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IN VITRO SOMATIC EMBRYOGENESIS IN BAEL [*Aegle marmelos* (L.) Corr.]

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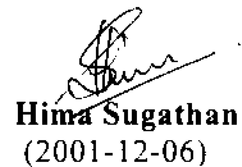
DECLARATION

I hereby declare that this thesis entitled "***In vitro*** **somatic embryogenesis in bael [*Aegle marmelos* (L.) Corr.]**" is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.



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LIST OF ABBREVIATIONS

ABA	–	Abscisic acid
AC	–	Activated charcoal
BA	–	6-benzyl amino purine
CW	–	Coconut water
GA_3	–	Gibberellic acid
IAA	–	Indole-3-acetic acid
IBA	–	Indole-3-butyric acid
MS	–	Murashige and Skoog
MT	–	Murashige and Tucker
NAA	–	Naphthalene acetic acid
SH	–	Schenk and Hildebrandt
WPM	–	Woody Plant Medium
2,4-D	–	2,4-dichlorophenoxy acetic acid
B_5	–	Gamborg *et al.*
PEDCS	–	Pre-embryogenic determined cells
IEDCS	–	Induced embryogenic determined cells
DNA	–	Deoxy ribonucleic acid
RNA	–	Ribonucleic acid
PGS	–	Plant growth substances
Fig.	–	Figure

INTRODUCTION

1. INTRODUCTION

Aegle marmelos (L.) Corr., commonly known as bael is an important medicinal fruit tree of India, belonging to the family Rutaceae, having considerable traditional and socio-cultural values. It is one among the thirty two medicinal plants identified by the National Medicinal Plants Board, Government of India for commercial cultivation, conservation and development (Rawat and Uniyal, 2003).

Bael has great demand in the indigenous systems of medicine especially, Ayurveda and is used for treating wide range of ailments (CSIR, 1985). The root is an ingredient of 'Dasamula' a medicine commonly used by the ayurvedic practitioners. The leaves are reported to have hypoglycaemic, antiasthmatic and cardiotonic properties. The unripe fruit is prescribed for chronic diarrhoea and dysentery. The pulp of the ripe fruit is used in the preparation of 'Sherbet', which is considered to be an aromatic summer drink in North India. Due to its high drought tolerance, bael can also be used in afforestation of dry areas.

Generally, bael is propagated by seed. But being a cross-pollinated species, seed propagation gives rise to highly heterozygous population. Moreover, the seeds have short viability and are prone to insect attack. Vegetative propagation through root suckers is slow, difficult and cumbersome (Ajithkumar and Seeni, 1998). Propagation by methods such as grafting and cutting are also not commercially feasible (Nambiar *et al.*, 2000). Since the supply of planting material is inadequate, more emphasis need to be given for large scale cultivation of this valuable tree.

Evolving protocols for the *in vitro* propagation of bael becomes relevant in this context as it can overcome the disadvantages of

conventional vegetative propagation methods and ensure high rate of multiplication.

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. On the contrary, plantlets obtained *via* other routes of *in vitro* propagation as well as from the conventional vegetative propagation methods like layering, bear only an adventitious root system, which causes uprooting of bael trees especially during heavy rains.

There are several reports on *in vitro* propagation of bael using different explants *via* enhanced release of axillary buds and somatic organogenesis (Arya *et al*., 1981; Bhati *et al*., 1992; Varghese *et al*., 1993; Hossain *et al*., 1994; Arumugam *et al*., 1997; Ajithkumar and Seeni, 1998; Hazeena, 2001). However, studies on the *in vitro* propagation of bael *via* somatic embryogenesis are very limited. Although somatic embryogenesis in bael has been attempted by a few scientists (Islam *et al*., 1996; Arumugam and Rao, 2000), production of normal plantlets was found to be difficult.

The present study '*In vitro* somatic embryogenesis in bael [*Aegle marmelos* (L.) Corr.]' was undertaken with the objective of evolving protocols for the *in vitro* propagation of bael *via* somatic embryogenesis, for high frequency plant regeneration system.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Tissue culture methods have been used as a tool for the propagation of genetically superior clones at a very rapid rate, compared to the conventional methods and for the *in vitro* conservation of valuable germplasm. Such plants are reported to grow faster and mature earlier than the seed propagated plants (Vasil and Vasil, 1980). They have been employed as an important aid to the conventional methods of plant improvement.

In vitro propagation is possible *via* enhanced release of axillary buds, *in vitro* organogenesis and *in vitro* somatic embryogenesis (Murashige, 1974). In shoot apex culture, genetic uniformity is favoured. Callus mediated somatic organogenesis is not recommended for clonal propagation, but may be ideal for the recovery of useful variants. Somatic embryogenesis, with its high rate of multiplication is promising for the *in vitro* propagation of many plant species.

Bael is an important indigenous medicinal fruit tree of India belonging to the family Rutaceae. Only very few reports are available on the *in vitro* propagation of bael *via* somatic embryogenesis. This review highlights the research on various aspects of *in vitro* propagation *via* somatic embryogenesis in bael and related crops

2.1 SOMATIC EMBRYOGENESIS

Somatic embryogenesis is a process in which single cell or small group of cells initiate the developmental pathway normally followed only by the pre-dominant embryo within the seed (Williams, 1987).

According to Mascarenhas (1989) somatic embryogenesis is the development of embryos from somatic cells.

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

Somatic embryogenesis was first reported by Rienert (1958) and Steward (1958) in carrot. Embryogenesis generally proceeds from globular to 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).

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 pathway is pre-determined and the cells need only the synthesis of an inducer to express its potential. IEDCS on the other hand requires 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.

Potentially embryoidal cells are differentiated from cellular aggregates by their differential staining properties, conspicuous presence of large number of nucleoli per nucleus (Konar *et al.*, 1972). The embryogenic cell cluster consists of small cells densely cytoplasmic, rich in ribosome and contains numerous starch grains (Staritsky, 1970). The developmental activation of somatic embryogenesis involve synthesis of ribosomal RNA and protein (Sussex, 1972). More RNA and protein are found in embryogenic cells than in non-embrogenic cells (Raghavan, 1983). Verma and Dougall (1978) observed that the DNA content of embryogenic cell was increasing

exponentially and the nutrient content of culture medium was a limiting factor for the process.

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

2.1.1 Factors Influencing Somatic Embryogenesis

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

2.1.1.1 Explants

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

A complete sexual apparatus is not an essential pre-requisite for embryogenesis. Sometimes individual cells or cells from group may escape and give rise to either embryoids or nodular embryogenic callus consisting of pro-embryoids which are bipolar structures and can germinate into plantlets under suitable condition (Williams, 1987).

Atree and Fowke (1991) reported that somatic embryos could be induced only from embryonic tissues. Somatic embryos have been reared from haploid microspores (Guha and Maheswari, 1964, Nitsch and Nitsch, 1969), diploid cell and triploid cells (Sita *et al.*, 1979).

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

Ling *et al.* (1990) could obtain somatic embryos from hypocotyl segments of satsuma mandarins (*Citrus unshiu*). In mandarins (*Citrus reticulata*), somatic embryogenesis and whole plant generation was achieved from epicotyl, cotyledon, leaf and root explants (Gill *et al.*, 1995). Belkoura *et al.* (1995) also reported that somatic embryogenesis could be achieved in Troyer citrange from hypocotyl segments and nucellar embryos.

In *Aegle marmelos* embryo axis containing one-fourth part of the cotyledon from decoated seeds were used as explant for inducing somatic embryogenesis (Islam *et al.*, 1996).

Tian and Iwamasa (1997) reported that somatic embryogenesis and plant regeneration could be achieved from immature seed in plants related to *Citrus* genera. According to Deb (2001) cotyledonary segments of *Melia azedarach* developed into somatic embryos.

In the case of yooza (*Citrus junos*) somatic embryogenesis and plant regeneration was achieved when shoot tips and immature ovules were cultured (Song *et al.*, 1991a).

Kato (1996) reported that somatic embryogenesis could be induced in tea from immature leaves of *in vitro* grown tea shoots.

In plants like *Chlorophytum borivilianum* (Arora *et al.*, 1999), *Clitoria ternatea* (Malabadi and Nataraja, 2002), *Curculigo orchioides* (Suri *et al.*, 1998) and *Tylophora indica* (Manjula *et al.*, 2002) leaf segments were used as explants for inducing somatic embryogenesis.

In Kinnow mandarin callus initiation occurred at cut end of the internode explants (Praveen *et al.*, 2003).

Plant regeneration through direct somatic embryogenesis was established in cashew using nucellus and embryo mass (Rekha. 1999) and also from seed coat (Martin, 2003).

2.1.1.2 Basal Medium

2.1.1.2.1 Induction

Somatic embryos have been grown on a wide range of media from relatively dilute White's medium (White, 1963) to the more concentrated formulation of B_5 (Gamborg *et al.,* 1968), SH (Schenk and Hildebrandt, 1972) and MS (Murashige and Skoog, 1962). The B_5, SH and MS media are classified as high salt media and MS in particular has about ten times the salt concentration than White's medium.

