evolution and dispersion of various gases can affect somatic embryogenesis. The effect of ethylene inhibitors was studied by Roustan *et al.* (1989). They observed that cobalt chloride at concentrations 10 µM to 50 µM effectively inhibited ethylene production by embryogenic cultures and significantly stimulated somatic embryogenesis in carrot. They also observed that increase in embryo number was proportional to the inhibition of ethylene production.

Sulekha (1996) used cobalt chloride and silver nitrate for inducing normal germination of mango somatic embryoids. According to her cobalt chloride was more effective in inducing near-normal germination

of somatic embryos than silver nitrate. Ramesh (1998) also opined that cobalt chloride 10.0 mg/l was able to initiate normal germination of the mango somatic embryoids. But he could not induce physiological maturity of the somatic embryoids by using ethylene inhibitors.

2.2.4.5. Sodium chloride

Sodium chloride was included in the germination medium as an osmoticum to provide stress for the developing embryoids. Sulekha (1996) reported that near normal growth with proper shoot and root development could be obtained in mango somatic embryoids, when sodium chloride was incorporated into the medium.

2.2.4.6. Glyphosate

According to Gowda and Prakash (1998), the herbicide glyphosate appears to promote the somatic embryo production in sweet potato by hastening the process of the maturation, germination, and conversion of embryos, possibly by promoting desiccation, an essential step for embryo quiescence. Somatic embryos developed earlier and matured faster on explants cultured on glyphosate (200 µM, 100 µM) than those cultured on ABA alone as control.

2.2.5. 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 (Tulecke, 1987). According to Simola (1987), the poor ability of regeneration in tree cultures is probably due to improper culture conditions.

2.2.5.1. Light and Darkness

The requirement of light for somatic embryogenesis vary 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 embryogenesis in mango occurred in suspension cultures kept in darkness (Dewald *et al.*, 1989a).

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

1993). According to Litz *et al.* (1993) germination of mango somatic embryoids occurred when somatic embryos were transferred to light.

Jha (1988) reported that induction of callus from explants of cashew occurred only in complete darkness. Explants exposed to 16 hours photoperiod did not form calli. The calli growing in complete darkness produced globular protuberances. This globular protuberances / embryoids turn green when subjected to subsequent exposure to light.

Keeping the cultures for callus induction in complete darkness for three weeks followed by 26 $\pm$ 2°C with 14 hours photoperiod with 1500 lux intensity had a profound effect on callusing from immature cotyledon bits of cashew (Thomas, 1995).

According to Kesavachandran (1998) more profuse callusing as well as higher percentage were obtained when the cultures of cashew were incubated in the dark.

2.2.5.2. Temperature

Yeoman (1986) found that the environmental temperature at the original habitat of a particular species should be taken into consideration while fixing the culture temperature under *in vitro* condition.

Somatic embryogenesis has been reported to occur at relatively higher temperature. Embryogenic potential was reduced in citrus cultures

when temperature was lowered from 27°C to 12°C (Ammirato, 1983). A temperature of 27°C was seen ideal for coffee (Michaux - Ferriere and Dublin, 1988) and eucalyptus (Muralidharan *et al.*, 1994).

Induction of somatic embryogenesis in mango was reported to occur when incubated at 25°C (Litz *et al.*, 1991). Somatic embryoids in maturation medium were incubated at 24°C to 26°C and germination occurred at 25°C (Dewald *et al.*, 1989b).

Bindu (1995) and Sulekha (1996) also reported that when the cultures were kept under darkness at a temperature of 26 ± 2°C, favoured somatic embryogenesis in mango.

2.2.6. Mode of culture

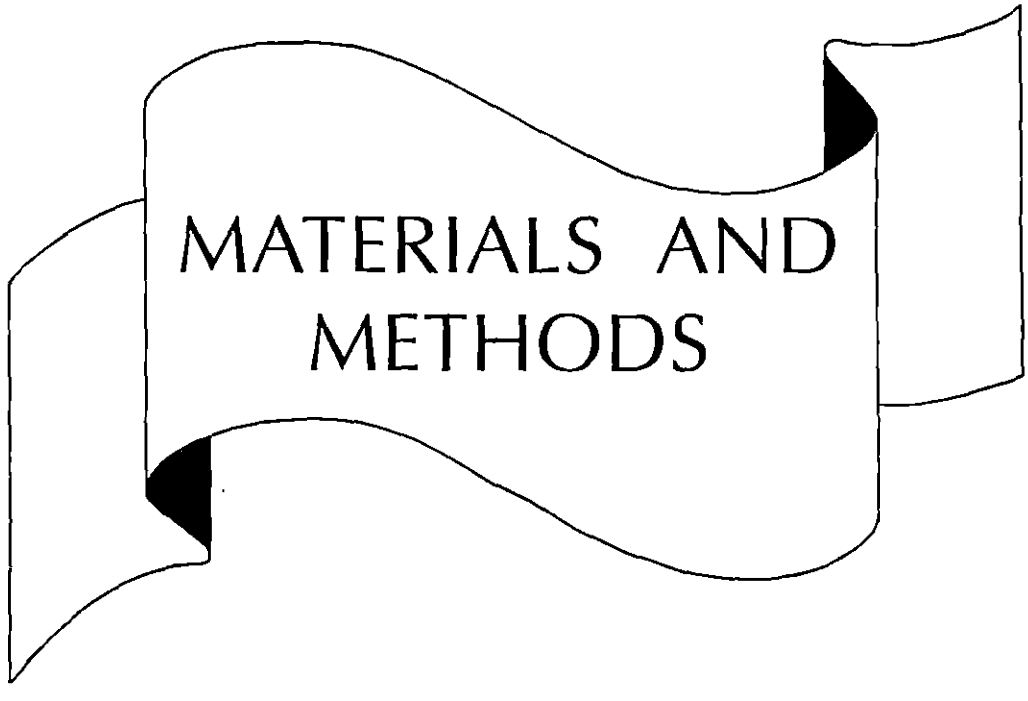
Somatic embryogenesis in mango occured in liquid medium (Litz *et al.*, 1982). The growth of callus was more rapid in liquid medium though it could be maintained on solid medium also (Litz, 1986a). Only fewer somatic embryoids developed on solid medium, but they were unable to advance to maturity (Litz *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, subculturing 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).

2.2.7. Frequency of subculture

Litz (1986a) reported that frequent subculturing was necessary to counteract polyphenol interference in mango somatic embryogenesis. Subculturing at shorter intervals was also found to be beneficial during somatic embryogenesis in apple (Paul *et al.*, 1994). Bindu (1995) and Sulekha (1996) observed that frequent subculturing was necessary to increase *in vitro* response of mango somatic embryoids.


MATERIALS AND
METHODS

3. MATERIALS AND METHODS

Investigations on improvement of *in vitro* somatic embryogenesis in cashew (*Anacardium occidentale* L.) were carried out at the Plant Tissue Culture Laboratory, Department of Horticulture, College of Agriculture, Vellayani during 1996-1999.

The materials used and the methods tried for the various stages of *in vitro* somatic embryogenesis namely, induction, initiation, maturation and germination of the somatic embryoids are described in this chapter.

3.1 Varieties

Local varieties of cashew grown in and around Vellayani were used in this study.

3.2 Explants

Nucellus and embryo mass from the ovules of tender cashew nuts were used. Embryo mass / cotyledons of six to eight weeks old nuts and nucellus of four to six weeks old nuts were used as explants (Plate 1).

3.2.1 Nucellus

In the ovule primordium, be it in angiosperm or gymnosperm, initially nucellus is the only recognizable component. Nucellus represents the wall of the megasporangium and is a nourishing tissue for the developing embryo. The nucellus grows by cell division and enlargement and appears as a clear watery mass enclosed within the ovule walls (integuments). Later as the embryo develops, the nucellus is digested or reduced to a remnant between the embryo and the integuments. Generally the nucellus remains within the confines of the inner integument. Rarely it projects into the micropyle or beyond forming a nucellar beak. As it is part of the mother plant and has the same ploidy it can be used for clonal propagation.

3.2.2 Cotyledons

The fertilized egg is called zygote. Following a predetermined mode of development, it gives rise to an embryo, which has the potential to form a complete plant. Irrespective of the mode of development, a mature embryo generally possesses an embryonic root (radicle), an embryonic shoot (plumule) and one or two cotyledons. Thus cotyledons are the first formed leaves, with a store of energy for the developing plant.

3.3 Collection and preparation of explants

Tender nuts (30-45 days and 45-60 days after fertilization) were collected from healthy cashew trees for taking nucellus and cotyledons respectively.

The nuts after removing the peduncle were washed out thoroughly in tap water with a few drops of the wetting agent labolene, followed by washing with double glass distilled water.

3.4 Surface sterilization

Surface sterilization of the plant materials was carried out inside a laminar air flow chamber. The nuts after initial cleaning were transferred to sterile beaker and surface sterilized using 70 per cent ethyl alcohol for ten minutes. After draining the alcohol, the nuts were washed with sterile double glass distilled water. They were then treated with freshly prepared sodium hypochlorite (1.0 per cent) for 45 min. This was followed by washing four to five times with sterile double glass distilled water.

3.5 Inoculation and incubation

The tools (needles, blades, forceps etc.) and the glasswares required for inoculation were washed thoroughly, rinsed with double glass distilled water, covered with aluminium foil and autoclaved at 121°C and 1.06 kg/cm^2 pressure for 40 min.

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

The distal one third portion of the surface sterilised nuts were cut open using dissection blade and cotyledons were pulled out using

Plate 1. Tender cashew nuts used as explant

Plate 2. Tender fruit of cashew and ovule dissected out showing the nucellar tissue

forceps. The tegmen was removed and the cotyledons were transversely cut into three-four segments. To culture the nucellus, the nuts were cut open without injuring the ovule, the ovule was taken out with forceps and cut longitudinally into two equal halves (Plate 2). After removing the embryo, nucellar tissue with a portion of the ovule was used for inoculation. The cotton plugs of the test tubes / Erlenmeyer flasks were removed and the rim was flamed. The nucellus and cotyledon were then inoculated on to the medium separately. The rim of the culture vessels was again flamed and closed with cotton plugs. The cultures were incubated either in light or in darkness as per the treatment.

3.6 Media

The basal media used for the study were MS (Murashige and Skoog, 1962), B_5 (Gamborg *et al.*, 1968), SH (Schenk and Hildebrandt, 1972) and Y_3 (Eeuwens and Blake, 1977). The chemicals used for the preparation of the culture media were of analytical grade from Sisco Research Laboratory, Bombay; Merck, Bombay; Sigma, U.S.A. and British Drug House, Bombay.

Standard procedures were followed for the preparation of the Basal media (Thorpe, 1980). Stock solutions of major and minor nutrients, amino acids and plant growth substances were prepared by dissolving the required quantity of chemicals in specific volume of double glass distilled water and were stored under refrigerated conditions (4°C).

The glasswares used for the preparation of the media were washed with diluted labolene and rinsed with double glass distilled water. Specific quantities of the stock solutions were pipetted out into 1000 ml beaker. Sucrose, glutamine, inositol were added fresh and dissolved. Coconut water collected from freshly harvested tender coconut (eight months old) was added and the volume made upto 950 ml, using double glass distilled water. With an electronic pH meter (Philips make, model PP 9046), the pH value of the solution was adjusted between 5.6 and 5.8 using 0.1 N NaOH or 0.1 N HCl. Agar (in the case of solid medium) was then added to the medium and the final volume was made upto 1000 ml. Agar was not added in the case of liquid medium.

The solution was then heated by placing the beaker on a heating mantle and stirred throughly for uniform mixing, till agar melted. Activated charcoal, when used in the medium, was added at this stage. The medium was poured to pre-sterilized culture vessels which were rinsed with double glass distilled water. Corning brand test tubes (25 x 150 mm) and Erlenmeyer Flasks (100 ml) were used as culture vessels. The test tubes and Erlenmeyer flasks were filled with 15 ml and 30 ml of the medium respectively. The culture vessels containing the medium were plugged tightly with cotton. They were then autoclaved at 121°C and 1.06 kg/cm^2 pressure for 20 min.

3.7 Somatic embryogenesis

3.7.1 Induction of somatic embryoids

3.7.1.1 Treatments

Explants were subjected to different treatments for induction of somatic embryogenesis. The treatments involved different levels of plant growth substances, sucrose, coconut water, activated charcoal, maltose, glutamine, polyvinyl pyrrolidone (PVP), yeast extract, casein hydrolysate and adenine sulphate. Different basal media like full as well as half strength of major salts of MS, SH and Y_3 with supplements were tried in inducing somatic embryogenesis. Thus twenty four treatments were tried for evaluating their ability to induce somatic embryoids (Table 1). The treatments were replicated 30 times.

Observations were recorded on the number of cultures initiating callus from nucellus as well as from cotyledons. Callus index (CI) was computed by multiplying per cent cultures initiating callus with growth score (G). Growth of the callus was assessed based on the visual rating (with score 1.0 to the smallest and score 4.0 to the largest). The mean score was expressed as growth score G. (Poor - 1, Medium - 2, Good - 3, Profuse - 4).

3.7.1.2 Culture conditions

Studies were conducted to find out the effect of light on the induction of somatic embryoids. Darkness was provided by placing the

Table 1. Treatments tried for the induction of embryogenic callus / somatic embryoids from nucellus and embryo mass of cashew

No.	Treatment
T_1	1/2 MS + 2,4-D 1.0 mg/l + NAA 1.0 mg/l + BA 1.0 mg/l + Sucrose 60.0 g/l + antibiotic (Norflox) 400.0 mg/l + agar 6.0 g/l
T_2	1/2 MS + 2,4-D 1.0 mg/l + GA 5.0 mg/l + BA 1.0 mg/l + antibiotic 400.0 mg/l + Sucrose 60.0 g/l agar 6.0 g/l
T_3	1/2 MS + 2,4-D 5.0 mg/l + NAA 1.0 mg/l + BA 1.0 mg/l + Sucrose 60.0 g/l + antibiotic 400.0 mg/l + agar 6.0 g/l
T_4	1/2 MS + BA 2.0 mg/l + GA 10.0 mg/l + glutamine 100.0 mg/l + CW 200.0 ml/l + AC 2.5 g/l + Sucrose 60.0 g/l + agar 7.0 g/l
T_5	1/2 MS + 2,4-D 1.0 mg/l + BA 1.0 mg/l + NAA 1.0 mg/l + Sucrose 30.0 g/l + AC 0.05 % + agar 6.0 g/l
T_6	1/2 MS + 2,4-D 1.0 mg/l + BA 1.0 mg/l + NAA 1.0 mg/l + Sucrose 30.0 g/l + PVP 250.0 mg/l + agar 6.0 g/l
T_7	1/2 MS + 2,4-D 1.0 mg/l + BA 1.0 mg/l + NAA 1.0 mg/l + CW 200.0 ml/l + CH 400.0 mg/l + Sucrose 40.0 g/l + AC 0.05 % + agar 6.0 g/l
T_8	1/2 MS + 2,4-D 2.0 mg/l + NAA 1.0 mg/l + BA 2.0 mg/l + CW 200.0 ml/l + CH 400.0 mg/l + AC 0.05 % + Sucrose 40.0 g/l + agar 6.0 g/l
T_9	1/2 MS + 2,4-D 1.0 mg/l + NAA 1.0 mg/l + BA 1.0 mg/l + CW 150.0 ml/l + CH 400.0 mg/l + AC 0.05 % + Sucrose 40.0 g/l + agar 6.0 g/l
T_{10}	1/2 MS + BA 2.0 mg/l GA 10.0 mg/l + glutamine 200.0 mg/l + CW 200.0 ml/l + AC 2.5 g/l + Sucrose 60.0 g/l + agar 7.0 g/l
T_{11}	SH + 2,4-D 1.0 mg/l + BA 1.0 mg/l + NAA 1.0 mg/l + glutamine 100.0 mg/l + Sucrose 30.0 g/l + agar 6.0 g/l

Table 1 (Contd...)

No.	Treatment
T_{12}	SH + 2,4-D 1.5 mg/l + BA 1.0 mg/l + NAA 1.0 mg/l + glutamine 100.0 mg/l + Sucrose 30.0 g/l + agar 6.0 g/l
T_{13}	Y_3 + 2,4-D 20.0 mg/l + NAA 10.0 mg/l + silver nitrate 5.0 mg/l + Sucrose 40.0 g/l + AC 10.0 g/l + agar 5.0 g/l
T_{14}	MS + BA 5.0 mg/l + Maltose 5.3 g/l + Sucrose 40.0 g/l + agar 6.0 g/l
T_{15}	MS + BA 2.0 mg/l + Maltose 5.3 g/l + Sucrose 60.0 g/l + agar 6.0 g/l
T_{16}	MS + BA 1.0 mg/l + GA 1.0 mg/l + Sucrose 30.0 g/l + agar 5.0 g/l
T_{17}	MS + 2,4-D 1.0 mg/l + BA 1.0 mg/l + NAA 1.0 mg/l + Sucrose 30.0 g/l + PVP 250.0 mg/l + agar 6.0 g/l
T_{18}	MS + BA 2.0 mg/l + GA 2.0 mg/l + Sucrose 30.0 g/l + agar 6.0 g/l
T_{19}	MS + BA 1.0 mg/l + GA 2.0 mg/l + Sucrose 30.0 g/l + agar 6.0 g/l
T_{20}	MS + BA 10.0 mg/l + GA 10.0 mg/l + Sucrose 60.0 g/l + agar 6.0 g/l
T_{21}	MS + BA 10.0 mg/l + GA 2.0 mg/l + Sucrose 60.0 g/l + agar 6.0 g/l
T_{22}	MS + BA 1.0 mg/l + 2,4-D 1.0 mg/l + NAA 1.0 mg/l + Sucrose 30.0 g/l + AC 0.05 % + agar 6.0 g/l
T_{23}	MS + inositol 1 mg/l + sucrose 30.0 g/l + PVP 250.0 mg/l + NAA 0.5 mg/l + kinetin 2.0 mg/l + adenine sulphate 40.0 mg/l + CH 200.0 mg/l + agar 5.5 g/l
T_{24}	MS + sucrose 30.0 g/l + NAA 4.0 mg/l + 2,4-D 4.0 mg/l + kinetin 4.0 mg/l + adenine sulphate 40.0 mg/l + PVP 250.0 mg/l + yeast extract 200.0 mg/l + agar 5.5 g/l

cultures in culture racks covered with black polythene sheets and by covering the culture vessels with aluminium foil. Light (photoperiod 16 hr) was provided by fluorescent tubes, giving an intensity of 3000 lux. The influence of temperature on induction of embryogenic callus was studied by keeping the culture inside the culture room (26 ± 2°C) and outside the culture room at ambient temperature (32 ± 2°C).

Observations

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

3.7.2 Initiation of somatic embryoids

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

3.7.2.1 Treatments

Fourteen treatments were tried for the initiation of somatic embryoids (Table 2). The treatments were replicated 15 times. The strength of the MS basal medium (full and half) was used. The treatments involved different levels and combinations of plant growth substances, sucrose, activated charcoal, PVP, yeast extract, adenine sulphate, glutamine, maltose, casein hydrolysate and coconut water.

Table 2. Treatments tried for the initiation of somatic embryoids from nucellus and embryo mass of cashew

No.	Treatment
I_1	1/2 MS + GA 5.0 mg/l + glutamine 400.0 mg/l + Sucrose 60.0 g/l + CH 500.0 mg/l + AC 2.5 g/l + CW 200.0 ml/l + Agar 6.0 g/l
I_2	1/2 MS + BA 1.0 mg/l + 2,4-D 2.0 mg/l GA_3 5.0 mg/l + Sucrose 60.0 g/l + CW 200 ml/l + glutamine 400.0 mg/l + AC 2.5 g/l + CH 500.0 mg/l + Agar 6.0 g/l
I_3	1/2 MS + BA 0.5 mg/l + IAA 2.0 mg/l + glutamine 400.0 mg/l + Sucrose 60.0 g/l + Agar 6.0 g/l
I_4	MS + NAA 0.5 mg/l + kinetin 2.0 mg/l + $AdSO_4$ 40.0 mg/l + yeast extract 200.0 mg/l + PVP 250.0 mg/l + Sucrose 30.0 g/l + Agar 5.5 g/l
I_5	1/2 MS + BA 1.0 mg/l + IAA 2.0 mg/l + glutamine 400.0 mg/l + Sucrose 60.0 g/l + Agar 6.0 g/l
I_6	1/2 MS + 2,4-D 4.0 mg/l + GA 5.0 mg/l + glutamine 400.0 mg/l + sucrose 60.0 g/l + agar 6.0 g/l
I_7	MS + BA 5.0 mg/l + Maltose 5.3 g/l + Sucrose 40.0 g/l + Agar 6.0 g/l
I_8	MS + BA 2.0 mg/l + Maltose 5.3 g/l + Sucrose 60.0 g/l + Agar 6.0 g/l
I_9	MS + BA 1.0 mg/l + GA 1.0 mg/l + Sucrose 30.0 g/l + Agar 5.0 g/l
I_{10}	1/2 MS + BA 1.0 mg/l + NAA 1.0 mg/l + Sucrose 30.0 g/l + AC 0.05 % + Agar 6.0 g/l
I_{11}	1/2 MS + 2,4-D 0.5 mg/l + NAA 0.5 mg/l + BA 0.5 mg/l + glutamine 400.0 mg/l + Sucrose 60.0 g/l + Agar 6.0 g/l
I_{12}	1/2 MS + 2,4-D 1.0 mg/l + NAA 1.0 mg/l + BA 1.0 mg/l + CW 200.0 ml/l + CH 400.0 mg/l + AC 0.05 % + Sucrose 40.0 g/l + Agar 6.0 g/l
I_{13}	1/2 MS + 2,4-D 0.5 mg/l + NAA 1.0 mg/l + BA 1.0 mg/l + CW 200.0 ml/l + CH 400.0 mg/l + AC 0.05 % + Sucrose 40.0 g/l + Agar 6.0 g/l
I_{14}	1/2 MS + glutamine 400 mg/l + CH 500.0 mg/l + Sucrose 60.0 g/l + CW 200 ml/l + AC 2.5 g/l + Agar 5.5 g/l

3.7.2.2 Culture conditions

The effect of light and temperature on the initiation of somatic embryoids was studied. Light was provided by keeping the cultures under the fluorescent tube lights at an intensity of 3000 lux. In order to provide darkness, culture vessels were kept in culture racks covered with black polythene sheets. The influence of temperature was studied by keeping the cultures inside the culture room (26 $\pm$ 2°C) and outside the culture room at room temperature.

Observations

Observations on the number of cultures initiating somatic embryoids from nucellus as well as embryo mass were recorded, four weeks after incubation.

3.7.3 Maturation of somatic embryoids

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

3.7.3.1 Treatments

For getting proper maturation of somatic embryoids, the somatic embryoids from initiation media were transferred to different basal media with supplements and their effect was studied. Then the response of various supplements in the maturation medium were tried.

Four treatments were tried to assess the effect of ABA, three each for sucrose and coconut water. Four treatments were tried to study the effect of ethylene inhibitors and three, for glyphosate. The treatments were replicated 6-15 times.

3.7.3.1.1 Basal media

The embryoids from initiation media were transferred to maturation media. Basal media such as MS, 1/2 MS, B_5 and B_5 major salts + MS minor salts were tried.

3.7.3.1.2 Abscisic acid

The somatic embryoids from the initiation medium were transferred to the maturation medium containing various concentrations of ABA (1.0, 2.0, 5.0 and 10.0 mg/l).

3.7.3.1.3 Sucrose

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

3.7.3.1.4 Coconut water

Different levels of coconut water (100.0, 150.0 and 200.0 ml/l)

were included in the medium to observe their effects on the maturation of somatic embryoids.

3.7.3.1.5 Glyphosate

The influence of glyphosate was tested by incorporating them in various concentrations (100 µM and 200 µM) along with the ABA.

3.7.3.1.6 Ethylene inhibitors

Ethylene inhibitors like cobalt chloride and silver nitrate at concentrations of 5.0 and 10.0 mg/l were tried.

3.7.3.2 Culture conditions

The performance of somatic embryoids was assessed in the presence and absence of light. Light (3000 lux, 16 hr photo period) was provided using cool white fluorescent tubes. Darkness was provided by keeping the cultures in a temperature controlled (26 $\pm$ 2°C) darkroom.

3.7.3.3 Frequency of subculture

Somatic embryoids were subcultured at five days, ten days and fifteen days interval in the same media.

3.7.3.4 Mode of culture

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

Observations

The effects of various treatments were observed over a period of four weeks. Observations were made on the percentage of cultures survived and the increment in size and colour of embryoids.

3.7.4 Germination of somatic embryoids

3.7.4.1 Treatments

Various basal media were tried for the germination of somatic embryoids. Eleven treatments involving plant growth substances (BA, GA_3, kinetin), four treatments with coconut water, three treatments with sucrose, three treatments each with sodium chloride and cobalt chloride and two treatments with silver nitrate, were tried for attaining normal germination of somatic embryoids. The treatments were replicated six times.

3.7.4.1.1 Basal media

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

3.7.4.1.2 Plant growth substances

The influence of plant growth substances like BA, GA_3 and kinetin

were tested by incorporating them alone and in various combinations. Eleven treatments were tried (Table 3).

Table 3. Treatments tried to assess the effect of plant growth substances on the germination of cashew somatic embryoids

Treatment No.	Plant growth substances (mg/l)
G_1	BA 0.05
G_2	BA 0.1
G_3	BA 0.5
G_4	BA 1.0
G_5	GA_3 1.0
G_6	GA_3 5.0
G_7	GA_3 10.0
G_8	kinetin 0.1
G_9	kinetin 0.2
G_{10}	BA 0.1 + GA_3 1.0
G_{11}	BA 0.1 + GA_3 5.0

Culture medium:

B_5 major salts + MS minor salts + sucrose 40.0 g/l + PVP 10.0 g/l + agar 5.0 g/l

3.7.4.1.3 Coconut water

Coconut water was tried at 0, 100.0, 150.0 and 200.0 ml/l in the germination medium.

3.7.4.1.4 Osmotic regulants

As an osmoticum, sucrose was incorporated into the medium at 40.0, 50.0 and 60.0 g/l. Different levels of sodium chloride (0.05, 0.10, 0.20 per cent were also supplemented in the medium as an osmotic regulant, to the medium to study their effects on germination.

3.7.4.1.5 Ethylene inhibitors

Cobalt chloride at concentrations of 5.0, 10.0, 15.0 mg/l and silver nitrate at 5.0, 10.0 mg/l were incorporated into the medium to study their effects on the germination.

3.7.4.2 Culture condition

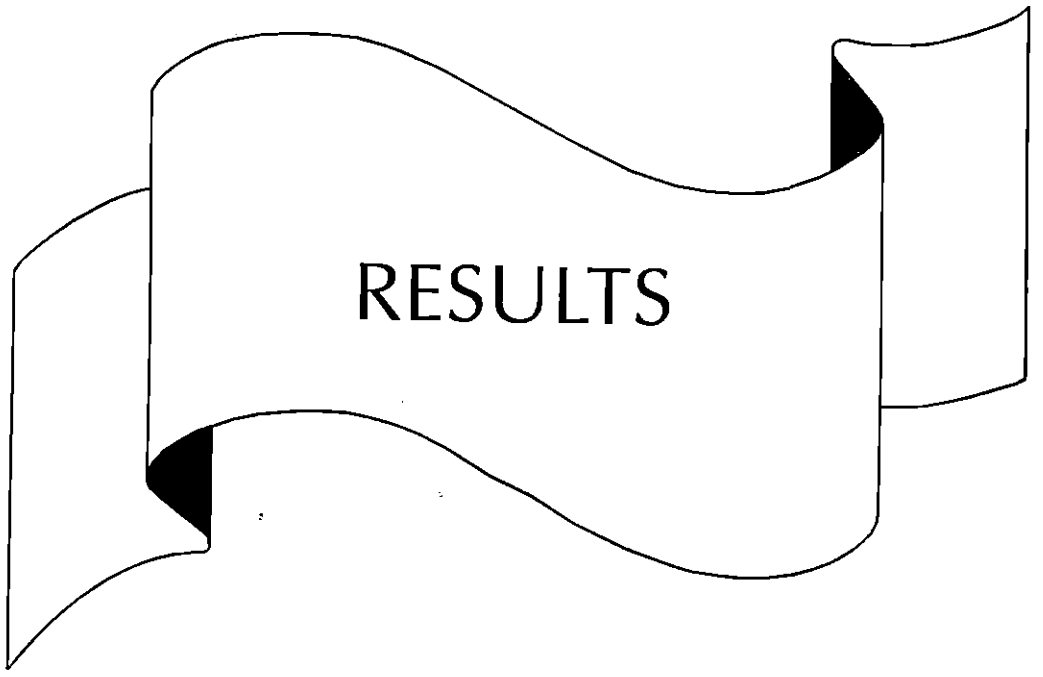
The cultures were kept in light (3000 lux, 16 hr photoperiod) provided by white fluorescent tubes or in darkness in order to assess the effect of light on the germination of somatic embryoids.

3.7.4.3 Frequency of subculture

Somatic embryoids were subcultured in the same media at an interval of five days, ten days and fifteen days.

Observations

Observations were recorded on the germination of somatic embryoids, formation of root and shoot, colour and abnormalities of the embryoids.


RESULTS

4. RESULTS

The results of the investigations carried out for improving somatic embryogenesis in cashew are presented in the following pages.

4.1 Explants

Two types of explants namely, nucellus and embryo mass were used to study the response of *in vitro* somatic embryogenesis in cashew. Out of these two explants tried, nucellus responded better than embryo mass for initiating embryogenic callus, when cultured in the same media in all the treatments tried. (Table 4).

Nucellar tissue initiated callus in 86.6 per cent cultures, where as embryo mass initiated callus in 26.6 per cent cultures only, when inoculated in half strength MS basal medium supplemented with 2,4.D 1.0 mg/l, BA 1.0mg/l + NAA 1.0 mg/l and sucrose 30.0 g/l + AC 0.5 g/l + agar 6.0 g/l. High growth score of 2.96 was recorded by nucellar tissue in this treatment, whereas embryo mass showed a poor callus index (59.85) with a growth score of 2.25. When inoculated in MS basal medium supplemented with BA 2.0 mg/l, maltose 5.3 g/l, sucrose 60.0 g/l, agar 6.0 g/l, (T_4) and NAA 0.5 mg/l kinetin 2.0 mg/l, adenine sulphate

Table 4. Response of different explants and treatments in inducing embryogenic callus

Treatments	Cultures initiating embryogenic callus from nucellus (%)	Growth score	Callus index	Cultures initiating embryogenic callus from embryo mass (%)	Growth score	Callus index
T_1	86.6	2.96	256.34	26.6	2.25	59.85
T_2	63.3	2.94	186.10	13.3	2.0	26.6
T_3	73.3	2.77	203.04	40.0	1.83	73.2
T_4	76.6	2.69	206.05	53.0	1.75	92.75

The data represent the average values of 30 replications.