In a survey of different studies conducted on somatic embryogenesis in various crop plants, Evans *et al.* (1981) noted 70 per cent of the explants were cultured on MS medium or modified MS medium. The key element of MS medium is the presence of high level of nitrogen in the form of ammonium nitrate. Another feature of MS media in inducing somatic embryogenesis, is the presence of chelated iron, in the form of iron EDTA. In the absence of iron, embryo development fails to pass from globular stage to heart shape stage (Heberle-Bors, 1980).

Induction treatment is required for redetermining the differentiated cells and the development of embryogenically determined cells (Ammirato. 1987). Different types of media were used for induction like MS (Murashige and Skoog, 1962), B_5 (Gamborg *et al.*, 1968), MT (Murashige and Tucker.1969) or SH (Schenk and Hildebrant. 1972) media. MS medium (Evans *et al.*, 1981) is the most widely used basal medium for induction of somatic embryogenesis.

Nito and Iwamasa (1990) induced callus from juice vesicles of satsuma on MS medium. In yooza (*Citrus junos*) MS and MT media were

proved to be ideal for induction (Song *et al.*, 1991a). Callus induction in mandarin oranges occurred in MS medium (Gill *et al.*, 1995). Globular somatic embryos of *Aegle marmelos* were also observed in MS medium (Islam *et al.*, 1996). Induction occurred in cultures of *Phoenix dactylifera* (Sharon and Shankar, 1998) in MS medium. This medium was also used for induction of somatic embryogenesis in mango (Muralidharan *et al.*, 1994) and jack (Rao *et al.*, 1981).

Somatic embryogenesis and whole plant generation were obtained in MS medium in the case of *Citrus reticulata* (Vijayakumari & Singh, 2001). In medicinal plants like safed musli (Arora *et al.*, 1999) and *Clitoria ternatea* (Malabadi and Nataraja, 2002) MS medium favoured the induction of embryogenic callus.

Induction was noticed in cultures of *Citrus unshiu* in MT basal medium (Ling *et al.*, 1990). Jumin and Nito (1996) reported induction of somatic embryos of citrus in MT basal medium. Fiore *et al.* (2002) could also obtain callus induction in lemon and sweet orange on MT basal medium.

Sy *et al.* (1991) used SH medium with supplements for induction of somatic embryogenesis from cashew cotyledon.

2.1.1.2.2 Initiation

Initiation of somatic embryos occurred when tissues were transferred from a medium containing high amount of auxin to a medium with low amount of auxin or no auxin at all. The medium contained nitrogenous compounds like amino acids (Reinert, 1958).

In the somatic embryogenesis of mandarins (*Citrus reticulata*), initiation occurred in MS medium (Gill *et al.*, 1995). Initiation of somatic embryos of *Aegle marmelos* was reported in MS basal medium (Islam *et al.*, 1996, Arumugam and Rao, 2000). Initiation of embryoids in sweet orange

occurred in MS medium (Carimi *et al.*, 1998). In *Holostemma ada-kodian* also MS medium favoured initiation (Martin, 2003).

According to Wu *et al.* (1990) citrus cultivars when inoculated in MT medium initiated somatic embryos. In the somatic embryogenesis of satsuma (*Citus unshiu*), embryo initiation occurred in MT medium (Ling *et al.*, 1990). Reports by Kunitake *et al.* (1991) also showed that MT medium was ideal for initiation in satsuma mandarins. Jumin and Nito (1995) initiated somatic embryogenesis of orange jessamine (*Murraya paniculata)* in MT medium.

For the production of mango somatic embryos modified B_5 medium was significantly more effective than WPM, MS or modified MS (Dewald *et al.*, 1989). Jaiswal (1990) and Litz *et al.* (1991) reported that a combination of B_5 major salts and MS minor salts could be used for the initiation of mango somatic embryos.

2.1.1.2.3 Maturation

For the normal germination of somatic embryos proper maturation is very important. Improper maturation will result in precocious germination, which is undesirable, since it may produce abnormal plants.

Tropical tree species possessing seeds with large embryos are found to have difficulty in controlling somatic embryo development to full maturity (Wang and Janik, 1986). Once the embryo is transferred to maturation medium, it is anticipated that the organizational events will proceed as in zygotic embryo maturation (Ammirato, 1987).

According to Nito and Iwamasa (1990), MS medium was found to be ideal for the maturation of satsuma (*Citrus unshiu*). In lemon, maturation occurred in MS medium (Carimi *et al.*, 1994).

Fiore *et al.* (2002) reported maturation of somatic embryos of lemon and sweet orange in MT medium.

2.1.1.2.4 Germination

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

Rey *et al.* (1995) observed that the germination of citrus was best in MS medium. Reports of Carimi *et al.* (1998) showed that higher germination of somatic embryos of sweet orange occurred in MS basal medium.

Islam *et al.* (1996) and Arumugam and Rao (2000) reported germination of somatic embryos of *Aegle marmelos* in half strength MS basal medium. Deb (2001) reported germination of somatic embryos of *Melia azaderach* in MS medium containing two per cent sucrose.

Germination of satsuma mandarin occurred in MT basal medium (Kunitake *et al.*, 1991). In yooza (*Citrus junos*) plantlet regeneration takes place in MT medium (Oh *et al.*, 1992). Similarly Fiore *et al.* (2002) could also obtain germination of somatic embryos of sweet orange and lemon in MT basal medium.

2.1.1.3 Plant growth substances

Growth and morphogenesis of plant tissue *in vitro* are largely governed by the composition of hormones in the culture media.

2.1.1.3.1 Induction

The presence of auxin in the medium is generally essential for the induction of somatic embryogenesis. In yooza (*Citrus junos*) induction medium supplemented with 2, 4-D as plant growth substance (Song *et al.*, 1991a) was found to be highly responsive.

In satsuma (*Citrus unshiu)* induction of embryogenic callus occurred in medium containing 2,4-D and BAP (Ling *et al.*, 1990). Embryogenic callus induction in *Citrus grandis* was the best in medium supplemented with 2,4-D. Of the combination treatments, 2,4-D and BA gave best results (Song *et al.,* 1991b). 2,4–D and BAP when supplemented in the media could also, induce somatic embryogenesis in lemon (Carimi *et al.*, 1994). In orange jessamine also 2,4-D and BAP combination induced somatic embryos (Jumin and Nito, 1995). Islam *et al.* (1996) reported the induction of somatic embryos in *Aegle marmelos* in cultures containing 2,4-D and BA.

Rekha (1999) reported that a combination of 2, 4-D, BAP and NAA was the best for inducing somatic embryogenesis in cashew cultures. Arumugam and Rao (2000) reported that a combination of 2, 4-D, BA and ABA favoured embryo induction in *Aegle marmelos.*

Auxins, other than 2, 4-D can also induce somatic embryogenesis in woody plants. In satsuma (*Citrus unshiu*), for induction, the basal media was supplemented with NAA and GA_3 (Nito and Iwamasa, 1990). Kunitake *et al.* (1991) reported induction of embryogenic callus in a medium supplemented with adenine, in the case of satsuma mandarins.

Nair *et al.* (1993) obtained callus from immature cotyledons of cashew when IBA and BA were incorporated in the basal medium. In mandarins, NAA and combination of NAA and Kinetin were reported to induce callus (Gill *et al.*, 1995). In Troyer citrange, embryo induction occurred in medium containing GA_3 (Belkoura *et al.*, 1995).

In citrus related species (*C. reticulata, C.sinensis, C. aurantifolium)*, medium containing BAP induced somatic embryos (Pimentel and Villegas, 1996)

Somatic embryos are observed to occur even without the application of exogenous plant growth regulators as in case of *Citrus sinensis* (Vardy, *et al.*,

1975) and *Camelia sinensis* (Wachira and Oganda, 1995). Ling *et al.* (1990) initiated somatic embryogenesis in satsuma mandarin in medium without any plant growth substances. Jumin and Nito (1996) induced somatic embryogenesis in citrus, cultured in a medium without any growth regulators.

2.1.1.3.2 Initiation

Initiation occurred in medium containing no auxin and sometimes with low concentration of auxin. Combinations of plant growth substances are important in initiating and maintaining growth and development of somatic embryos.

Cytokinins also help to initiate somatic embryos in a number of plant species (Ammirato, 1983). The requirement is often specific. Zeatin is the only cytokinin which could promote somatic embryogenesis in carrot (Fujimura and Komamine, 1975).

In cultures of lemon, initiation of somatic embryos occurred in medium supplemented with BA or NAA (Carimi *et al.*, 1994). In studies conducted on six plant species related to citrus, Jumin and Nito (1996) observed that BA alone initiated somatic embryos. Gill *et al.* (1995) reported initiation of callus of mandarin in medium supplemented with BA. Vijayakumari and Singh (2001) could initiate somatic embryogenesis of Nagpur mandarins in medium supplemented with NAA. GA_3 was found to be the most effective in initiation of somatic embryogenesis in cultures of Mexican lime (*Citrus aurantiifolia*) (Ghazvini and Shirani, 2002).

Ling *et al.* (1990) could initiate somatic embryogenesis of satsuma mandarins in hormone free medium. Song *et al.* (1991b) reported that initiation of somatic embryos in dangyooza (*C. grandis*) occurred in a medium devoid of any plant growth substances.