T_1 1/2 MS + 2,4-D 1.0 mg/l + BA 1.0 mg/l + NAA 1.0 mg/l + Sucrose 30.0 g/l + AC 0.05% + agar 6.0 g/l

T_2 1/2 MS + 2,4-D 1.0 mg/l + BA 1.0 mg/l + NAA 1.0 mg/l + Sucrose 30.0 g/l + PVP 250.0 mg/l + agar 6.0 g/l

T_3 MS + BA 5.0 mg/l + Maltose 5.3 g/l + Sucrose 40.0 g/l + agar 6.0 g/l

T_4 MS + BA 1.0 mg/l + GA 1.0 mg/l + Sucrose 30.0 g/l + agar 5.0 g/l

4.0 g/l, yeast extract 200.0 mg/l, PVP 250.0 mg/l, sucrose 30.0 g/l, agar 5.5 g/l (T_5), 76.6 per cent cultures initiated embryogenic callus from nucellus. But the callus index was higher in T_4 (206.05) than in T_5, recording a callus index of 191.5. The embryo mass when cultured in T_4 and T_5 gave only 53.0 and 63.3 per cent initiation of embryogenic callus, respectively.

4.2 Induction of somatic embryogenesis

4.2.1 Treatments

Twenty four treatments involving various concentrations of plant growth substances, glutamine, sucrose, activated charcoal, coconut water and agar were tried for the induction of somatic embryogenesis.

The best treatment for initiating embryogenic callus from nucellar tissue was half strength MS basal medium supplemented with 2,4-D 1.0 mg/l + BA 1.0 mg/l + NAA 1.0 mg/l + sucrose 30.0 g/l + AC 0.5 g/l + agar 6.0 g/l (Plate 3). The treatment recorded 86.6 per cent initiation of embryogenic callus from nucellus. The maximum growth score of 2.96 and callus index value of 256.34 were recorded by this treatment. The treatments T_7, T_9, T_{15}, T_{16} and T_{24} recorded 76.6 per cent response from nucellus. These treatments had good callus index with high growth score (Table 5).

The treatments T_6, T_8, T_{10}, T_{11}, T_{13}, T_{17} also responded. The least response from nucellus was recorded by the treatment T_1 (23.3 %) with a low growth score of 1.3.

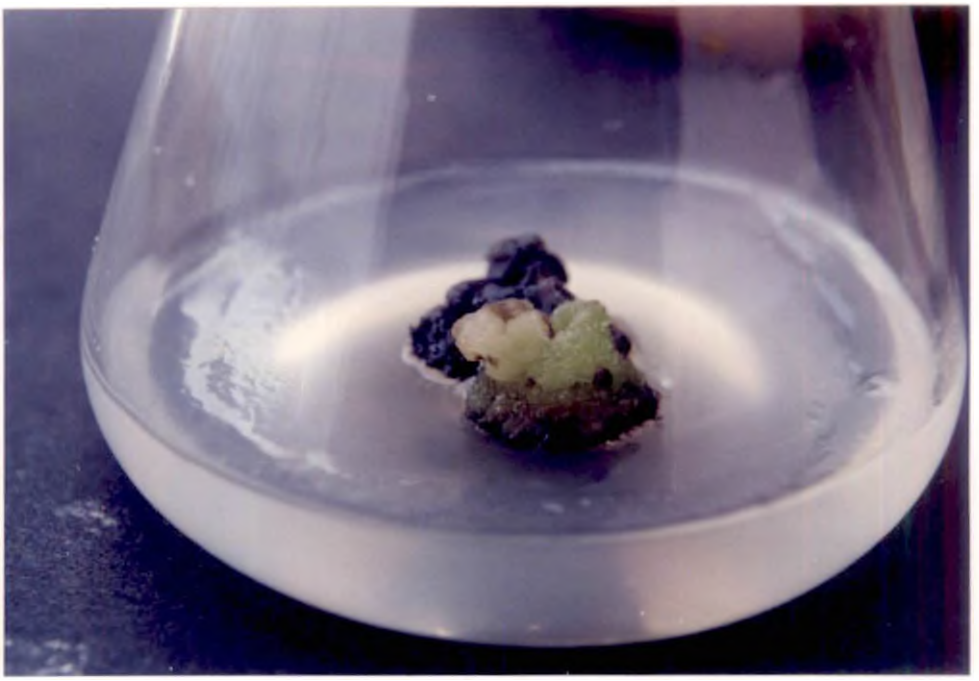
Table 5. Effect of culture media on the induction of embryogenic callus / somatic embryoids from nucellus of cashew

Treatment	Cultures initiating embryogenic callus/ somatic embryoids (%)	Growth score	Callus index
T_1	23.3	1.38	32.15
T_2	53.3	1.31	69.82
T_3	40.0	1.66	66.4
T_4	40.0	1.25	50.0
T_5	86.6	2.96	256.34
T_6	63.3	2.94	186.10
T_7	76.6	2.95	225.97
T_8	66.6	2.8	186.48
T_9	76.6	2.7	206.82
T_{10}	60.0	1.83	109.80
T_{11}	63.3	1.89	119.64
T_{12}	50.0	2.1	105.00
T_{13}	66.6	1.95	129.87
T_{14}	73.3	2.77	203.04
T_{15}	76.6	2.65	202.99
T_{16}	76.6	2.69	206.05
T_{17}	60.0	1.8	108.00
T_{18}	26.6	1.62	43.09
T_{19}	46.6	1.42	66.17
T_{20}	26.6	1.42	37.77
T_{21}	26.6	1.25	33.25
T_{22}	53.3	1.5	79.95
T_{23}	73.3	2.8	233.24
T_{24}	76.6	2.5	191.5

The data represent the average value of 30 replications. Treatment details given in Table 1.

Plate 3. Induction of embryogenic callus from nucellus

Plate 4. Induction of embryogenic callus from embryo mass



The treatment T_{24} was the best for initiating embryogenic callus from embryo mass (Table 6). In this treatment 63.3 per cent cultures initiated embryogenic callus with a growth score of 2.47 and callus index of 156.35 (Plate 4).

The treatments T_{16}, T_{14} and T_{15} showed better response in initiating embryogenic callus from embryo mass. Least response was observed in the treatment T_6 which recorded initiation only in 13.3 per cent cultures.

T_1, T_2, T_3, T_4, T_{10}, T_{17}, T_{18}, T_{19}, T_{20} and T_{21} did not respond at all, in initiating embryogenic callus from embryo mass.

4.2.2 Culture conditions

Darkness combined with low temperature was found to be ideal for induction of somatic embryogenesis, both from nucellus and embryo mass. When the cultures were kept under darkness plus low temperature (26 $\pm$ 2°C), 80.0 per cent cultures initiated embryogenic callus from nucellus and 50.0 per cent cultures from embryo mass (Table 7). When the cultures were kept under light (3000 lux, 16 hr photoperiod) at low temperature, 60.0 per cent cultures initiated embryogenic callus from nucellus and 33.3 per cent from embryo mass. In dark conditions plus room temperature (32 $\pm$ 2°C), the per cent cultures initiating embryogenic callus was only 20.0 from nucellus whereas none of the cultures of embryo mass responded. In culture conditions of light and room temperature, embryogenic callus did not initiate either from the nucellus or the embryo mass.

Table 6. Effect of culture media on the induction of embryogenic callus / somatic embryoids from embryo mass of cashew

Treatment	Cultures initiating embryogenic callus/ somatic embryoids (%)	Growth score	Callus index
T_1	—	—	—
T_2	—	—	—
T_3	—	—	—
T_4	—	—	—
T_5	26.6	2.25	59.85
T_6	13.3	2.0	26.6
T_7	—	—	—
T_8	—	—	—
T_9	—	—	—
T_{14}	40.0	1.83	73.2
T_{15}	40.0	1.66	66.4
T_{16}	53.0	1.75	92.75
T_{22}	33.3	1.8	59.94
T_{24}	63.3	2.47	156.35

The data represent the average value of 30 replications.

Treatment details given in Table 1.

Table 7. Effect of culture conditions on the induction of embryogenic callus / somatic embryoids from nucellus and embryo mass

	Culture condition	Cultures initiating embryogenic callus / somatic embryoids	
		from nucellus (%)	from embryo mass (%)
1	Light (3000 lux, 16 hr photoperiod) + low temperature (26 ± 2°C)	60.0	33.3
2.	Light (3000 lux, 16 hr photoperiod) + room temp. (32 ± 2°C)	0.0	—
3.	Darkness + low temp. (26 ± 2°C)	80.0	50.0
4.	Darkness + room temp. (32 ± 2°C)	20.0	—

Culture medium:

MS + BA 2.0 mg/l + Maltose 5.3 g/l + Sucrose 60.0 g/l + agar 6.0 g/l

The data represent the average value of 6 replications.

4.3 Initiation of somatic embryoids

4.3.1 Treatments

The explants from the induction media were subjected to fourteen treatments for the initiation of somatic embryoids (Plate 5 and 6). The highest percentage initiation of somatic embryoids (60.0) was obtained when the embryogenic callus of nucellus was subcultured in MS basal media supplemented with NAA 0.5 mg/l, kinetin 2.0 mg/l, adenine sulphate 40.0 mg/l, yeast extract 200.0 mg/l, PVP 250.0 mg/l, sucrose 30.0 g/l, agar 5.5 g/l (I_4) followed by half strength MS basal medium supplemented with 2,4-D 0.5 mg/l + NAA 0.5 mg/l + BA 0.5 mg/l + glutamine 400.0 mg/l + sucrose 60.0 g/l + agar 6.0 g/l (I_{11})

In I_{11} initiation medium, 53.3 per cent initiation of somatic embryoids was noticed. When the embryogenic callus was subcultured in I_3 and I_6 media, initiation of somatic embryoids did not occur (Table 8).

In initiating somatic embryoids from embryo mass also, the treatment I_4 was found to be the best. 60.0 per cent cultures initiated somatic embryoids in this treatment followed by I_9 (46.6 per cent) and I_{11} (46.6 per cent). There was no initiation of somatic embryoids in the treatments I_1, I_2, I_3, I_5, I_{12} and I_{13}. Only few somatic embryoids were found initiated in I_6, I_7, I_{10} and I_{14} treatments (Table 9).

Plate 5. Initiation of somatic embryoids from the embryogenic callus of nucellus

Plate 6. Initiation of somatic embryoids from the embryogenic callus of nucellus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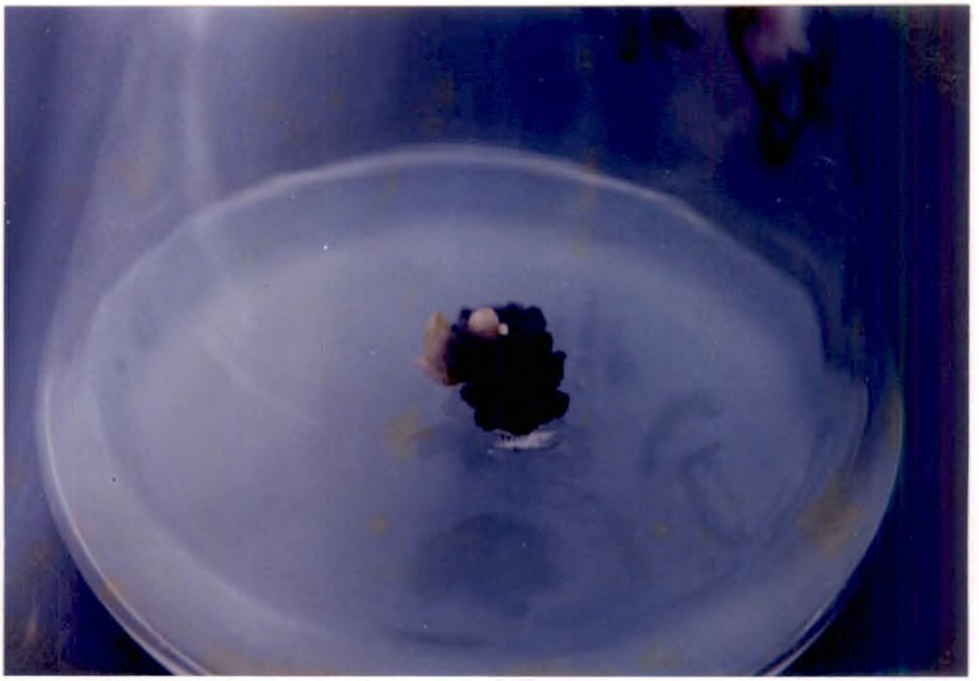


Table 8. Effect of culture media on the initiation of somatic embryoids from nucellus of cashew

Treatment	Cultures initiating somatic embryoids from nucellus (%)
I_1	6.6
I_2	6.6
I_3	—
I_4	60.0
I_5	—
I_6	—
I_7	33.3
I_8	33.3
I_9	40.0
I_{10}	13.3
I_{11}	53.3
I_{12}	13.3
I_{13}	13.3
I_{14}	20.0

The data represent the average value of 15 replications.

Treatment details given in Table 2.

Table 9. Effect of culture media on the initiation of somatic embryoids from embryo mass of cashew

Treatment	Cultures initiating somatic embryoids from embryo mass (%)
I_1	—
I_2	—
I_3	—
I_4	60.0
I_5	—
I_6	6.6
I_7	6.6
I_8	33.3
I_9	46.6
I_{10}	6.6
I_{11}	46.6
I_{12}	—
I_{13}	—
I_{14}	6.6

The data represent the average value of 15 replications.

Treatment details given in Table 2.

4.3.2 Culture conditions

The influence of light and temperature on the initiation of somatic embryoids was studied. When the cultures were incubated in a culture condition of darkness and low temperature (26± 2° C), 33.3 per cent cultures initiated somatic embryoids. In light (3000 lux, 16 hr photoperiod) plus low temperature, 16.6 per cent cultures initiated somatic embryoids. The culture conditions of dark plus room temperature (32 ± 2° C) and light plus room temperature did not initiate somatic embryoids (Table 10).

4.4 Maturation of somatic embryoids

4.4.1 Basal medium

It was observed that basal medium consisting of B_5 major salts and MS minor salts with supplements was the best for the maturation of somatic embryoids. 50.0 per cent cultures survived in this medium (Table 11). In full strength and half strength MS basal medium, the survival percentage was same (33.3 per cent). But in B_5 basal medium with supplements, the survival percentage was only, 16.6 (Fig. 1 and Plate 7).

4.4.2 Abscisic acid (ABA)

Different levels of ABA were tried to study its effect on the maturation of somatic embryoids. When ABA 1.0 mg/l was incorporated into the medium, 50.0 per cent cultures survived. The treatment produced

Table 10. Effect of culture conditions on the initiation of somatic embryoids from nucellus of cashew

Treatment	Cultures initiating somatic embryoids (%)
Light (3000 lux, 16 hr photo period) + low temperature (26 ± 2°C)	16.6
Dark + low temperature (26 ± 2°C)	33.3
Dark + room temperature (32 ± 2°C)	0
Light (3000 lux, 16 hr photo period) + room temperature (32 ± 2°C)	0

Culture media:

MS + BA 1.0 mg/l + GA 1.0 mg/l + Sucrose 30.0 g/l + agar 5.0 g/l

The data represent the average value of 6 replications.

Table 11. Effect of basal medium on the maturation of somatic embryoids of cashew

Basal medium	Cultures survived (%)	Colour of embryoids
MS	33.3	Cream
1/2 MS	33.3	Cream
B_5	16.6	Brown
B_5 major + MS minor	50.0	Cream

Other supplements :

Sucrose 50.0 g/l + coconut water 200.0 ml/l + CH 100.0 mg/l + PVP 10.0 g/l + ABA 1.0 mg/l + agar 5.0 g/l

The data represent the average value of 6 replications.

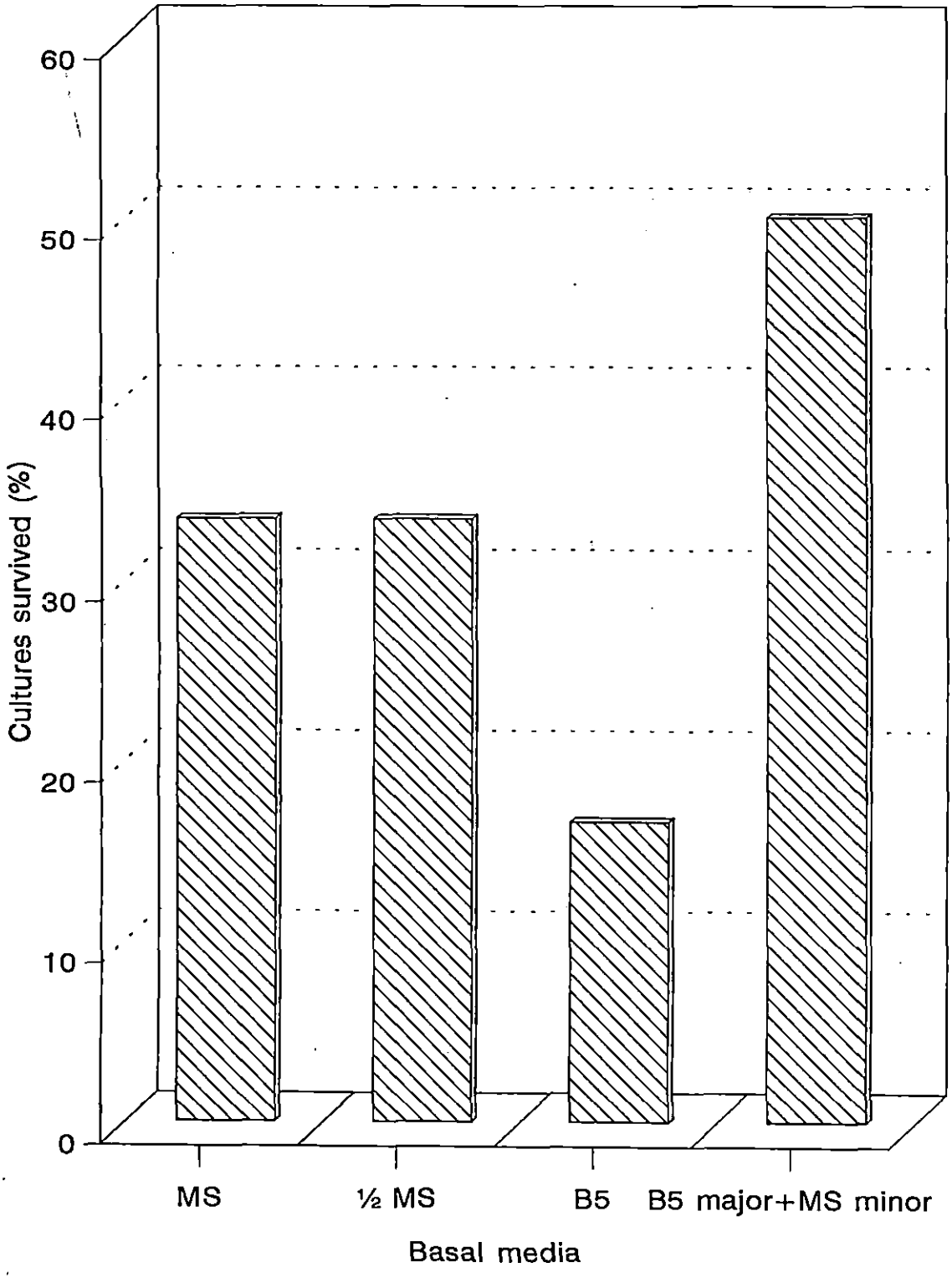


Fig. 1. Effect of basal media on the maturation of somatic embryoids of cashew

cream coloured embryoids. Though a higher per cent survival could be observed, no significant growth or increase in size was observed (Table 12). When ABA 2.0 mg/l and 5.0 mg/l were used, 33.3 per cent cultures survived and there was also no increase in the size of the embryoids. The colour of the embryoids was dull cream in these treatments. On the other hand, at the highest level of ABA (10.0 mg/l), the embryoids could not survive at all.

4.4.3 Sucrose

Three treatments involving different sucrose levels (40.0 g, 50.0 g, 60.0 g/l) were tried for the maturation of somatic embryoids. With 40.0 g/l sucrose, 50.0 per cent cultures survived, whereas, with 50.0 g/l and 60.0 g/l, only 33.3 per cent cultures survived (Table 13). When sucrose 40.0 g/l was added to the medium, the somatic embryoids produced were cream in colour. Though it appeared to be fresh, only a slight increase in size of embryoid was observed.

4.4.4 Coconut water

In order to study the influence of coconut water on maturation of somatic embryoids, three treatments involving coconut water 100.0, 150.0 and 200.0 ml/l in the medium were attempted. When coconut water 200.0 ml/l was added in the maturation medium, the percentage survival was 50.0 with cream coloured embryoids. With coconut water 150.0 ml/l,

Table 12. Effect of ABA on the maturation of somatic embryoids of cashew

Treatments ABA (mg/l)	Cultures survived (%)	Description of embryoids
1.0	50.0	Cream in colour with slight growth
2.0	33.3	Dull cream colour, no increase in growth
5.0	33.3	Dull cream - brown in colour, no growth
10.0	—	—

Culture medium :

B_5 major salts + MS minor salts + casein hydrolysate 100.0 mg/l + sucrose 40.0 g/l, coconut water 200.0 ml/l + PVP 10.0 g/l + agar 5.0 g/l

The data represent the average value of 6 replications.

Table 13. Effect of sucrose on the maturation of somatic embryoids of cashew

Treatments Sucrose (g/l)	Cultures survived (%)	Description of embryoids
40.0	50.0	Cream colour, slight growth
50.0	33.3	Green with creamy patches, no growth
60.0	33.3	Dull cream colour, no growth

Culture medium :

B_5 major salts + MS minorsalts + coconut water 200.0 ml/l + CH 100.0 mg/l + PVP 10.0 g/l + ABA 1.0 mg/l + agar 60.0 g/l

The data represent the average value of 6 replications.

only 33.3 per cent cultures survived and colour of the embryoids ranged between green to cream. But when coconut water 100.0 ml/l was included in the medium, the embryoids changed from cream to brown in colour and gradually dried up (Table 14).

4.4.5 Glyphosate

To study the effect of glyphosate on the maturation of somatic embryoids, the combinations of glyphosate and ABA were tried. Mortality of the cultures resulted in all the treatments. Only the treatment without glyphosate survived (Table 15).

4.4.6 Ethylene inhibitors

Different levels of cobalt chloride and silver nitrate were tried as ethylene inhibitors (Table 16). With 5.0 mg/l and 10.0 mg/l cobalt chloride in the medium, the survival of embryoids was same (16.6 per cent). No growth was observed in both the treatments. Silver nitrate 5.0 mg/l as well as 10.0 mg/l were included in the media. Both the treatments couldn't support the maturation of somatic embryoids.

4.4.7 Culture conditions

Culture conditions did not influence the maturation process of the somatic embryoids. In both cases (light as well as dark culture conditions) survival percentage remained the same (33.3 per cent) (Table 17).

Table 14. Effect of coconut water on the maturation of somatic embryoids of cashew

Treatments Coconut water (ml/l)	Cultures survived (%)	Description of embryoids
200.0	50.0	Cream colour, slight growth
150.0	33.3	Green to cream, no growth
100.0	—	—

Culture medium:

B_5 major salts + MS minor salts + Sucrose 60.0 g/l + PVP 10.0 g/l + CH 100.0 mg/l + ABA 5.0 mg/l + Agar 5.0 g/l

The data represent the average value of 6 replications.

Table 15. Effect of glyphosate on the maturation of somatic embryoids of cashew

Treatments Glyphosate (μM)	Cultures survived (%)	Description of embryoids
0.0 control	16.6	Cream colour, slight growth
100.0	—	turned brown and got dried up
200.0	—	turned brown and got dried up

Culture medium:

B_5 major salts + MS minor salts + Sucrose 40.0 g/l + CW 200.0 ml/l + CH 100.0 mg/l + PVP 10.0 g/l + ABA 5.0 mg/l + Agar 5.0 g/l

The data represent the average value of 6 replications.

Table 16. Effect of ethylene inhibitors on the maturation of somatic embryoids of cashew

Ethylene inhibitors (mg/l)	Cultures survived (%)	Description of embryoids
Cobalt chloride 5.0	16.6	dull cream colour no growth
Cobalt chloride 10.0	16.6	cream colour no growth
Silver nitrate 5.0	—	—
Silver nitrate 10.0	—	—

Culture medium:

B_5 major salts + MS minor salts + Sucrose 40.0 g/l + PVP 10.0 g/l + ABA 1.0 mg/l + Agar 5.0 g/l

The data represent the average value of 6 replications.

Table 17. Effect of culture conditions on the maturation of somatic embryoids of cashew

Culture conditions	Cultures survived (%)	Colour of embryoid	Description of embryoids
Light (3000 lux + 16 hr photoperiod) + low temperature (26 ± 2°C)	33.3	dull cream	no growth
Darkness + room temperature (32 ± 2°C)	33.3	cream	slight growth

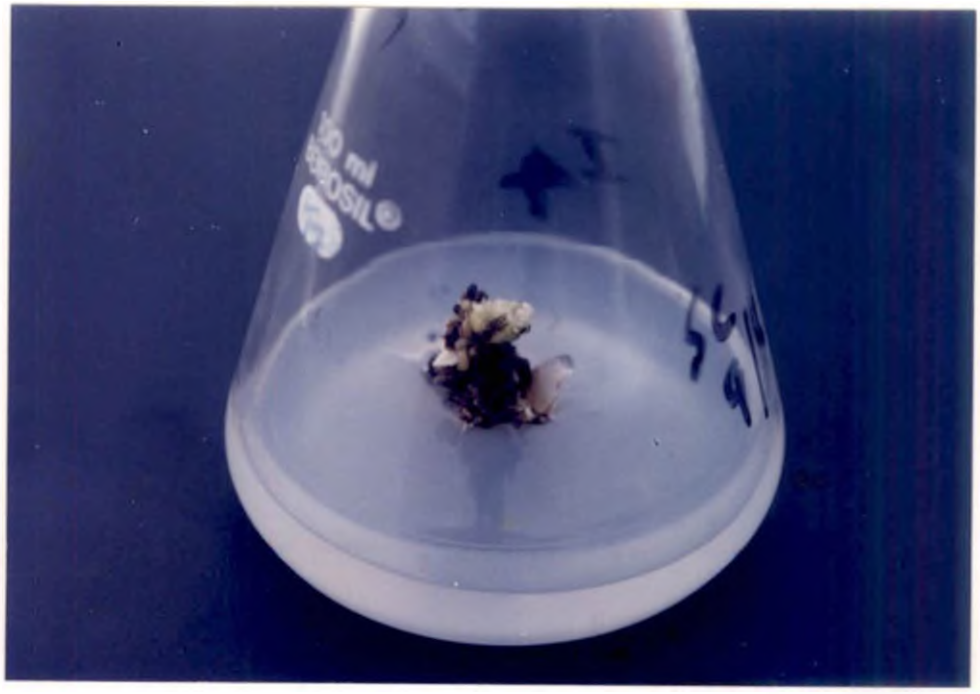
Culture medium:

B_5 major salts + MS minor salts + Sucrose 40.0 g/l + Coconut water 200.0 ml/l + CH 100.0 mg/l + ABA 1.0 mg/l + PVP 10.0 g/l + Agar 5.0 g/l.

The data represent the average value of 6 replications.

Plate 7. Somatic embryoids in maturation medium

Plate 8. Abnormal growth of somatic embryoid in germination medium with proper root development and poor organisation of shoot portion


250 ml
PYREX

The general appearance of the embryoids was good when kept under darkness. Under room temperature, the cultures turned black and did not survive.

4.4.8 Frequency of subculture

Subculturing at an interval of ten days and fifteen days produced cream coloured embryoids. In both cases, 50.0 per cent of the cultures survived. When subcultured at fifteen days interval, slight growth was observed, but the phenolic interference was more than the cultures with ten days subculturing frequency (Table 18). When subcultured at an interval of five days, survival percentage was less (16.6 per cent). No growth was observed.

4.4.9 Mode of culture

Effect of solid and liquid media were tried. Solid media was found to be good for the maturation of somatic embryoids. In liquid medium, though a large amount of phenolics could be washed out easily, the embryoids got vitrified and gradually mortality resulted.

4.5 Germination of somatic embryoids

4.5.1 Basal medium

Somatic embryoids in the maturation media were transferred to different germination media. The study revealed that basal medium with

Table 18. Effect of frequency of subculture on the maturation of somatic embryoids of cashew

Subculturing interval (days)	Cultures survived (%)	Browning of cultures	Description of the embryoids
5	16.6	Very less	Cream colour, No growth
10	50	Less	Cream colour, No growth
15	50	More	Cream colour, Slight growth

Culture medium:

B_5 major salts + MS minor salts + Sucrose 40.0 g/l + Coconut Water 200.0 ml/l + CH 100.0 mg/l + PVP 10.0 g/l + ABA 1.0 mg/l + Agar 5.0 g/l

The data represent the average value of 6 replications.

B_5 major salts plus MS minor salts supported 50.0 per cent survival of cultures (Table 19). In this medium embryoids produced simultaneous shoot and root development and 16.6 per cent cultures germinated with near normal growth. In MS basal medium having half strength major salts, though 50.0 per cent cultures survived, none germinated. But when grown in MS basal medium and B_5 basal medium the embryoids produced malformed shoots.

4.5.2 Plant growth substances

The somatic embryoids in maturation media were transferred to germination media supplemented with various plant growth substances alone and in combinations. The effect of plant growth substances on germination of somatic embryoids from nucellus is presented in Table 20.

In basal medium with B_5 major salts plus MS minor salts supplemented with BA 1.0 mg/l (G_4), the embryoids showed 83.3 percentage survival. Out of this 33.3 per cent cultures showed simultaneous shoot and root development and near normal growth. In germination media with BA 0.1 mg/l (G_2), though 66.6 per cent cultures survived, only roots developed in 33.3 per cent cultures and only shoots developed in the remaining half. Other treatments of BA, eventhough produced simultaneous shoot and root growth, the shoots were rudimentary and malformed.