2.1.1.3.4 Maturation

The plant growth substance which aid the maturation process, is the natural growth inhibitor, ABA. It permits embryo maturation to continue but inhibits proliferation and 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). ABA can prevent induction of embryogenic competence and thereby inhibit the formation of secondary embryoids. It provided a high frequency of embryos and medium lacking ABA resulted in large number of multiple embryos developing from single proembryos (Ammirato, 1987).

Dewald *et al.* (1989) reported that ABA was effective for maturation of mango somatic embryos and could control developmental abnormalities. Jana *et al.* (1994) could obtain mature somatic embryos of mango by supplementing ABA 1.0 mg l^{-1} in the medium According to Sulekha (1996) maturation of somatic embryos of mango variety 'Neelum' was attained when ABA 5.0 mg l^{-1} was supplemented in the medium.

Monsalud *et al.* (1995) observed that ABA could reverse hyper hydricity of secondary somatic embryos 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.

In citrus, Kochba *et al.* (1974) initiated maturation using GA_3 as the plant growth substance. Ling *et al.* (1990) reported maturation of somatic embryos of satsuma mandarins in media supplemented with GA_3.

In yooza (*Citrus junos*) plant regeneration from somatic embryos was found to be maximum when the medium was supplemented with zeatin (Oh

et al.,1992). In *Citrus reticulata*, Kinetin alone or in combination with IAA promoted plantlet formation (Vijayakumari and Singh, 2001).

Jumin and Nito (1995) could develop plantlets of orange jessamine in medium without any plant growth regulator.

2.1.1.3.5 Germination

Germination of somatic embryos will be normal, only if they are physiologically matured. If embryos are not properly matured, precocious germination will take place. Somatic embryos can even germinate on agar medium without plant growth substance (Razdan, 1993). Jaiswal (1990) obtained germination of mango somatic embryos without exogenous application of plant growth substances.

Germination medium supplemented with GA_3 caused germination of somatic embyos in *Citrus sinesis* (Rangaswami, 1961) and *Santalum album* (Sita *et al.*, 1979).

In certain cases cytokinins are required for the growth of embryos into plantlets (Kavathekar and Johri, 1978). Jana *et al.* (1994) employed BA for the germination of mango somatic embryos.

In *Citrus paradisi* germination of embryoids was reported in medium containing $GA_3$and BA (Dhillon *et al.*, 1989). Germination of satsuma mandarin somatic embryos occurred in medium containing GA_3 (Kunitake *et al.*, 1991). Arora *et al.* (1999) reported the germination of *Chlorophytum borivilianum* in medium containing BAP alone.

Somatic embryos of *Aegle marmelos* germinated in a medium supplemented with a combination of BA and GA_3 (Arumugan and Rao, 2000).

2.1.1.4 Other supplements

2.1.1.4.1 Coconut water

Use of organic supplements proved to be better when chemically defined mixture fail to induce somatic embryogenesis. The earliest success in somatic embryogenesis was achieved in media supplemented with coconut milk or coconut water (Steward *et al.*, 1958). Coconut water is extremely useful both in embryo induction and maturation (Vasil and Vasil, 1980).

Reduced form of nitrogen is required for embryo initiation and maturation. Coconut water is a good source of reduced nitrogen in the medium (Tulecke, *et al.*, 1961 and Razdan, 1993)

In *Clitoria ternatea*, induction and maturation occurred in medium supplemented with coconut water (Malabadi and Nataraja, 2002). Giri *et al.* (1993) reported induction of somatic embryos of Aconitum in medium containing 10 per cent coconut water.

According to Dewald *et al.* (1989) maturation and germination of somatic embryo of mango was achieved by sequential transfer of somatic embryos on to medium containing 20 percent coconut water.

Somatic embryogenesis of *Aegle marmelos* occurred in medium supplemented with 20 per cent coconut water (Islam *et al.*, 1996).

2.1.1.4.2 Malt extract

In yooza (*Citrus junos*), malt extract proved to be a better supplement than yeast extract and coconut milk (Song *et al.*, 1991a). Addition of malt extract induced higher percentage of cotyledonary embryos in cultures of Nagpur mandarins (Parthasarathy and Nagaraju, 2000; Vijayakumari and Singh, 2001). According to Ghazvini and Shirani (2002) malt extract increased embryo formation in Mexican lime (*Citrus aurantiifolia*).

2.1.1.4.3 Carbon source

All culture media requires presence of carbon as energy source. Choice of sugar depends on cultured plant tissue and purpose of experiment. Sucrose appears to be the most effective reduced carbon source for somatic embryogenesis (Ammirato, 1983)

By increasing the sucrose concentration in carrot somatic embryo cultures, precocious germination was prevented (Ammirato and Steward, 1971).

In satsuma mandarins, somatic embryogenesis occurred in medium containing lactose, but somatic embryos developed to plantlets in media supplemented with sucrose (Ling *et al.*, 1990). Kunitake *et al.* (1991) could also obtain embryoids of satsuma in lactose containing media and germination in sucrose containing media. Rey *et al.* (1995) reported that 50.0 g l^{-1} sucrose produced somatic embryogenesis in citrus. Reports by Jumin and Nito (1996) revealed that 50.0 g l^{-1} sucrose initiated embryogenesis in many citrus species.

In *Aegle marmelos*, 40.0 g l^{-1} sucrose induced somatic embryo formation (Islam *et al.*, 1996). Kumar *et al.* (2002) reported the induction of embryogenic callus of *Gymnema* in 20.0 g l^{-1} sucrose cultures.

In certain cases, sugars other than sucrose have been used. In satsuma (*Citrus unshiu*), medium with lactose produced somatic embryos (Ling *et al.*, 1990). Plantlet formation in orange jessamine occurred in a medium containing lactose as the carbon source (Jumin and Nito, 1995). In citrus related species 50.0 g l^{-1} lactose proved to be ideal for somatic embryogenesis (Thian and Iwamasa, 1997). Ricci *et al.* (2002) reported induction of higher number of somatic embryos with galactose or lactose in citrus plants.

2.1.1.4.4 Activated Charcoal

The addition of activated charcoal to the medium has proven useful for somatic embryo development. Activated charcoal acts by absorbing substances inhibitory to somatic embryo formation and their further growth.

Analysis had shown that media with charcoal substantially lowered the level of phenyl acetate and p-OH benzoic acid, compounds that inhibited somatic embryogenesis (Fridborg *et al.*, 1978). Activated charcoal also adsorb 5-hydromethyl furfural, an inhibitor formed by sucrose degradation during autoclaving as well as substantial amount of auxins and cytokinins (Weatherhead *et al.*, 1978).

Activated charcoal hastened the differentiation of somatic embryos in mango (Litz, 1986). Bindu (1995) and Sulekha (1996) observed that activated charcoal was essential in mango somatic embryogenesis .

Pan and Stadon (2001) reported that in carrot cultures somatic embryos were produced in medium containing activated charcoal, whereas no somatic embryos were formed in the absence of activated charcoal.

2.1.1.5 Culture conditions

The environmental and physical conditions are 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 (Tulecke, 1987). According to Simola (1987), the poor ability of regeneration in tree cultures was probably due to improper culture conditions.

2.1.1.6 Light and Darkness

Somatic embryogenesis has been known to occur under a wide variety of light and dark regime. For somatic embryogenesis, the requirement of light varies according to the crop and stage of development. Rao *et al.* (1981)

reported induction of callus in jack occurred in complete darkness. Initiation of somatic embryogenesis in sweet orange occurred in suspension cultures kept under darkness (Carimi *et al.*, 1998).

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

In *Aegle marmelos* for initiation of somatic embryos, cultures were kept in light (Islam *et al.*, 1996)

Deb (2001) reported induction of somatic embryos of *Melia azaderach* in 12h photoperiod.

2.1.1.7 Temperature

The growth rate of tissue explants closely relates to the prevailing temperature. Growth may occur at temperature below 20°C. A temperature range of 26-28°C is considered as optimum.

In lemon, a temperature range of 25-27°C induced somatic embryogenesis (Carimi *et al.*, 1994). Jumin and Nito (1995) also reported that when the cultures of orange jessamine were kept under darkness at a temperature 25°C, it favored somatic embryogenesis. Islam *et al.* (1996) observed that 26±1°C was ideal for the formation of somatic embryos in *Aegle marmelos.*

A high temperature of 28 ± 1°C favoured somatic embryogenesis in date palm (Sharon and Shankar, 1998).

2.1.1.8 Mode of Culture

The original reports showed that, in carrot somatic embryogenesis will proceed on callus cultures grown on semi-solid medium (Rienert, 1958) and in liquid suspension cultures (Steward *et al.*, 1958).

Somatic embryogenesis in citrus occurred in solid culture (Rey *et al.*, 1995). In sweet orange, explants were maintained in semi solid medium. The friable callus were used to initiate suspension cultures. Eventhough, in sweet orange high rate of embryogenic callus occurred in liquid medium, subculturing onto solid medium was necessary for high frequency production of normal somatic embryos.(Carimi *et al.*, 1998).

Litz *et al.* (1998) reported embryogenic nurse culture technique for the induction of embryogenic potential in mango cultures.

Filter paper bridge technique was developed by Goodwin (1966) for the rapid growth of potato buds. Smith (1973), Mc William *et al.* (1974) and Drew (1979) also observed the formation somatic embryos on filter paper bridges perched above the liquid media.