GA_3 alone did not support the germination of somatic embryoids. When GA_3 1.0 mg/l and GA_3 5.0 mg/l was supplemented in the germination

Table 19. Effect of basal media on the germination of somatic embryoids of cashew

Treatment	Surviving cultures (%)	Cultures with simultaneous shoot and root growth (%)	Cultures with shoot only (%)	Cultures with root only (%)	Description of embryoids
MS	33.3	—	—	33.3	Malformed shoots
1/2 MS	50.0	—	—	—	Remained as such without any further growth
B_5	33.3	—	16.6	16.6	Malformed shoots
B_5 major + MS minor	50.0	16.6	16.6	16.6	Near normal growth

Other supplements :

Sucrose 50.0 g/l + CH 100.0 mg/l + PVP 10.0 g/l + CW 200.0 ml/l + BA 0.1 mg/l + $COCl_2$ 10.0 mg/l + Agar 6.0 g/l

The data represents the average values of 6 replications

Table 20. Effect of plant growth substances on the germination of somatic embryoids of cashew

Treatment	Surviving cultures (%)	Cultures with simultaneous shoot and root growth (%)	Cultures with shoot only (%)	Cultures with root only (%)	Description of embryoids
G_1	50.0	33.3	—	16.6	Abnormal
G_2	66.6	—	33.3	33.3	Malformed
G_3	33.3	16.6	—	16.6	Abnormal
G_4	83.3	33.3	16.6	33.3	Near normal
G_5	33.3	—	16.6	16.6	Malformed
G_6	33.3	—	—	—	No growth
G_7	0.0	—	—	—	—
G_8	33.3	33.3	—	—	Abnormal
G_9	33.3	—	—	—	No growth
G_{10}	33.3	16.6	—	16.6	Abnormal
G_{11}	50.0	—	—	—	No growth

The data represents the average values of 6 replications Treatment details given in Table 3.

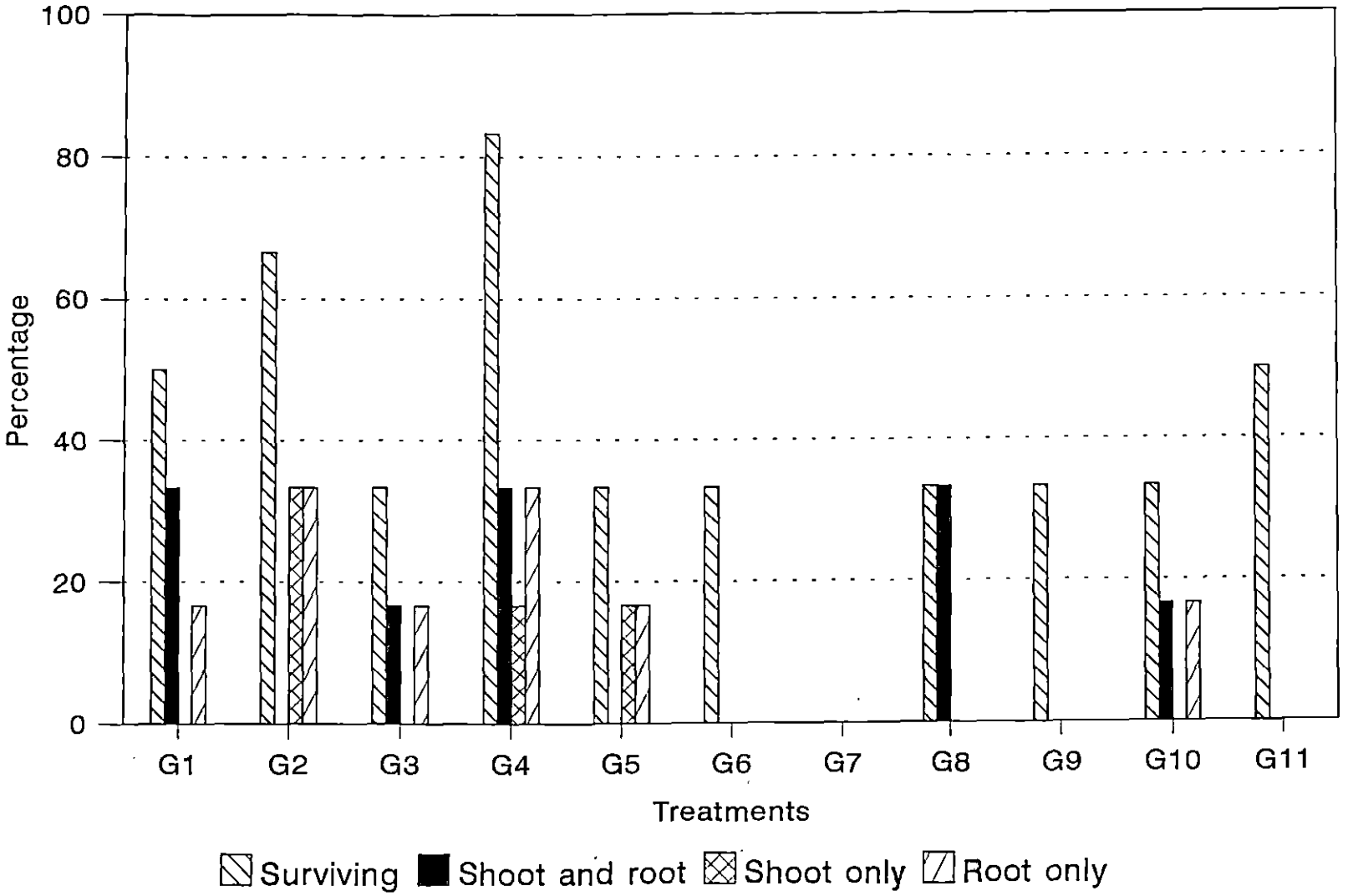


Fig. 2. Effect of plant growth substances on the germination of somatic embryoids of cashew

media, though the cultures survived, none of the embryoids showed normal germination. But when GA_3 10.0 mg/l was supplemented in the germination medium (G_7), the cultures did not survive at all. Germination treatments with kinetin also failed to support the normal germination of the somatic embryoids (G_8 and G_9).

Abnormal growth was observed when kinetin 0.1 mg/l was used in the germination medium whereas at 0.2 mg/l kinetin in the germination medium, none of the cultures germinated. In the treatment having GA_3 1.0 mg/l and BA 0.1 mg/l in combination (G_{10}), simultaneous shoot and root development with abnormal growth was obtained (Fig. 2). On the otherhand somatic embryoids were found alive but did not germinate when transferred to the germination media supplemented with BA 0.1 mg/l and GA_3 5.0 mg/l in combination (G_{11}).

4.5.3 Coconut water

Germination medium with 200.0 ml/l coconut water, 50.0 per cent cultures survived. Out of it, 33.3 per cent cultures showed simultaneous shoot and root growth and near normal germination (Table 21). When 150.0 ml/l coconut water was included in the medium, 83.3 per cent cultures survived. Eventhough 33.3 per cent cultures showed simultaneous shoot and root growth, only the root system was well developed, whereas, the shoot system was malformed. When 100.0 ml/l coconut water was added in the germination medium, 33.3 per cent survival of the cultures was observed. Only root emergence was observed in 16.6 per cent cultures

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

Treatment coconut water (ml/l)	Surviving cultures (%)	Cultures with simultaneous shoot and root growth (%)	Cultures with shoot only (%)	Cultures with root only (%)	Description of embryoids
0	50.0	16.6	—	33.3	Embryoids with long root
100.0	33.3	—	16.6	16.6	Malformed
150.0	83.3	33.3	16.6	33.3	root well developed shoot pole remained under developed
200.0	50.0	33.3	—	16.6	near normal

Culture medium:

B_5 Major salts + MS minor salts + sucrose 40.0 g/l + CH 100.0 mg/l + PVP 10.0 g/l + BA 0.1 mg/l + agar 6.0 g/l

The data represents the average values of 6 replications

and shoot emergence in 16.6 per cent. Eventhough 50.0 per cent cultures survived in the media without coconut water, simultaneous root and shoot growth was observed only in 16.6 per cent cultures. But the roots produced by the embryoids grown in this treatment were long, thin and hair like.

4.5.4 Osmotic regulants

4.5.4.1 Sucrose

The effect of sucrose on germination of somatic embryoids is shown in Table 22. Sucrose at concentrations of 40.0 g/l and 50.0 g/l supported germination in 66.6 per cent cultures. At 40.0 g/l sucrose, 16.6 per cent cultures showed root emergence and 33.3 per cent cultures formed leaf like structures and 16.6 per cent showed simultaneous shoot and root development. When 50.0 g/l sucrose was added in the germination medium, eventhough 50.0 per cent cultures had simultaneous shoot and root development, only 16.6 per cent cultures produced near normal growth. The remaining 33.3 per cent cultures showed abnormal development, such as formation of cotyledonary leaves and unequal root / shoot ratios ie., well developed root portion and poor organisation of shoot portion (Plate 8). When sucrose 60.0 g/l was added to the germination medium, 50.0 per cent cultures survived with abnormal growth.

4.5.4.2 Sodium Chloride

The influence of sodium chloride in the germination of somatic embryoids was studied by incorporating 0.05, 0.1 and 0.2 per cent sodium

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

Treatment sucrose (g/l)	Surviving cultures (%)	Cultures with simultaneous shoot and root growth (%)	Cultures with shoot only (%)	Cultures with root only (%)	Description of embryoids
40.0	66.6	16.6	33.3	16.6	leaf like structures were observed in few cultures
50.0	66.6	50.0	—	16.6	A few embryoids showed near-normal growth
60.0	50.0	50.0	—	—	abnormal development

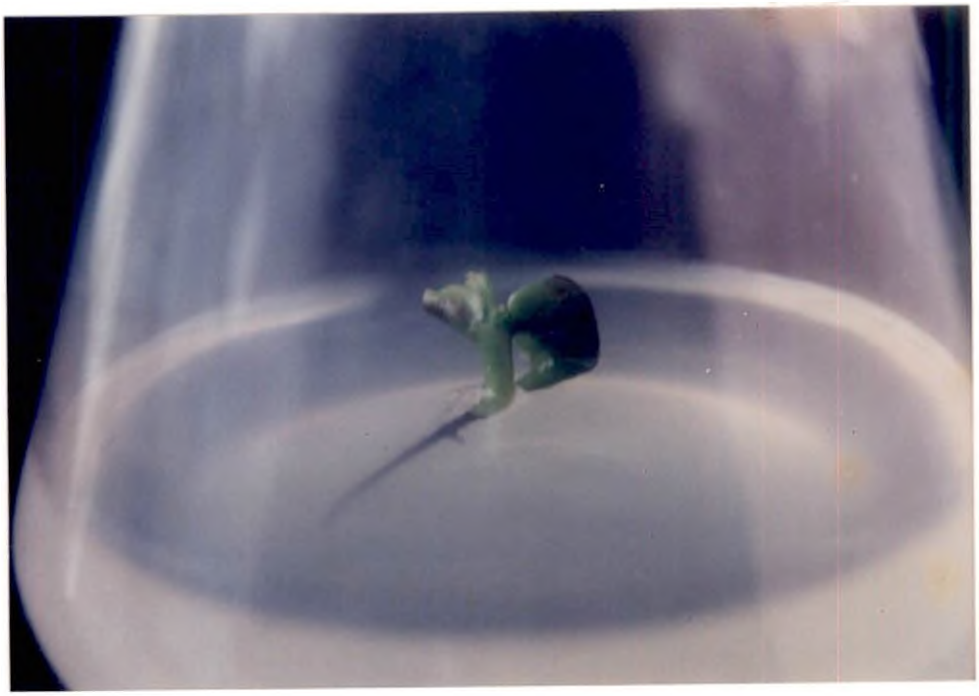
Culture medium:

B_5 major salts + MS minor salts + CH 100.0 mg/l + PVP 10.0 mg/l + BA 0.2 mg/l + $Cocl_2$ 10.0 mg/l + agar 5.0 g/l

The data represents the average values of 6 replications

Plate 9. Growing somatic embryoid in germination medium supplemented with sodium chloride 0.1 per cent

Plate 10. Somatic embryoid in germination medium supplemented with cobalt chloride 10.0 mg/l



chloride to the germination medium. The study revealed that sodium chloride could initiate germination of cashew somatic embryoids (Fig. 3). In germination medium supplemented with sodium chloride 0.1 per cent, 66.6 per cent cultures survived and 50.0 per cent germination could be obtained (Table 23). Near normal growth of the embryoids could be observed in this treatment (Plate 9). Sodium chloride at 0.2 per cent in the germination medium was also effective in initiating near normal growth of a few somatic embryoids. On the otherhand, germination medium supplemented with 0.05 per cent sodium chloride produced malformed shoot in the germinating embryoids.

4.5.5 Ethylene inhibitors

The effect of ethylene inhibitors such as cobalt chloride and silver nitrate on the germination of somatic embryoids is presented in Table 24. Cobalt chloride 10.0 mg/l supported near normal germination in 33.3 per cent cultures (Fig. 4 and Plate 10). Cobalt chloride 15.0 mg/l when added to the germination medium, though 33.3 per cent cultures survived, none germinated. No proper germination was observed when cobalt chloride 5.0 mg/l was added to the medium. The embryoids showed abnormal growth.

Silver nitrate did not support the germination of somatic embryoids and all the treatments either failed to make the cultures respond or caused abnormalities.

Table 23. Effect of sodium chloride on the germination of somatic embryoids of cashew

Treatment (Sodium chloride)	Surviving cultures (%)	Cultures with simultaneous shoot and root growth (%)	Cultures with shoot only (%)	Cultures with root only (%)	Description of embryoids
0.05 %	50.0	—	16.6	33.3	Malformed
0.1 %	66.6	50.0	—	16.6	Near normal
0.2 %	50.0	33.3	—	16.6	Near normal

Culture medium :

B_5 Major salts + MS minor salts + BA 0.1 mg/l sucrose 50.0 g/l + PVP 10.0 g/l + agar 5.0 g/l

The data represents the average values of 6 replications

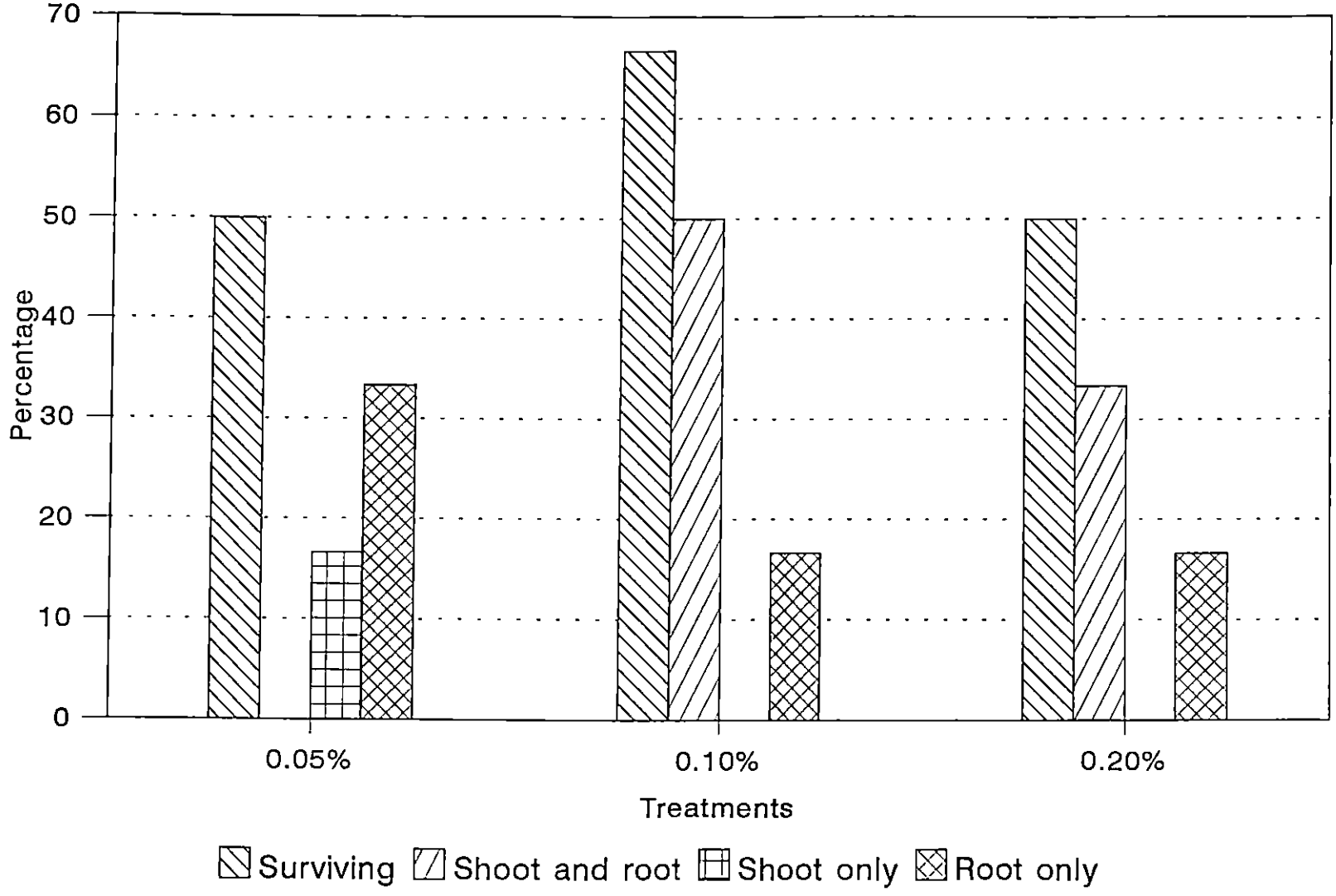


Fig. 3. Effect of sodium chloride on the germination of somatic embryoids of cashew

Table 24. Effect of ethylene inhibitors on the germination of somatic embryoids of cashew

Treatment Ethylene inhibitors (mg/l)	Surviving cultures (%)	Cultures with simultaneous shoot and root growth (%)	Cultures with shoot only (%)	Cultures with root only (%)	Description of embryoids
Silver nitrate					
5.0	33.3	—	—	—	No growth
10.0	—	—	—	—	—
Cobalt chloride					
5.0	33.3	—	—	33.3	Malformed
10.0	66.6	33.3	—	33.3	Near normal
15.0	33.3	—	—	—	No growth (remained as such)

Culture medium :

B_5 major salts + MS minor salts + sucrose 40.0 g/l + PVP 10.0 g/l + BA 0.1 mg/l + agar 5.0 g/l

The data represents the average values of 6 replications

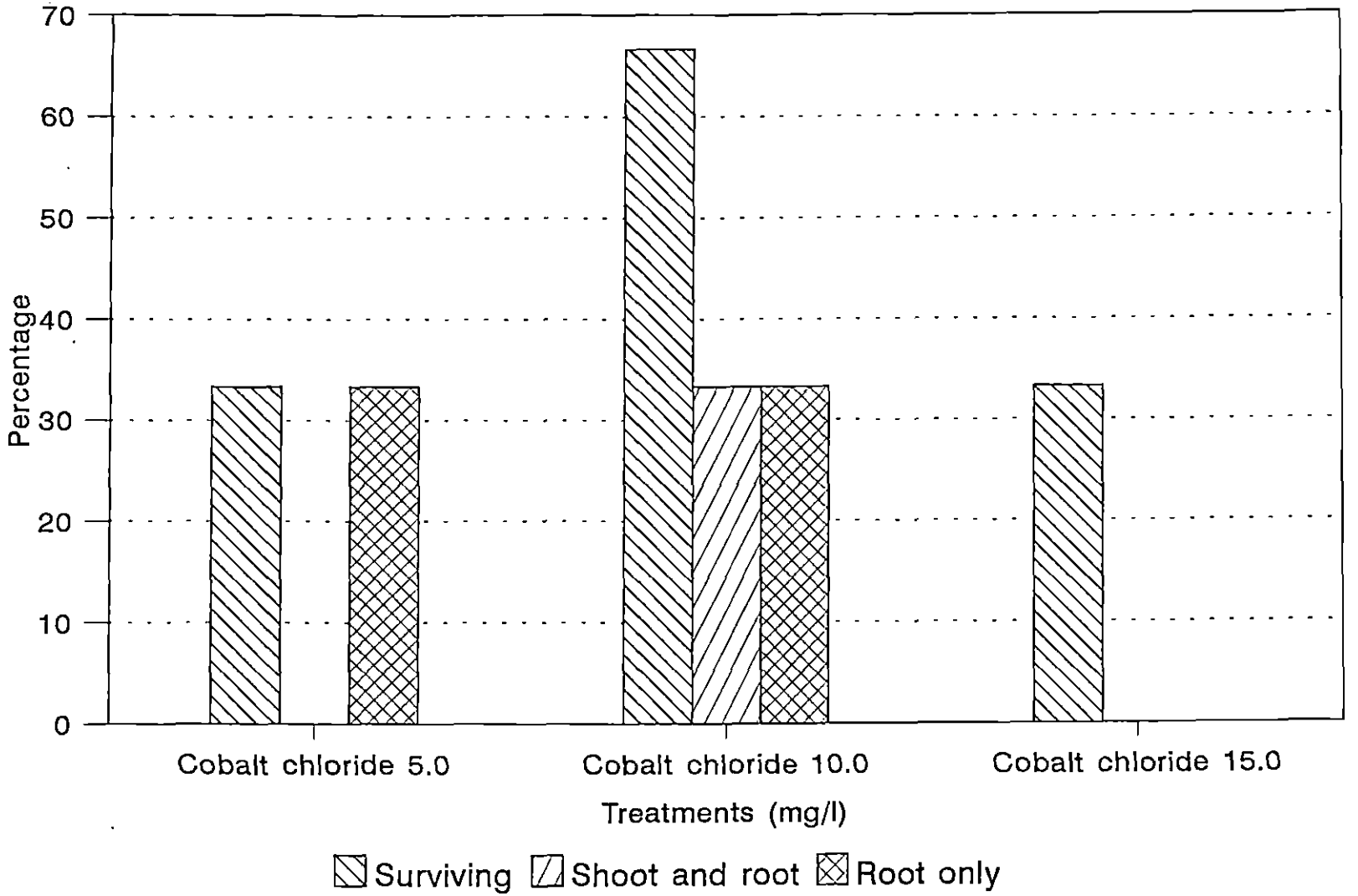


Fig. 4. Effect of cobalt chloride on the germination of somatic embryoids of cashew

Table 25. Effect of culture conditions on the germination of somatic embryoids of cashew

Treatment	Surviving cultures (%)	Cultures with simultaneous shoot and root growth (%)	Cultures with shoot only (%)	Cultures with root only (%)	Description of embryoids
Light (3000 lux + 16 hr photoperiod) + low temperature (26 ± 2°C)	66.6	33.3	16.6	16.6	Near normal
Dark + low temperature (26 ± 2°C)	33.3	—	16.6	16.6	Abnormal

Culture medium:

B_5 major salts + MS minor salts + BA 1.0 mg/l + CH 100.0 mg/l + PVP 10.0 mg/l + agar 6.0 g/l

The data represents the average values of 6 replications

Table 26. Effect of frequency of subculture on the germination of somatic embryoids of cashew

Treatment (days)	Surviving cultures (%)	Cultures with simultaneous shoot and root growth (%)	Cultures with shoot only (%)	Cultures with root only (%)	Description of embryoids
5	16.6	—	—	—	No growth
10	50.0	—	33.3	16.6	Long narrow root
15	50.0	33.3	—	16.6	Near normal
20	50.0	33.3	—	16.6	Near normal

Culture medium :

B_5 major salts + MS minor salts + PVP 10.0 g/l + Sucrose 40.0 g/l + NaCl 0.1 % + BA 0.1 mg/l + agar 5.0 g/l

The data represents the average values of 6 replications

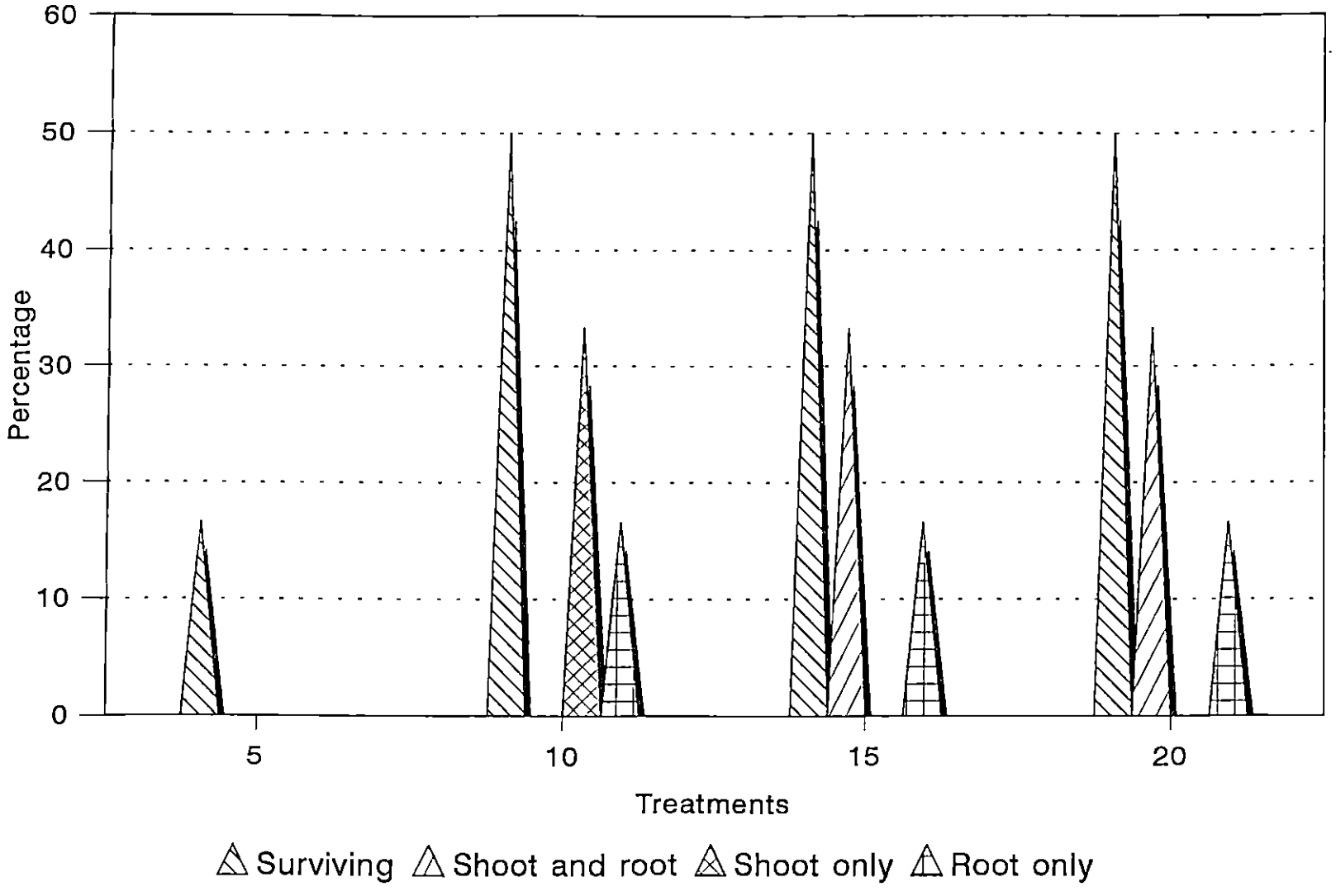


Fig. 5. Effect of frequency of subculture on the germination of somatic embryoids of cashew

4.5.6 Culture conditions

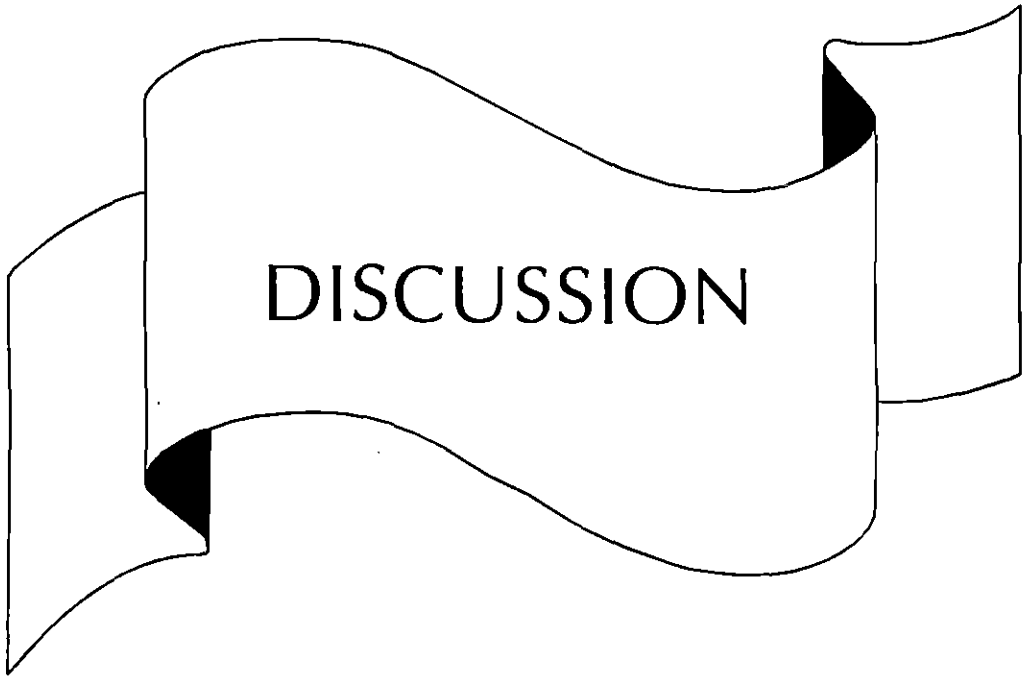
Light was found to be essential for normal germination of somatic embryoids. Under light, survival of embryoids was 66.6 percentage with 33.3 per cent cultures showing near normal growth. Few cultures kept under darkness remained as such without any growth, whereas 16.6 per cent cultures showed root emergence (Table 25).

4.5.7 Frequency of subculture

Subculturing at an interval of 15 days and 20 days supported 50.0 per cent survival of cultures. Under both the conditions, 33.3 per cent cultures had simultaneous shoot and root growth with near normal germination. But when subcultured at an interval of five days, germination of the embryoids could not be observed (Fig. 5). At ten days subculturing interval, the embryoids showed abnormal growth with poor organisation of shoot and root portion (Table 26).

Plate 11. A fully germinated somatic embryoid with proper shoot and root development

Plate 12. Germinated somatic embryoid of cashew planted out in potting media (sterilised sand)


DISCUSSION

5. DISCUSSION

Cashew (*Anacardium occidentale* L.) is commercially grown for its kernels, although cashewnut shell liquid and apples are also valuable byproducts. It is considered to be the most important edible nut in the world trade. Cashew crop which has been considered only useful for soil conservation, afforestation and for development of wastelands, has today assumed a status of an important dollar earning horticultural crop in India.

Although cashew is both seed propagated as well as vegetatively multiplied through veneer grafting, budding, air layering and epicotyl grafting, the rate of multiplication and field establishment possible are by no means commensurate with the demand for superior planting materials. Being a highly cross pollinated crop, seed propagation gives a highly variable progeny resulting in high instability in yields.