In coffee, the endosperm extract of coffee in MS medium with different concentration of kinetin helped in the rapid germination of somatic embryos (Sreenath *et al.*, 2002). In *Holostemma ada-kodian* suspension cultures were found to be superior to static cultures (Martin, 2003).

2.1.1.9 **Frequency of subculture**

Subculturing at shorter interval was found to be beneficial during somatic embryogenesis in apple (Paul *et al.*, 1994). Carimi *et al.* (1998) observed that frequent subculturing was necessary to increase the *in vitro* response of sweet orange somatic embryos.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

Studies on *in vitro* somatic embryogenesis in bael [*Aegle marmelos* (L.) Corr.] were carried out at the Plant Molecular Biology and Biotehnology Centre, College of Agriculture, Vellayani, during 2001-2003.

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

3.1 VARIETIES

Local collections of bael seen in South Kerala were used.

3.2 EXPLANT

Cotyledons, integuments, hypocotyls, nucellus, *in vitro* and *ex vitro* leaves, in *vitro* roots and internodes were used for the study.

3.2.1 Cotyledons

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

3.2.2 Integuments

Integument is the outer envelope of an ovule, which, with other parts form the seed coat, enclosing the nucellus. Integuments are believed to be formed as a result of reduced and fused branches or leaves. In some species, the cells of inner

Plate 1. Fruit of bael

integument serves as nutritional support for the developing embryo sac and later hardens and act as a protective layer for the ovule.

3.2.3 Hypocotyl

Hypocotyl is the part of embryo or seedling below the cotyledonary node and above the root, the transition region connecting the stem and root. Thus hypocotyl is the first formed stem unit.

3.2.4 Nucellus

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

3.3 COLLECTION AND PREPARATION OF EXPLANTS

Tender as well as mature ripe fruits were collected from healthy bael trees for taking the cotyledons, nucellus and integuments (Plate 1). Internodes, hypocotyls and leaves of *in vitro* grown plants were also used for the study. *Ex vitro* leaves were also inoculated.

The fruits were cut open and seeds were extracted by washing out the mucilaginous coating. The seeds were again washed thoroughly with few drops of labolene. The seed coat was removed and cotyledons were separated.

For taking the hypocotyls, the seeds were germinated in a medium with low concentration of plant growth substance. After proper germination of the seed, the hypocotyls were cut off and used for inoculation.

Immature *ex vitro* leaves (15 -20 days old) were collected from current season's growth of mature six year old tree. After removing the petiole, the leaves were washed thoroughly in tap water followed by distilled water and sterile

water. Each leaf was cut into two or three pieces and then subjected to surface sterilization.

3.4 SURFACE STERILIZATION

Surface sterilization of plant materials was carried out inside a laminar airflow chamber. The cotyledons were surface sterilized using 0.08 per cent mercuric chloride solution for five minutes. After draining out the solution, the explants were washed 4-5 times in sterile double glass distilled water.

The tender *ex vitro* leaves were surface sterilized in a sterile beaker using mercuric chloride (0.08 per cent) for ten minutes and then washed with sterile double glass distilled water for four to five times.

3.5 INOCULATION AND INCUBATION

Inoculation operations were carried out inside laminar airflow chamber. The tools and glasswares required for inoculation were washed thoroughly, rinsed with double glass-distilled water, covered with aluminium foil and then autoclaved at 121°C and 1.06 kg cm^{-2} pressure for 40 minutes.

The cotyledons were cut transversely and inoculated after removing the embryonic portion. *Ex vitro* leaves, *in vitro* leaves, roots, internodes and hypocotyls were also inoculated. The cotton plugs of the test tubes were removed and the rim was flamed. The explants were then inoculated on to the medium. The rim of the culture vessels was again flamed and closed with cotton plugs. The cultures were incubated either in light or in dark as per the treatment.

3.6 MEDIA

The basal media used for the study were full and half strength major salts of MS (Murashige and Skoog, 1962), B_5 (Gamborg *et al.*, 1968), SH (Schenk and Hildebradndt, 1972) and MT (Murashige and Tucker, 1969). The chemicals used for the preparation of the media were of analytical grade from British Drug House, Mumbai, Sisco Research Laboratory, Mumbai.

Standard procedures were followed for the preparation of basal media (Thorpe, 1980). Stock solutions of major and minor nutrients were prepared by dissolving the required quantity of chemicals in specific volume of double glass distilled water and stored at refrigerated conditions. The required quantity of each stock solution was pipetted out to a 1000 ml, thoroughly washed and rinsed beaker. Sucrose, glutamine, inositol were added fresh and dissolved. Tender coconut water was also added as per treatments and the volume was made up to 950 ml using double glass distilled water. With an electronic pH meter (Philips make, model pp9046) the pH of the solution was adjusted to values ranging between 5.6 and 5.8 using 0.1 N NaOH or 0.1 N HCl.

In the case of solid medium, agar was added and the final volume was made upto 1000 ml.

The solution was thoroughly stirred for uniform mixing and boiled till agar melted. Activated charcoal, when used in the medium was added at this stage. The medium was poured to pre-sterilized culture vessels. 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 minutes.

3.6.1 Liquid Culture

For liquid culture, media without solidifying agent was prepared with appropriate quantity of growth supplements. They were autoclaved at 121°C and 1.06 kg cm^{-2} pressure for 20 minutes. The explants were inoculated on to the medium under aseptic conditions.

3.6.1.1 Filter Paper Bridge Technique

The filter paper bridge prevents direct contact of the plant material with the media. A filter paper bridge in the shape of letter 'M' was made

and inserted into the test tube. The test tube was then plugged tightly with cotton and autoclaved at 121°C and 1.06 kg cm^{-2} pressure for 40 minutes. Liquid medium was prepared with specific quantity of plant growth substances. The sterilized medium was then poured into the test tube containing paper bridge in such a way that two arms of the filter paper dip into the medium. The bridge with the explant was kept above the medium.

3.6.2 Nurse Culture

In this method the explant was kept in the medium surrounded by calli already initiated in a medium. Medium was prepared with suitable plant growth substances and other supplements and then autoclaved at 121°C and 1.06 kg cm^{-2} pressure for 20 minutes. Calli were inoculated on to the medium. The explant (cotyledon) after surface sterilization was inoculated in the center of the calli.

Nurse culture with plant extract was also tried. Mature seed was inoculated in a medium without plant growth substance. After germination, the seedling was taken out and immersed in distilled water and kept for three days. The solution was then filter sterilized. For filter sterilization the membrane was fitted into filter assembly holders. The entire assembly along with filter was autoclaved at 121°C and 1.06 kg cm^{-2} pressure for 40 minutes. A graduated syringe carrying the liquid was fixed to one end of the filter assembly and the solution gradually pushed through the membrane present in the middle of the assembly. The filtered sterile solution dripping out from the other end was added directly to the melted medium. All these operations were carried out inside laminar airflow chamber. After setting of the medium the explant was inoculated into it.

3.7 SOMATIC EMBRYOGENESIS

3.7.1 Induction of Somatic Embryogenesis

3.7.1.1 Treatments

Explants were subjected to different treatments for the induction of somatic embryogenesis. The treatments involved different levels of plant growth substances, sucrose, coconut water, malt extract and activated charcoal. Different basal media like MS (full and half strength), MT, B_5, SH and WPM were tried in inducing somatic embryogenesis. The treatments were replicated 12 times.

3.7.1.1.1 Plant Growth Substances

Hundred and eight treatments involving 2, 4-D, BA, GA_3, ABA, NAA, IAA, IBA and kinetin in combination and alone were tried for inducing somatic embryogenesis (Table 1a, b, c and d).

3.7.1.1.2 Basal Media

Comparison on the effect of various basal media such as MS (full and half strength), MT, SH, B_5 and WPM on induction of somatic embryogenesis was made. The treatments were replicated 12 times.

3.7.1.1.3 Sucrose

Varying levels of sucrose (20.0, 30.0, 40.0 and 60.0 g l^{-1}) were tried to study their effects on induction of somatic embryogenesis.

3.7.1.1.4 Coconut Water

The effect of coconut water at different levels (0.0, 100.0 and 200.0 ml l^{-1}) was studied in inducing somatic embryogenesis.

3.7.1.1.5 Activated Charcoal

Three levels of activated charcoal (0.5, 1.0, 1.5 g l^{-1}) along with zero level were tried to study its impact on induction of somatic embryogenesis.