Evolving techniques for the *in vitro* propagation of cashew will help rapid clonal propagation and early establishment of trees having superior traits. Rapid multiplication of scion materials and large scale production of clonal root stocks are also possible. As the root stock influences the growth and quality of scion, clonal root stocks would be

helpful in ensuring uniform performance of the grafts. Rare and endangered cultivars can be multiplied and prevented from being extinct.

Hence studies were undertaken to standardise more rapid and prolific rate of vegetative multiplication, using modern techniques of cell, tissue and organ culture, in order to generate adequate clonal planting materials of high yielding hybrids and selections. Thus tissue culture techniques are expected to supplement the conventional clonal propagation methods in cashew in order to meet the ever increasing demand for superior planting materials.

Somatic embryogenesis has been found to be a 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. With its immense rate of multiplication it is able to provide large number of plants having uniform size within a short time.

The present studies were conducted for improving *in vitro* somatic embryogenesis in cashew. The results of the investigations are discussed in the following pages.

Embryogenic potential is largely a function of the explant, its stage of development and the interaction of the explant with the growth medium. Choice of appropriate explant is, therefore critical for morphogenesis.

Out of the two explants tried for inducing somatic embryogenesis, nucellus responded better than embryo mass. Nucellar tissue initiated embryogenic callus in 86.6 per cent cultures, whereas embryo mass initiated callus in 26.6 per cent cultures only, when inoculated in half strength MS basal medium supplemented with 2,4-D 1.0 mg/l + BA 1.0 mg/l + NAA 1.0 mg/l and sucrose 30.0 g/l + AC 0.5 g/l + agar 6.0 g/l. High growth score of 2.96 was recorded by nucellar tissue in this treatment, whereas embryo mass showed a poor callus index (59.85) with a growth score of 2.25. The present studies showed the superiority of nucellar tissue for inducing somatic embryogenesis. Nucellus represents the wall of megasporangium and is a nourishing tissue for the developing embryo. As it is a part of mother plant and has the same ploidy, it can be used for clonal propagation. In perennial fruit crops, somatic embryogenesis was reported from the nucellar tissue of *Citrus* spp. for the first time (Stevenson, 1956). Somatic embryogenesis was subsequently obtained from the nucellar tissue in *Pyrus communis* (Janick, 1982), *Syzigium* spp. (Litz, 1984b), *Eriobotrya japonica* (Litz, 1985), *Theobroma cacao* (Sondahl, 1991). These studies confirmed the morphogenetic potential of the nucellus for a broad range of woody plant species. In mango nucellus has been identified as the best explant for inducing somatic embryogenesis by Litz *et al.* (1982, 1984 and 1993), Dewald *et al.* (1989a and 1989b), Sulekha (1996) and Ramesh (1998). In cashew also Nair *et al.* (1993) could induce somatic embryogenesis from nucellar tissue.

The present study revealed that embryo mass could also be used as explant for inducing somatic embryogenesis. In cashew, somatic embryogenesis has been reported from embryo mass by Hegde (1988), Jha (1988), Sy *et al.* (1991), Nair *et al.* (1993), Lakshmisita (1994), Thomas (1995) and Kesavachandran (1998). Somatic embryogenesis has also been reported from embryo mass explant in *Theobroma cacao* (Pence *et al.*, 1979), *Persea americana* (Pliego - Alfaro and Murashige, 1988) and *Mangifera indica* (Sulekha, 1996 and Ramesh, 1998).

The various steps involved in somatic embryogenesis are induction of embryogenic callus / somatic embryoids, initiation of somatic embryoids, maturation and germination of the somatic embryoids.

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

Twenty four treatments involving various concentrations of plant growth substances, glutamine, sucrose, activated charcoal, coconut water and agar were tried for the induction of somatic embryogenesis. The best treatment for initiating embryogenic callus from nucellar tissue was half strength MS basal medium supplemented with 2,4-D 1.0 mg/l, BA

1.0 mg/l, NAA 1.0 mg/l, Sucrose 30.0 g/l, AC 0.5 g/l, agar 6.0 g/l. The same results were obtained by Nair *et al.* (1993) in cashew. They obtained callusing from immature cotyledons of cashew when inoculated in half strength MS basal medium with plant growth substances namely, 2,4-D, NAA and BA. Thomas (1995) reported maximum callusing with initiation of somatic embryoids in MS basal medium with 2,4-D, NAA and BA combination as supplements, when cashew cotyledons were used as explant.

Different components of induction media were not studied in detail since this has already been standardised under the USDA project of the College of Agriculture, Vellayani during 1988-1993 by Nair *et al.* (1993).

Along with the culture media, culture condition also influence somatic embryogenesis. When the cultures were incubated in induction medium and kept under darkness at low temperature (26 $\pm 2^{o}$C), 80.0 per cent cultures induced somatic embryoids from nucellus and 50.0 per cent from embryo mass. The culture conditions of light and room temperature, embryogenic callus did not initiate either from nucellus or from embryo mass. Jha (1988) reported that induction of callus from explants of cashew occurred only in complete darkness. According to Kesavachandran (1998) more profuse callusing as well as higher percentage were obtained when cashew explants were incubated in dark. In mango also the similar results were reported by Rao *et al.* (1981), Litz *et al.* (1993), Jana *et al.* (1994), Bindu (1995), Sulekha (1996) and Ramesh (1998). The probable

reason may be that under darkness, photo oxidation of auxin in the medium is reduced and hence the potentiality for inducing somatic embryogenesis is increased. Dark conditions can also reduce polyphenol oxidation which may accelerate the response.

Embryogenic calli from the induction medium were transferred to the initiation / expression medium for furthering somatic embryogenesis. MS basal medium supplemented with NAA 0.5 mg/l, kinetin 2.0 mg/l, adenine sulphate 40.0 mg/l, yeast extract 200.0 mg/l, PVP 250.0 mg/l, sucrose 30.0 g/l, agar 5.5 g/l was the best for the initiation of somatic embryoids from cashew nucellar explant. 60.0 per cent initiation of embryoids was obtained from this treatment. This has been supported by the findings of Jha (1988) in cashew. He observed that removal of 2,4-D in the initiation medium enhanced the number of embryoids produced in MS basal medium. 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 embryoids did not proceed beyond a stage in the presence of 2,4-D. According to Ramesh (1998) cytokinins were found to promote initiation of somatic embryoids from embryogenic callus of mango varieties. The present studies confirm with the findings of Atree and Fowke (1991) who also reported that somatic embryoids initiated on a medium containing auxin and cytokinin.

Culture conditions influenced the initiation of somatic embryoids. When kept under darkness and low temperature,33.3 per cent cultures initiated somatic embryoids. None of the cultures initiated somatic embryoids when kept under room temperature either at light or at dark conditions. These results are supported by earlier reports. Dewald *et al.* (1989a) kept mango cultures in the initiation medium only in dark. Bindu (1995), Sulekha (1996) and Ramesh (1998) could initiate somatic embryoids from the nucellus of mango varieties when the cultures were kept under darkness at low temperature. The increased *in vitro* response under dark condition might be due to the reduced polyphenol oxidation and changes in the endogenous ratio of plant growth substances as the photo oxidation of auxin is reduced.

Somatic embryoids from the initiation medium were transferred to the maturation medium. According to Litz *et al.* (1993), control of somatic embryo maturation was the most critical and difficult process in mango regeneration. If the embryos are not physiologically mature, they cannot germinate normally and usually cannot survive. In the present investigations, different treatments were given to initiate maturation. The treatments included plant growth substance ABA, basal medium, media composition, osmotic regulants, culture conditions and frequency of subculturing. Various types of basal media and the concentration of inorganic and organic salts in the basal medium have been shown to influence the maturation of somatic embryoids.

In the present study modified basal medium containing B_5 major salts plus MS minor salts with organics was identified as the best for the maturation of cashew somatic embryoids. 50.0 per cent cultures survived in this medium. Dewald *et al.* (1989b), Sulekha (1996) and Ramesh (1998) also obtained mature mango somatic embryoids on incubation of the cultures in a maturation medium consisting B_5 major salts + MS minor salts and organics.

Abscisic acid (ABA) is a growth retardant and has been demonstrated to inhibit somatic embryo development beyond the early stages and initiate maturation (Nodel *et al.*, 1990). It inhibited abnormal proliferations and repressed precocious germination (Ammirato, 1973 and 1974). In the present studies, various concentrations of ABA were tried for obtaining maturation of cashew somatic embryoids. When ABA 1.0 mg/l was incorporated into the medium, 50.0 per cent cultures survived. The treatment produced cream coloured embryoids. Though a higher per cent survival could be observed, there was no significant growth or increase in size. When ABA 2.0 mg/l and 5.0 mg/l were incorporated in the maturation medium, 33.3 per cent cultures survived with no increase in the size of the embryoids. At the highest level of ABA tried (10.0 mg/l), the embryoids could not survive at all. Jana *et al.* (1994) could obtain matured somatic embryoids of the mango varieties, when ABA 1.0 mg/l was used in the medium. But Litz (1992), Bindu (1995) and Sulekha (1996) obtained matured somatic embryoids of mango, when higher level of ABA was incorporated into the medium. The normalising effect of

ABA on embryo maturation has also been observed with *Dacus carota* (Kamada and Harada, 1981) and *Pennistum americanum* (Vasil and Vasil, 1981). But in the present study though some cultures survived, physiologically mature large sized somatic embryoids were difficult to obtain. The size of the embryoids was not at all influenced by different concentrations of ABA in the maturation medium.

Sucrose was provided in the medium for optimising the maturation process of somatic embryoids. In the present studies attempts were made to find out the effects of concentration of sucrose in the maturation medium on the response of the embryoids. With 40.0 g/l sucrose, 50.0 per cent cultures survived. The somatic embryoids produced were cream in colour. Though it appeared to be fresh, only a slight growth was observed, whereas with 50.0 g/l and 60.0 g/l, only 33.3 per cent survived with no increment in size. Previous reports have shown that a moderate concentration of sucrose is necessary for embryo maturation. According to Sulekha (1996) and Ramesh (1998) effective concentrations of sucrose, for maturation of somatic embryoids of mango were 40.0 g/l and 50.0 g/l. According to Dewald *et al.* (1989b), sucrose 60.0 g/l was essential for the maturation of mango somatic embryoids. Sucrose can act as the carbon source and energy source causing increment in size. It also act as an osmoticum that can stimulate and regulate morphogenesis.

Coconut water was reported to prevent necrosis in mango somatic embryoids in the maturation medium (Litz, 1984b) and to mediate normal

germination along with ABA (Dewald *et al.*, 1989b). When coconut water 200.0 ml/l was added in the maturation medium, the per cent survival was 50.0, with cream coloured embryoids. With 150.0 ml/l, only 33.3 per cent cultures survived and colour of the embryoid ranged between green to cream. But when coconut water 100.0 ml/l was included in the medium, the embryoids dried up gradually. Jana *et al.* (1994), Bindu (1995), Sulekha (1996) and Ramesh (1998) also identified 200.0 ml/l coconut water to be the best in getting matured mango somatic embryoids. Coconut water was identified as an essential component in the maturation of mango somatic embryoids by many workers.

Eventhough glyphosate was found to influence the maturation process of the somatic embryoids in certain crops, this chemical had a negative influence in the maturation of cashew somatic embryoids. Glyphosate 200.0 µM and 100.0 mM had a positive response in bringing physiological maturity of sweet potato somatic embryos (Gowda and Prakash, 1998), by promoting desiccation of the embryoids, which is an essential step for embryo quiescence. But cashew being recalcitrant could not survive desiccation beyond a certain level may be the reason for its ill effects.

Endogenous ethylene level above critical concentration can cause inhibitory effects on maturation process of somatic embryoids. In order to check this, ethylene inhibitors like cobalt chloride and silver nitrate were tried in the medium. Though 16.6 per cent survival was observed

when cobalt chloride was incorporated in the medium, no growth was observed. Treatments having silver nitrate failed to aid maturation of somatic embryoids. These results are in conformation with the reports of Ramesh (1998). According to him cobalt chloride did not record any significant improvement in maturation.

When the cultures were subcultured at 15 days interval, slight growth was observed, but the phenolic interference was more than the cultures with ten days and five days subculturing frequency. Litz *et al.* (1991) and Sulekha (1996) maintained the cultures of mango in maturation medium by subculturing at two weeks intervals.

Solid medium was found to be ideal for maturation of nucellus derived embryoids of cashew. In liquid medium, though a large amount of phenolics could be washed out easily, the embryoids got vitrified and gradually mortality resulted. Dewald *et al.* (1989b) also reported more abnormalities, such as ploycotyledony in liquid medium. There are other reports also on the fasciation and loss of bipolarity (Litz *et al.*, 1993) and vitrification (Ramesh, 1998) especially in highly embryogenic cultures of mango somatic embryoids in liquid medium.

Germination of somatic embryoid is a critical factor. Poor germination is typical in many embryogenic culture systems (Litz *et al.*, 1982). In the present study also normal germination could not be obtained except in a few instances.

The choice of basal medium could influence the germination of somatic embryoids. The concentration of organic and inorganic salts in the basal medium influenced the germination of somatic embryoids. Basal medium having B_5 major salts and MS minor salts supported good germination. Near normal growth of the embryoids could be obtained in this basal medium. Sulekha (1995) and Ramesh (1998) could also get near normal growth of mango of somatic embryoids in this medium. They reported that germination was accompanied by slight enlargement and progressive greening of the somatic embryoids when cultured in the above basal medium.

The cytokinin,BA at concentration of 1.0 mg/l was found to be very effective to promote normal germination in the present study. Litz *et al.* (1984b) and Jana *et al.* (1994) reported the use of BA for normal germination of mango somatic embryoids. Sulekha (1995) reported that BA 1.0 mg/l favoured normal germination. According to Ramesh (1998) BA 0.1 mg/l when supplied in the germination medium was found to produce near normal germination of somatic embryoids.

Steward and Shantz (1959) suggested that reduced nitrogen could be supplied in the form of complex addenda such as coconut water. In the present study also, treatments involving different concentrations of coconut water were tried. In the treatment with coconut water (200.0 ml/l), near normal germination of embryoids could be achieved in few cultures with a good survival percentage. Previous reports have also

supported the importance of coconut water in the germination of somatic embryoids. Jana *et al.* (1994), Bindu (1995) and Sulekha (1996) had also reported the importance of coconut water in the germination of mango somatic embryoids.

Sucrose at a concentration of 50.0 g/l was found to be ideal for germination of somatic embryoids. Sulekha (1996) was able to attain normal germination of mango somatic embryoids by using 50.0 g/l sucrose in the medium. However many scientists had reported near normal somatic embryo development and germination in mango when 60.0 g/l sucrose was used in the medium (Litz, 1985 and Dewald *et al.*, 1989b). But in the present study, 60.0 g/l sucrose when added to the medium, cultures showed abnormal development.

Sodium chloride was included in the germination medium as an osmoticum to provide stress for the germinating embryoids. Sodium chloride 0.1 per cent supplemented in the germination medium effected near normal germination in 50.0 per cent cultures. Sodium chloride 0.2 per cent in the germination medium was also effective in the germination of few somatic embryoids. Earlier reports support the present investigations. Sulekha (1996) obtained near normal germination of mango somatic embryoids when sodium chloride 0.05 per cent was incorporated in the germination medium. The beneficial effect of osmotic stress have been reported in carrot by Kamada *et al.*(1986). He concluded that factors which influenced the physiology of cultured cells have a

positive effect on embryogenesis. The influence of sodium chloride in effecting near normal germination can be attributed to its ability in preventing secondary somatic embryogenesis and reducing developmental abnormalities by regulating the osmotic potential.

Ethylene can influence the process of germination. Endogenous ethylene level above the critical level can be inhibitory. For addressing this problem, ethylene inhibitors were used. Silver nitrate and cobalt chloride at 5.0, 10.0, 15.0 mg/l were tried. It was observed that cobalt chloride (10.0 mg/l) could induce germination in 33.3 per cent cultures. A near normal growth of the embryoids, with a terminal shoot primordium was observed in some of the embryoids. A few plantlets were planted out. Silver nitrate was not beneficial in initiating normal growth of the embryoids. These results agree with the findings of Sulekha (1996) and Ramesh (1998). According to Tisserat and Murashige (1977), ethylene suppressed embryo germination in citrus and *Daucus* cultures. The effect of ethylene inhibitors was studied by Roustan *et al.* (1989). They observed that cobalt chloride at concentrations of 10 Mm to 50 Mm effectively inhibited ethylene production by embryogenic cultures and significantly stimulated somatic embryogenesis in carrot.

Light was found to be essential for normal germination of somatic embryoids of cashew. In the presence of light, cultures showed near normal growth. Dewald *et al.* (1989b) and Ramesh (1998) reported near normal germination of mango somatic embryos in the light of cool white fluorescent tubes with a sixteen hour photoperiod.

Frequent subculturing could be identified as a beneficial step in the process of germination of somatic embryoids. The present studies revealed that subculturing at 15 days and 20 days interval supported near normal germination with 50.0 per cent survival. But, when the subculturing interval was five days, even if the phenolic interference is reduced, no germination was observed. According to Litz (1986a), frequent subculturing was necessary to counteract polyphenol interference in mango somatic embryoids. Bindu (1995) and Sulekha (1996) also observed that frequent subculturing was necessary to increase *in vitro* response of mango somatic embryoids by reducing phenolic interference.

A few plantlets that showed near normal germination were planted out in plastic pots containing sterilized sand after treatment with systemic and contact fungicide (Plate 11 and 12) • The pots were then placed in a mist chamber having 25.0 per cent shade, a relative humidity of 80.0 per cent and temperature of 26-27° C. The plants survived for a maximum of three weeks. *Ex vitro* establishment is a very critical step in somatic embryogenesis. Rajmohan (1986) and Sulekha (1996) reported that *ex vitro* establishment is difficult in woody perennials. Special treatments like inclusion of VAM (Vesicular Arbuscular mycorrhyza) in potting media and carbondioxide enrichment might become essential in overcoming the *ex vitro* establishment problems.

From the present investigations, culture media and culture conditions for the first two stages of somatic embryogenesis viz.

induction, initiation could be refined although these were standardised under the USDA project of the Dept. of Horticulture, College of Agriculture, Vellayani during 1988-1993. Proper maturation and normal germination of the embryoids could be successfully induced and near normal germination could be obtained in certain treatments. Though a few plantlets that showed near normal germination and growth were planted out, the *ex vitro* establishment was not successful.

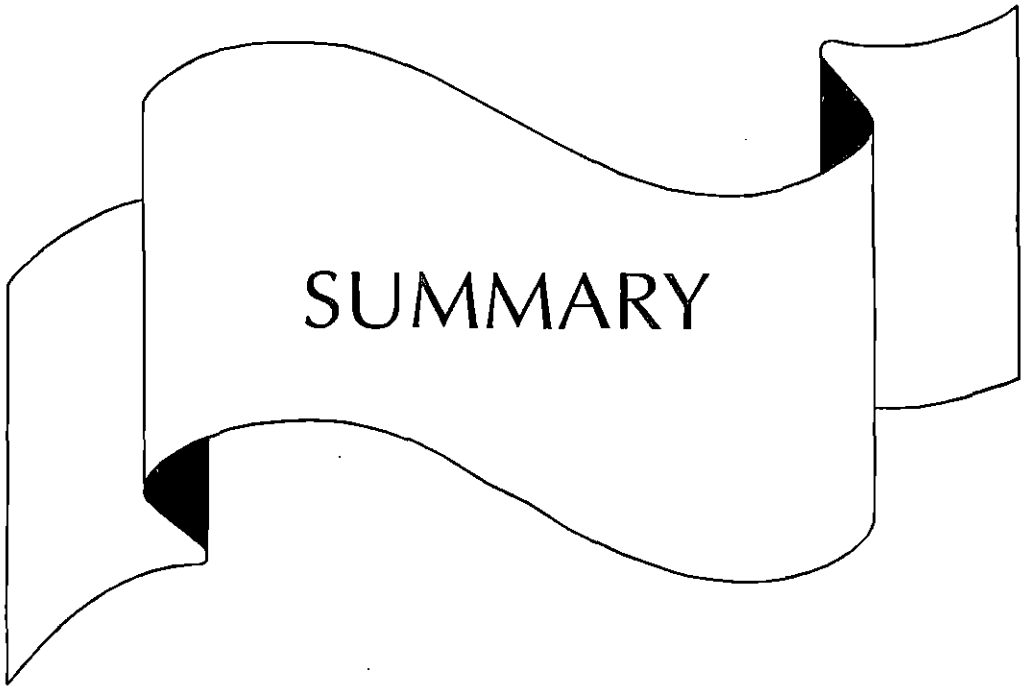
Cashew somatic embryoids seem to be peculiar in several respects. Maturation treatments were not found to influence the germination process.

Embryoids in the initiation medium were subcultured directly into the maturation media and also to the germination media skipping the maturation treatments. The results showed that the embryoids which were directly transferred to the germination medium showed better growth response than those transferred from the maturation media to the germination media. These results revealed that maturation treatments were not essential for initiating the germination process of cashew somatic embryoids. Anyway further studies are necessary for confirming the results.

Since a positive response could be obtained from the ethylene inhibitors studied in germination, this needs special attention. Different types and concentrations of ethylene inhibitors should be resorted to,

which might be useful for success. Based on the earlier report eventhough high concentration of glyphosate was not found to influence the maturation process, lower levels of these compounds, alone or in combination with ABA could also be resorted to, for inducing normal germination of embryoids. So also triazoles compounds could be resorted to, for getting physiologically matured normal embryoids. Better understanding of the inherrent or induced inhibitors during the course of embryogenesis may also be helpful.

Methods have to be standardised for obtaining a sequence of growth media to achieve high frequency production of morphologically normal somatic embryos. Methods should also be standardised to increase the size of embryoids and to attain correct physiological maturity so that normal germination occurs. Further refinement of media is thus necessary for standardising *in vitro* propagation of cashew *via* somatic embryogenesis.


SUMMARY

6. SUMMARY

Attempts were made in the Plant Tissue Culture Laboratory of the Department of Horticulture, College of Agriculture, Vellayani during 1996-'99 for improving somatic embryogenesis in cashew. Nucellus and embryo mass from the ovules of tender nuts of cashew were used as explants. Standarisation of basal media, media components and culture conditions during various stages of somatic embryogenesis, namely, induction, initiation and germination were attempted.

The salient findings of the studies are summarised below :

1. Two types of explants *viz.*, nucellus and embryo mass / cotyledon were tried. Nucellus responded better than embryo mass for initiating embryogenic callus.

2. Out of the twenty four treatments tried, the best treatment for initiating embryogenic callus from nucellar tissue (86.6 per cent) was half strength MS basal medium supplemented with 2,4-D 1.0 mg/l, BA 1.0 mg/l, NAA 1.0 mg/l, sucrose 30.0 g/l, AC 0.5 g/l and agar 6.0 g/l.

3. The best treatment for initiating embryogenic callus from embryo mass was MS basal medium supplemented with 2,4-D 4.0 mg/l, NAA

4.0 mg/l, kinetin 4.0 mg/l, adenine sulphate 40.0 mg/l, yeast extract 200.0 mg/l, PVP 250.0 mg/l, sucrose 30.0 g/l and agar 5.5 g/l.

4. Incubating the cultures in dark rather than light at regulated temperature (26 ± 2°C) was ideal for inducing somatic embryogenesis.

5. The highest percentage initiation of somatic embryoids from nucellus as well as embryo mass was obtained when the callus / somatic embryoids were subcultured on to MS basal medium supplemented with NAA 0.5 mg/l, kinetin 2.0 mg/l, adenine sulphate 40.0 mg/l, yeast extract 200.0 mg/l, PVP 250.0 mg/l, sucrose 30.0 g/l and agar 5.5 g/l.

6. Initiation of somatic embryoids was better when the cultures were kept under a culture condition of darkness plus low temperature (26 ± 2°C).

7. In maturation media supplemented with Abscisic acid 1.0 mg/l, 50.0 per cent cultures survived with cream coloured embryoids

8. Basal media containing B_5 major salts and MS minor salts with supplements was found better for initiating maturation of the somatic embryoids.

9. Sucrose 40.0 g/l in the medium was found to be better for the maturation of somatic embryoids which effected 50.0 per cent survival of cultures.

10. Coconut water 200.0 ml/l in the maturation medium supported maximum survival of cultures.

11. None of the cultures survived when different levels of glyphosate was supplemented in the maturation media.

12. Ethylene inhibitors such as cobalt chloride and silver nitrate were not beneficial for the maturation of cashew somatic embryoids.

13. Culture conditions did not influence the maturation process of somatic embryoids.

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

15. When subcultured at fifteen days interval in the maturation medium, cream coloured embryoids with 50.0 per cent survival was observed.

16. When the cultures were subcultured at five days interval phenolic interference could be remarkably reduced.

17. Basal medium with B_5 major salts plus MS minor salts supplemented with cytokinin was found to be the best for the near normal germination of somatic embryoids.

18. Among the plant growth substances tried, normal germination of somatic embryoids was aided by BA (1.0 mg/l) in the germination medium.

19. GA_3 alone without any cytokinin in the germination medium did not effect the germination of somatic embryoids.

20. Supplementing kinetin in the germination medium was also not beneficial in the germination of somatic embryoids.

21. Coconut water 200.0 ml/l could effect near normal germination of somatic embryoids.

22. Sucrose 50.0 g/l could initiate near normal growth of the somatic embryoids.

23. Silver nitrate had no effect in the germination process of somatic embryoids.

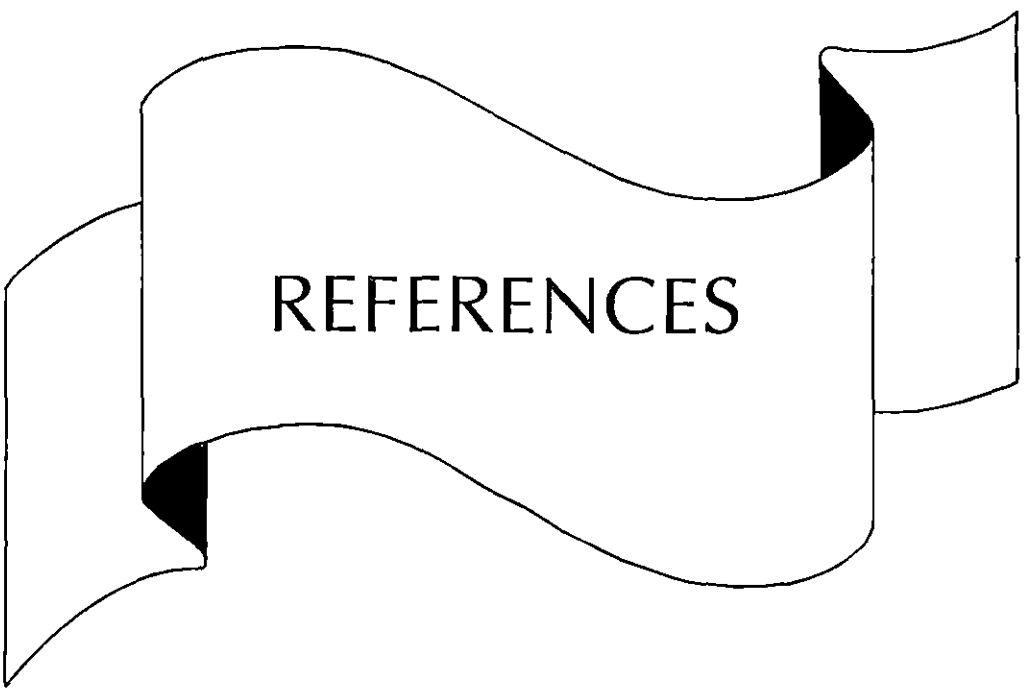
24. Cobalt chloride 10.0 mg/l supplemented in the germination medium could induce near normal growth in 33.3 per cent cultures.

25. Sodium chloride 0.1 per cent supplemented in the germination medium could induce near normal growth in a few embryoids.

26. Light was essential for germination of somatic embryoids.

27. Subculturing at an interval of 15 days and 20 days supported 50.0 per cent survival of cultures with near normal germination.

28. Though a few plantlets that showed near normal germination were planted out in plastic pots containing sterilized sand, *ex vitro* establishment was not successful.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IMPROVEMENT OF *IN VITRO* SOMATIC EMBRYOGENESIS IN CASHEW *(Anacardium occidentale* L.)

By

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ABSTRACT OF THE THESIS

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ABSTRACT

Studies were conducted for improving techniques for *in vitro* somatic embryogenesis in cashew during 1996-1999 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.

Out of the two explants tried, nucellus responded better than embryo mass in initiating embryogenic callus/somatic embryoids.

Induction of somatic embryogenesis from nucellus was found to occur at its maximum when cultured in darkness on MS basal medium having half strength major salts, supplemented with 2,4-D 1.0 mg/l, BA 1.0 mg/l, NAA 1.0 mg/l, sucrose 30.0 g/l, Activated charcoal 0.5 g/l and agar 6.0 g/l.

The best treatment identified for the induction of embryogenic callus / somatic embryoids from embryo mass was MS basal medium supplemented with 2,4-D 4.0 mg/l, NAA 4.0 mg/l, kinetin 4.0 mg/l, adenine sulphate 40.0 mg/l, yeast extract 200.0 mg/l, PVP 250.0 mg/l, sucrose 30.0 g/l and agar 5.5 g/l, in dark culture condition at regulated temperature (26 $\pm$ 2°C).

Initiation of somatic embryoids from nucellus as well as embryo mass occurred at its best in darkness on MS basal medium supplemented with NAA 0.5 mg/l, kinetin 2.0 mg/l, adenine sulphate 40.0 mg/l, PVP 250.0 mg/l, yeast extract 200.0 mg/l, sucrose 30.0 g/l and agar 5.5 g/l.

.Among the treatments tried for inducing proper maturation of the somatic embryoids, the maximum survival of embryoids was recorded on a combination of basal media with B_5 major salts and MS minor salts supplemented with ABA 1.0 mg/l, coconut water 200.0 ml/l, casein hydrolysate 100.0 mg/l, PVP 10.0 g/l, sucrose 40.0 g/l and agar 5.0 g/l.

Culture conditions did not influence the maturation process of the somatic embryoids.

Maturation process was not found to be essential in inducing normal germination of the somatic embryoids. The cultures showed good response when subcultured from initiation media to germination media without a maturation phase.

Germination of somatic embryoids occurred only in the presence of light, on a combination of basal media with B_5 major salts and MS minor salts supplemented with BA 1.0 mg/l, PVP 10.0 g/l, coconut water 200.0 ml/l, sodium chloride 0.1 per cent, cobalt chloride 10.0 g/l, sucrose 50.0 g/l and agar 6.0 g/l.