Table 1a. Plant growth substances tried for inducing somatic embryogenesis in bael

Treatments	Plant growth substances (mg l^{-1})
I_1	2,4-D 0.1, BA 0.1
I_2	2,4-D 0.2, BA 0.2
I_3	2,4-D 0.3, BA 0.3
I_4	2,4-D 0.4, BA 0.4
I_5	2,4-D 0.1, BA 0.2
I_6	2,4-D 0.1, BA 0.3
I_7	2,4-D 0.1, BA 0.4
I_8	2,4-D 0.2, BA 0.1
I_9	2,4-D 0.2, BA 0.3
I_{10}	2,4-D 0.2, BA 0.4
I_{11}	2,4-D 0.3, BA 0.1
I_{12}	2,4-D 0.3, BA 0.2
I_{13}	2,4-D 0.3, BA 0.4
I_{14}	2,4-D 0.4, BA 0.1
I_{15}	Kinetin 1.0, NAA 1.0
I_{16}	Kinetin 1.0, NAA 2.0
I_{17}	BA 1.0, NAA 1.0
I_{18}	Kinetin 1.0, IAA 1.0
I_{19}	BA 1.0, IAA 1.0
I_{20}	BA 0.25
I_{21}	BA 0.5
I_{22}	BA 0.75
I_{23}	BA 1.0
I_{24}	BA 1.25
I_{25}	BA 1.5
I_{26}	BA 1.75
I_{27}	BA 2.0
I_{28}	2,4-D 0.5, BA 0.5
I_{29}	2,4-D 1.0, BA 0.5
I_{30}	2,4-D 1.5, BA 0.5
I_{31}	2,4-D 2.0, BA 0.5
I_{32}	2,4-D 0.5, BA 1.0
I_{33}	2,4-D 1.0, BA 1.0
I_{34}	2,4-D 1.5, BA 1.0
I_{35}	2,4-D 2.0, BA 1.0
I_{36}	2,4-D 0.5, BA 1.5
I_{37}	2,4-D 1.0, BA 1.5
I_{38}	2,4-D 1.5, BA 1.5
I_{39}	2,4-D 2.0, BA 1.5
I_{40}	2,4-D 0.5, BA 2.0
I_{41}	2,4-D 1.0, BA 2.0

Table 1a Continued

I_{42}	2.4-D 1.5, BA 2.0
I_{43}	2,4-D 2.0, BA 2.0
I_{44}	2,4-D 0.1, BA 0.5, ABA 0.5
I_{45}	2,4-D 0.5, BA 0.5, ABA 0.5
I_{46}	2,4-D 1.0, BA 0.5, ABA 0.5
I_{47}	2,4-D 0.1, BA 1.0, ABA 0.5
I_{48}	2,4-D 0.1, BA 1.5, ABA 0.5
I_{49}	2,4-D 0.5, BA 0.5, ABA 1.0
I_{50}	2,4-D 1.0, BA 0.5, ABA 1.0
I_{51}	2,4-D 1.5, BA 0.5, ABA 1.0
I_{52}	2,4-D 2.0, BA 0.5, ABA 1.0
I_{53}	2,4-D 0.5, BA 1.0, ABA 1.0
I_{54}	2,4-D 0.5, BA 0.5, ABA 1.5
I_{55}	2,4-D 1.0, BA 0.5, ABA 1.5
I_{56}	2,4-D 1.5, BA 0.5, ABA 1.5
I_{57}	2,4-D 0.1
I_{58}	2,4-D 0.2
I_{59}	2,4-D 0.3
I_{60}	2,4-D 0.4
I_{61}	2,4-D 0.5
I_{62}	2,4-D 1.0
I_{63}	2,4-D 1.0, NAA 1.0, BA 1.0
I_{64}	BA 1.0, GA_3 1.0
I_{65}	NAA 0.5, kinetin 2.0
I_{66}	2,4-D 4.0, NAA 4.0, kinetin 4.0
I_{67}	2,4-D 3.0, NAA 3.0

Culture medium-MS + Sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1} and Agar 6.0 g l^{-1}

Table 1b. Plant growth substances tried for inducing somatic embryogenesis in bael

Treatments	Plant growth substances (mg l^{-1})
I_{68}	2,4-D 0.1, BA 0.1
I_{69}	2,4-D 0.1, BA1.0
I_{70}	2,4-D 0.5, BA 0.1
I_{71}	2,4-D 0.5, BA 1.0
I_{72}	2,4-D 1.0, BA 0.1
I_{73}	2,4-D 1.0, BA 1.0
I_{74}	2,4-D 2.0, BA 0.1
I_{75}	2,4-D 2.0, BA 1.0
I_{76}	2,4-D 1.0, BA 2.0
I_{77}	2,4-D 0.5, BA 1.5
I_{78}	2,4-D 1.0, BA 0.5 GA_3 5.0
I_{79}	2,4-D 0.5, BA 0.5 NAA 1.0
I_{80}	2,4-D 1.0, BA 1.0 ABA 1.0
I_{81}	2,4-D 1.0, BA 1.0 NAA 0.1
I_{82}	2,4-D 1.0, BA 1.0 GA_3 5.0
I_{83}	2,4-D 5.0, BA 1.0 NAA 1.0
I_{84}	BA 2.0 GA_3 10
I_{85}	2,4-D 1.0, BA 1.0 NAA 1.0
I_{86}	GA_3 1.5
I_{87}	IBA 0.5
I_{88}	IBA 1.0
I_{89}	GA_3 0.5
I_{90}	GA_3 1.0

Culture medium:

Half strength MS + Sucrose 40.0g l^{-1}, CW 200.0ml l^{-1} and Agar 6.0g l^{-1}

Table 1c. Plant growth substances tried for inducing somatic embryogenesis in bael

Treatments	Plant growth substances (mg l^{-1})
I_{91}	2,4-D 0.1, BA 0.1
I_{92}	2,4-D 0.2, BA 0.1
I_{93}	2,4-D 0.3, BA 0.1
I_{94}	2,4-D 0.4, BA 0.1
I_{95}	2,4-D 0.5, BA 0.1
I_{96}	2,4-D 0.1, BA 0.2
I_{97}	2,4-D 0.1, BA 0.3
I_{98}	2,4-D 0.1, BA 0.4
I_{99}	2,4-D 0.1, BA 0.5

Culture medium: MT + Sucrose 40.0 g l^{-1}, CW 200.0 ml $l^{-1}$and Agar 6.0 g l^{-1}

Table 1d. Plant growth substances tried for inducing somatic embryogenesis in bael

Treatments	Plant growth substances (mg l^{-1})
I_{100}	2,4-D 0.1, BA 0.1
I_{101}	2,4-D 0.2, BA 0.1
I_{102}	2,4-D 0.3, BA 0.1
I_{103}	2,4-D 0.4, BA 0.1
I_{104}	2,4-D 0.5, BA 0.1
I_{105}	2,4-D 0.1, BA 0.2
I_{106}	2,4-D 0.1, BA 0.3
I_{107}	2,4-D 0.1, BA 0.4
I_{108}	2,4-D 0.1, BA 0.5

Culture medium : SH + Sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1} and Agar 6.0g l^{-1}

3.7.1.1.6 Malt Extract

Two different levels of malt extract (250.0 and 500.0 mg l^{-1}) were compared for studying its effect in inducing somatic embryogenesis.

3.7.1.3 Culture Conditions

The culture were kept under light (3000 lux,16 hours photoperiod) provided by cool white fluorescent light or in darkness in order to study the effect of light on induction of somatic embryogenesis.

3.7.1.4 Frequency of Subculture

Subculturing was done at an interval of 10, 15 and 30 days in the medium of same composition to study its effect on induction of somatic embryogenesis.

Observations were recorded on the number of cultures survived and number of cultures initiating callus. Callus index (CI) was computed by multiplying per cent cultures initiating callus with growth score (G). Growth of the callus was assessed based on visual rating, with score 1.0 to the smallest and 4.0 to the largest. The mean score was expressed as growth score G.

3.7.2 Initiation of Somatic Embryos

The cultures from the induction media were transferred to initiation media.

3.7.2.1 Treatments

The treatments involved different levels of plant growth substances such as BA and GA_3 and varying levels of sucrose. Different basal media such as MS (full and half strength), MT, SH, B_5 and WPM were tried.

3.7.2.1.1 Plant Growth Substances

Fifteen treatments involving different levels of BA and GA_3 along with one treatment without plant growth substances were tried for initiation or expression of somatic embryos (Table 2). The treatments were replicated 12 times.

3.7.2.1.2 Basal media

Different basal media such as MS (full and half strength major salts), MT, SH, B_5 and WPM were tried.

3.7.2.1.2 Sucrose

The calli induced in induction media were transferred to initiation media with different levels of sucrose (20.0, 30.0, 40.0 and 60.0 g l^{-1}) for studying its effect on initiation of somatic embryos.

3.7.2.3 Observations

Observations were taken on the number of cultures survived, number of cultures initiating somatic embryos and other salient features of the embryoids.

Table 2. Plant growth substances tried for initiating somatic embryos

Treatments	Plant growth substances (mg l^{-1})
T_1	GA_3 0.5
T_2	GA_3 1.0
T_3	GA_3 1.5
T_4	GA_3 2.0
T_5	BA 0.1
T_6	BA 0.2
T_7	BA 0.3
T_8	BA 0.4
T_9	BA 0.5
T_{10}	BA 0.6
T_{11}	BA 0.7
T_{12}	BA 0.8
T_{13}	BA 0.9
T_{14}	BA 1.0
T_{15}	BA 2.0
T_{16}	No PGS

Culture medium: Half strength MS + Sucrose 40.0 g l^{-1} and Agar 6 g l^{-1}

RESULTS

4. RESULTS

Investigations were carried out for standardizing *in vitro* techniques *via* somatic embryogenesis for rapid clonal propagation of *Aegle marmelos*. at Plant Molecular Biology and Biotechnology Centre, College of Agriculture, Vellayani, during 2001-2003. The results of studies are presented in this chapter.

4.1 EXPLANTS

Different explants such as cotyledons, nucellus, integuments. internode of *in vitro* plants, hypocotyls, *in vitro* leaves, *in vitro* roots and *ex vitro* leaves were used for the study (Table 3, Fig. 1).

4.1.1 Cotyledons

The survival rate of culture in the different media tried varied from 75.00 to 91.60 per cent (Table 3). I_4 medium (MS + 2,4-D 0.4 mg l^{-1}, BA 0.4 mg l^{-1}, sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1}) recorded the highest percentage of survival (91.60 per cent). The lowest survival rate of 75.00 per cent was recorded in I_1 and I_2 medium (MS + 2,4-D 0.1 mg l^{-1}, BA 0.1 mg l^{-1}, sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1} and MS + 2,4-D 0.2 mg l^{-1}, BA 0.2 mg l^{-1}, sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1}).

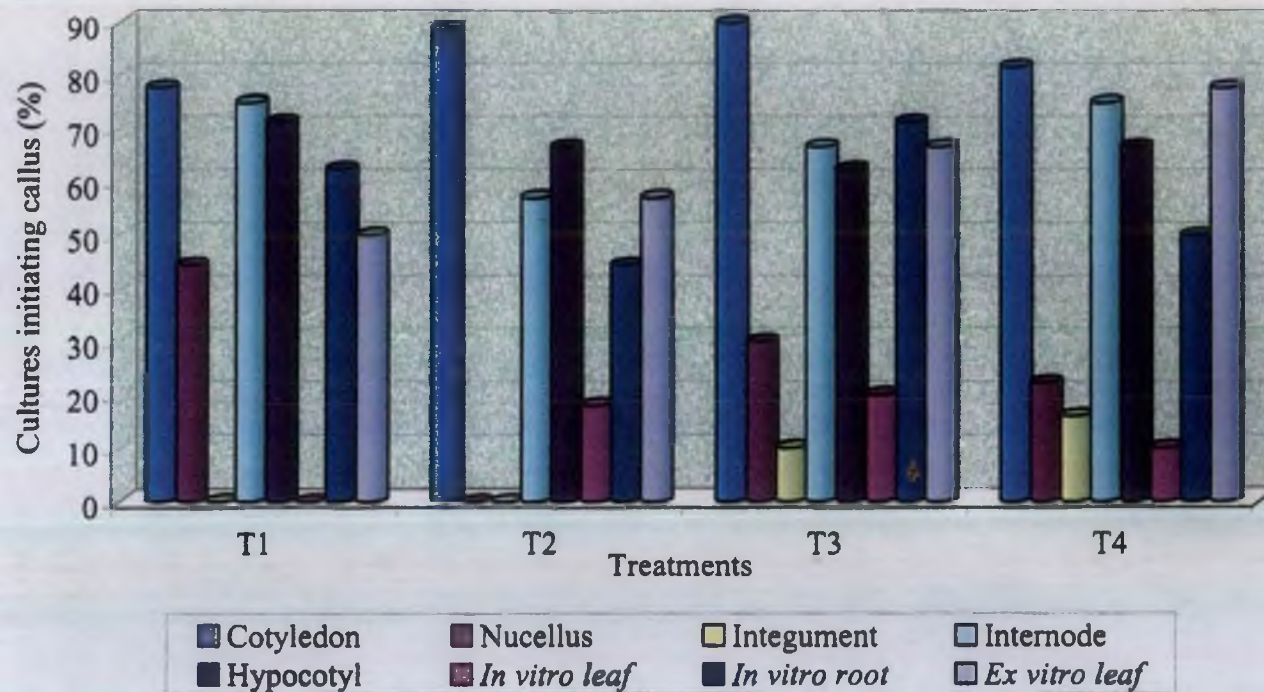
The highest per cent of cultures initiating callus (90.00 per cent) was obtained when cotyledons were inoculated in I_3 medium (MS + 2.4-D 0.30 mg l^{-1}, BA 0.3 mg l^{-1}, sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1}). Cultures in I_2 and I_4 (MS + 2,4-D 0.2 mg l^{-1}, BA 0.2 mg l^{-1}, sucrose 40.0 g l^{-1}. CW 200.0 ml l^{-1} and MS + 2,4-D 0.4 mg l^{-1}, BA 0.4 mg l^{-1}, sucrose 40.0 g l^{-1}. CW 200.0 ml l^{-1}) initiated 88.80 and 81.80 per cent callus respectively. The least response (77.78 per cent) was recorded in I_1 medium.

Table 3. Response of various explants of *Aegle marmelos* on the induction of somatic embryogenesis in four different culture media

Explants	Survival rate (%)				Cultures initiating callus (%)				Growth score				Callus index			
	I_1	I_2	I_3	I_4	I_1	I_2	I_3	I_4	I_1	I_2	I_3	I_4	I_1	I_2	I_3	I_4
Cotyledon	75.00	75.00	83.00	91.60	77.78	88.80	90.00	81.80	2.98	2.80	3.30	2.68	231.78	248.64	297.00	219.22
Nucellus	75.00	66.67	83.00	75.00	44.40	0.00	30.00	22.20	1.10	0.00	1.00	1.00	48.84	0.00	30.00	22.22
Integument	83.00	93.00	83.00	100.00	0.00	0.00	10.00	16.00	0.00	0.00	1.00	1.00	0.00	0.00	10.00	16.00
Internode	66.67	58.30	75.00	67.00	75.00	57.10	66.67	75.00	1.56	1.06	1.22	1.34	117.00	60.53	81.34	100.50
Hypocotyl	66.67	50.00	66.67	75.00	71.40	66.67	62.50	66.67	1.44	1.33	1.06	1.20	102.82	88.58	66.25	80.00
In vitro leaf	92.00	92.00	83.00	83.30	0.00	18.00	20.00	10.00	0.00	1.13	1.00	1.25	0.00	20.25	20.00	12.50
In vitro root	83.00	75.00	58.30	50.00	62.50	44.40	71.40	50.00	1.29	1.25	1.39	1.00	80.63	55.55	99.25	50.00
Ex vitro leaf	66.67	58.30	75.00	75.00	50.00	57.10	66.67	77.78	1.00	1.25	1.15	1.30	50.00	71.38	76.67	101.10

The data represents the average value of 12 replications

I_1 – MS + 2,4-D 0.1 mg l^{-1}, BA 0.1 mg l^{-1}, Sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1} and Agar 6.0 g l^{-1}
I_2 – MS + 2,4-D 0.2 mg l^{-1}, BA 0.2 mg l^{-1}, Sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1} and Agar 6.0 g l^{-1}
I_3 – MS + 2,4-D 0.3 mg l^{-1}, BA 0.3 mg l^{-1}, Sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1} and Agar 6.0 g l^{-1}
I_4 – MS + 2,4-D 0.4 mg l^{-1}, BA 0.4 mg l^{-1}, Sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1} and Agar 6.0 g l^{-1}



T1 – MS + 2,4-D 0.1 mg l^{-1}, BA 0.1 mg l^{-1}, Sucrose 40.0 g l^{-1}, Agar 6.0 g l^{-1} and CW 200.0 ml l^{-1}

T2 – MS + 2,4-D 0.2 mg l^{-1}, BA 0.2 mg l^{-1}, Sucrose 40.0 g l^{-1}, Agar 6.0 g l^{-1} and CW 200.0 ml l^{-1}

T3 – MS + 2,4-D 0.3 mg l^{-1}, BA 0.3 mg l^{-1}, Sucrose 40.0 g l^{-1}, Agar 6.0 g l^{-1} and CW 200.0 ml l^{-1}

T4 – MS + 2,4-D 0.4 mg l^{-1}, BA 0.4 mg l^{-1}, Sucrose 40.0 g l^{-1}, Agar 6.0 g l^{-1} and CW 200.0 ml l^{-1}

Fig. 1 Response of various explants of *Aegle marmelos* on induction of somatic embryogenesis in four different culture media

The highest growth score of 3.30 was obtained when the cotyledons were cultured in I_3 medium with a callus index of 297.0 whereas in I_1 and I_2 medium the growth score recorded were 2.98 and 2.80 respectively. These treatments recorded a callus index of 231.78 in I_1 medium and 248.64 in I_2 medium. The least growth score of 2.68 was recorded in I_4 medium with a callus index of 219.22.

4.1.2 Nucellus

When nucellus was used as explants the survival rate of cultures varied between 66.67 and 83.00 per cent (Table 3). Cultures in I_3 medium (MS + 2, 4-D 0.3 mgl^{-1}, BA 0.3 mg l^{-1}, sucrose 40.0 gl^{-1}, CW 200.0 ml l^{-1}) showed 83.00 per cent survival. Only 66.67 per cent cultures inoculated in I_2 medium (MS + 2, 4-D 0.2 mgl^{-1}, BA 0.2 mg l^{-1}, sucrose 40.0 gl^{-1}, CW 200.0 ml l^{-1}) survived. 75.00 per cent of cultures in I_1 (MS + 2, 4-D 0.1 mgl^{-1}, BA 0.1 mg l^{-1}, sucrose 40.0 gl^{-1}, CW 200.0 ml l^{-1}) and I_4 media (MS + 2, 4-D 0.4 mgl^{-1}, BA 0.4 mg l^{-1}, sucrose 40.0 gl^{-1}, CW 200.0 ml l^{-1}) survived.

None of the cultures initiated callus when inoculated in I_2 medium. The highest callus formation (44.40.0 per cent) was given by the cultures in I_1 medium. Per cent initiation of callus was only 30.00 and 22.20 in cultures inoculated in I_3 and I_4 media.

Growth score of 1.10 was recorded by the culture inoculated in I_1 medium whereas a growth score of one was recorded by cultures in I_3 and I_4 media. The highest callus index of 48.84 was noticed in I_1 medium.

4.1.3 Integument

The cultures inoculated with integument also showed a higher survival rate between 83.00 and 100.00 per cent. All the cultures in I_4 medium (MS + 2, 4-D 0.4 mgl^{-1}, BA 0.4 mg l^{-1}, sucrose 40.0 gl^{-1}, CW 200.0 ml l^{-1}) survived whereas only 83.00 per cent cultures survived in I_3 (MS + 2, 4-D

0.3 mgl^{-1}, BA 0.3 mg l^{-1}, sucrose 40.0 gl^{-1}, CW 200.0 ml l^{-1}) and I_1 (MS + 2, 4-D 0.1 mgl^{-1}, BA 0.1 mg l^{-1}, sucrose 40.0 gl^{-1}, CW 200.0 ml l^{-1}) media.

No callus initiation noted in I_1 and I_2 media. Only 10.00 and 16.00 per cent cultures initiated callus in I_3 and I_4 media. The growth score of cultures in this media was one. Callus indices of the cultures in I_3 and I_4 were 10.00 and 16.00 respectively.

From the above result it has been proved beyond doubt that the cotyledon showed the higher response in initiating callus. So for further detailed studies cotyledon was used as the explants.

4.1.4 Internodes

In the initial studies, percentage survival of callus varied from 58.30 to 75.00 per cent when internode was used as explant (Table 3). The highest survival rate (75.00 per cent) was recorded in I_3 medium (MS + 2, 4-D 0.3 mg l^{-1}, BA 0.3 mg l^{-1}, sucrose 40g l^{-1}, CW 200ml l^{-1}) whereas in I_2 medium (MS + 2, 4-D 0.2mg l^{-1}, BA 0.2 mg l^{-1}, sucrose 40gl^{-1}, CW 200.0 ml l^{-1}) it was least (58.30 per cent).

In treatments I_1 and I_4 (MS + 2, 4-D 0.1mg l^{-1}, BA 0.1 mg l^{-1}, sucrose 40.0 gl^{-1}, CW 200.0 ml l^{-1} and MS + 2, 4-D 0.4mgl^{-1}, BA 0.4 mg l^{-1}, sucrose 40.0 gl^{-1}, CW 200.0 ml l^{-1}) 75.00 per cent cultures initiated callus whereas only 57.10 per cent cultures initiated callus in I_2 medium. In I_3 medium 66.67 per cent culture initiated callus.

A highest growth score of 1.56 was obtained when the internodes were cultured in I_1 medium with a callus index of 117.00. A least growth score of 1.06 was recorded in I_2 medium with a callus index of 60.53. In I_3 and I_4 media the growth score recorded were 1.22 and 1.34 respectively. The callus index obtained in I_3 medium was 81.34 whereas it was 100.50 in I_4 medium.

4.1.5 Hypocotyls

The survival rate of cultures varied between 50.00 to 75.00 per cent when inoculated with hypocotyls (Table 3). A highest survival rate of 75.00 per cent was recoded by the cultures in I_4 medium (MS + 2, 4-D 0.4 mgl^{-1}, BA 0.4 mg l^{-1}, sucrose 40.0 gl^{-1}, CW 200.0 ml l^{-1}) whereas only 50.00 per cent cultures survived in I_2 medium (MS + 2, 4-D 0.2 mgl^{-1}, BA 0.2 mg l^{-1}, sucrose 40.0 gl^{-1}, CW 200.0 ml l^{-1}). In I_3 and I_1 media the cultures recorded a survival rate of 66.67 per cent.

Per cent cultures initiating callus was high in I_1 medium (71.40.0 per cent) (MS + 2, 4-D 0.1 mgl^{-1}, BA 0.1 mg l^{-1}, sucrose 40.0 gl^{-1}, CW 200.0 ml l^{-1}) whereas it was only 62.50 per cent in I_3 medium (MS + 2, 4-D 0.3 mgl^{-1}, BA 0.3 mg l^{-1}, sucrose 40.0 gl^{-1}, CW 200.0 ml l^{-1}). Callus initiation was 66.67 per cent in I_2 and I_4 media.

A comparison of growth score of callus in different media showed that I_1 treatment was the best. The cultures in this media recorded a growth score of 1.44 with a callus index of 102.82. The least growth score was given by the cultures in I_3 medium (1.06). Cultures in I_2 and I_4 media recorded a growth score of 1.33 and 1.20 respectively.

4.1.6 *In vitro* Leaves

A high survival rate of 83.30 and 92.00 per cent was observed in cultures where *in vitro* leaf was inoculated as explant (Table 3). A survival rate of 92.00 per cent was recorded by the cultures in I_1 (MS + 2, 4-D 0.1 mgl^{-1}, BA 0.1 mg l^{-1}, sucrose 40.0 gl^{-1}, CW 200.0 ml l^{-1}) and I_2 (MS + 2, 4-D 0.2 mgl^{-1}, BA 0.2 mg l^{-1}, sucrose 40.0 gl^{-1}, CW 200.0 ml l^{-1}) media and 83.30 per cent in I_3 (MS + 2, 4-D 0.3 mgl^{-1}, BA 0.3 mg l^{-1}, sucrose 40.0 gl^{-1}, CW 200.0 ml l^{-1}) and I_4 (MS + 2,4-D 0.4 mg l^{-1}, BA 0.4 mg l^{-1}, sucrose 40.0 g l^{-1}, CW 200.0 ml^{-1}) media.

Even though 92.00 per cent of the cultures survived in I_1 medium none of them produced any callus. But a callus initiation of 20.00 per cent was given by cultures in I_3 medium. Per cent cultures initiating callus were 18.00 and 10.00 in I_2 and I_4 media respectively.

The callus initiation in I_3 was higher than I_2 and I_4 but the growth score (1.00) was lower. The growth score of I_2 and I_4 were 1.13 and 1.25 respectively. Callus index was higher in I_2 (20.25) when compared to I_3 and I_4 (20.00 and 12.50 respectively).

4.1.7 *In vitro* Roots

The range of survival rate in media inoculated with *in vitro* root was between 50.00 and 83.00 per cent (Table 3). Cultures in I_4 medium (MS + 2, 4-D 0.4 mgl^{-1}, BA 0.4 mg l^{-1}, sucrose 40.0 gl^{-1}, CW 200.0 ml l^{-1}) recorded the lowest survival rate of 50.00 per cent. But 83.00 per cent cultures inoculated in I_1 medium (MS + 2, 4-D 0.1 mgl^{-1}, BA 0.1 mg l^{-1}, sucrose 40.0 gl^{-1}, CW 200.0 ml l^{-1}) survived. In the case of I_2 (MS + 2, 4-D 0.2 mgl^{-1}, BA 0.2 mg l^{-1}, sucrose 40.0 gl^{-1}, CW 200.0 ml l^{-1}) and I_3 (MS + 2, 4-D 0.3 mgl^{-1}, BA 0.3 mg l^{-1}, sucrose 40.0 gl^{-1}. CW 200.0 ml l^{-1}) media, the survival rate recorded were 75.00 and 58.30 per cent respectively.

It was observed that the per cent cultures initiating callus was the highest (71.40.0 per cent) in I_3 medium. But a lower per cent initiation of callus (44.40.0 per cent) was recorded by the cultures in I_2 medium. Cultures initiating callus were 62.50 and 71.40 per cent in I_1 (MS + 2, 4-D 0.1 mgl^{-1}, BA 0.1 mg l^{-1}, sucrose 40.0 gl^{-1}, CW 200.0 ml l^{-1}) and I_3 media respectively.

The growth scores and callus indices of the cultures in different media, when analysed, it was observed that the cultures in I_3 medium recorded the highest growth score and callus index of 1.39 and 99.25 respectively. On the other hand, the cultures in I_4 medium recorded a low

growth score and callus index value of 1.00 and 50.00. But the cultures in I_2 and I_1 media recorded a growth score and callus index of 1.25. 55.55 and 1.39. 80.63 respectively.

4.1.8 *Ex vitro* Leaves

When *ex vitro* leaves were inoculated in different media, the survival rate varied between 58.30 to 75.00 per cent (Table 3). Cultures in I_3 and I_4 media recorded a survival rate of 75.00 per cent whereas only 58.30 per cent cultures survived in I_2 medium.

The highest per cent of cultures initiating callus (77.78 per cent) was recorded in I_4 medium (MS + 2, 4-D 0.4 mgl^{-1}, BA 0.4 mg l^{-1}, sucrose 40.0 gl^{-1}, CW 200.0 ml l^{-1}). The response was low (50.00 per cent) in I_1 medium (MS + 2, 4-D 0.1 mgl^{-1}, BA 0.1 mg l^{-1}, sucrose 40.0 gl^{-1}, CW 200.0 ml l^{-1}). Only 57.10 per cent and 66.67 per cent cultures initiated callus in I_2 and I_3 media respectively.

The growth score and callus indices of these cultures when analysed revealed that they were the highest in I_4 medium (1.30 and 101.10 respectively). Even though the cultures in I_2 medium recorded a growth score of 1.25, which was higher than I_3 (1.15), the callus index of I_2 (71.38) was lower than I_3 (76.67). The cultures in I_1 medium recorded the least growth score and callus index value of 1.00 and 50.00 respectively.

4.2 INDUCTION OF SOMATIC EMBRYOGENESIS

4.2.1 Basal Medium

Six different basal media were tried to assess its effect on induction of somatic embryogenesis using cotyledon as explant. All the media were supplemented with 2, 4-D 0.5 mg l^{-1}, BA 0.1 mg l^{-1}, sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1} and Agar 6.0 g l^{-1}. The response of cultures in various basal media are given in the Table 4 and Fig. 2.

Table 4. Effect of basal media in inducing somatic embryogenesis from cotyledon explants of *Aegle marmelos*

Basal medium	Survival rate (%)	Cultures initiating callus (%)	Growth score	Callus index
MS	83.00	90.00	2.80	252.00
½ MS	75.00	88.80	2.50	222.00
MT	91.60	27.00	1.50	40.50
SH	66.67	12.50	1.33	16.63
B_5	83.00	0.00	0.00	0.00
WPM	75.00	0.00	0.00	0.00

The data represents the average value of 12 replications
Supplements: 2,4-D 0.5 mg l^{-1}, BA 0.1 mg l^{-1}, Sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1} and Agar 6.0 g l^{-1}

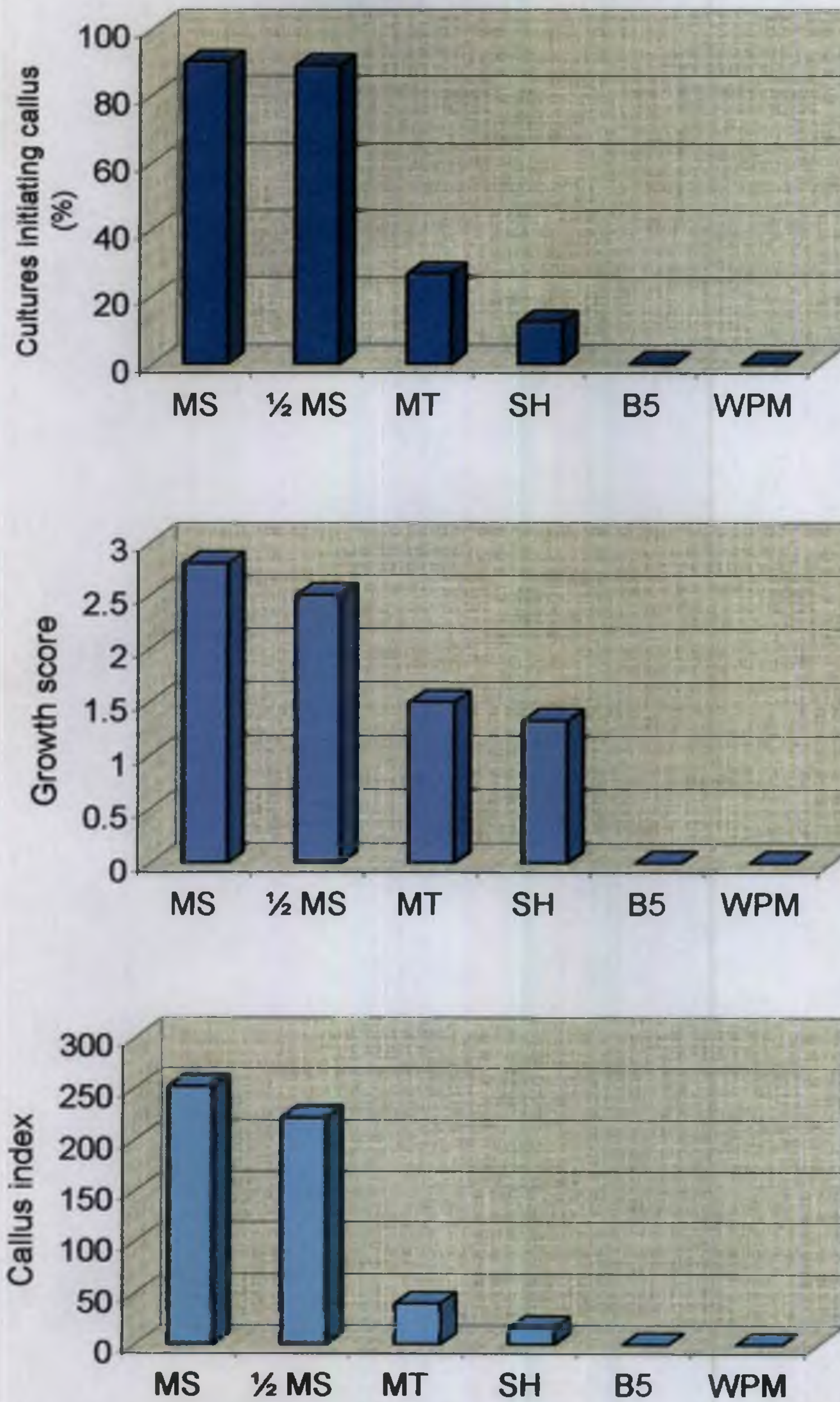


Fig. 2 Effect of basal media in inducing somatic embryogenesis from cotyledon explants of *Aegle marmelos*

The survival rate of cultures varied between 66.67 to 91.60 per cent. On analysing the values it was observed that 91.60 per cent of cultures survived in MT medium, whereas only 66.67 per cent of cultures survived when grown in SH medium. The survival rate of cultures in MS and B_5 medium was 83.00 per cent. In the case of half strength MS basal medium and WPM the per cent survival was 75.00.

Callus initiation of cultures in this different media was also noted. The data revealed that a high value of per cent initiation of callus was recorded by cultures in MS (full and half strength) basal media. Among them the cultures in MS recorded the highest callus initiation per cent (90.00) closely followed by half strength MS basal medium (88.80 per cent). The cultures in B_5 and WPM did not respond at all. On the other hand 27.00 and 12.50 per cent cultures initiated callus in MT and SH basal medium respectively.

Highest value for the growth score and callus index were registered by the cultures kept in MS basal medium (2.80 and 252.00 respectively).

Even though MT medium proved to be successful for somatic embryogenesis in various plants related to bael, especially *Citrus* sp., here the response was not satisfactory. The cultures in MT media recorded a growth score of 1.50 and a callus index of 40.50.

In SH medium the cultures recorded a growth score and callus index of 1.33 and 16.63 respectively.

4.2.2 Plant Growth Substances

4.2.2.1 MS Basal Medium

Sixty seven treatments of plant growth substance incorporated in MS basal media were tried for inducing somatic embryos from bael. Cotyledon was used as explant in all the treatments (Table 5 a).

Table 5a. Effect of plant growth substances in inducing somatic embryogenesis from cotyledon explants of *Aegle marmelos*

Plant growth substances (mgl^{-1})		Survival rate (%)	Cultures initiating callus (%)	Growth score	Callus index
I_1	2,4-D 0.1, BA 0.1	91.67	72.73	2.98	216.74
I_2	2,4-D 0.2, BA 0.2	91.67	90.90	2.80	254.52
I_3	2,4-D 0.3, BA 0.3	75.00	88.89	3.30	293.94
I_4	2,4-D 0.4, BA 0.4	91.67	90.10	2.68	241.47
I_5	2,4-D 0.1, BA 0.2	75.00	77.78	1.89	147.00
I_6	2, 4-D 0.1, BA 0.3	41.60	80.00	2.89	231.20
I_7	2,4-D 0.1, BA 0.4	83.00	70.00	2.42	169.40
I_8	2,4-D 0.2, BA 0.1	83.00	16.67	1.87	31.17
I_9	2,4-D 0.2, BA 0.3	91.67	81.80	2.23	182.40
I_{10}	2,4-D 0.2, BA 0.4	83.00	70.00	1.68	117.60
I_{11}	2,4-D 0.3, BA 0.1	91.67	81.80	2.25	184.05
I_{12}	2,4-D 0.3, BA 0.2	83.00	80.00	2.15	172.00
I_{13}	2,4-D 0.3, BA 0.4	91.67	81.80	2.60	212.68
I_{14}	2,4-D 0.4, BA 0.1	83.00	70.00	2.43	170.10
I_{15}	Kinetin 1.0 NAA 1.0	83.00	90.00	1.65	148.50
I_{16}	Kinetin 1. 0, NAA 2.0	83.00	80.00	1.55	124.00
I_{17}	BA 1.0, NAA 1.0	75.00	77.78	1.81	140.78
I_{18}	Kinetin 1.0, IAA 1.0	91.67	72.70	1.80	130.86
I_{19}	BA 1.0, IAA 1.0	66.67	75.00	1.92	144.00
I_{20}	BA 0.25	75.00	77.78	1.95	151.67
I_{21}	BA 0.5	83.00	80.00	2.13	170.40
I_{22}	BA 0.75	83.00	70.00	1.58	110.60
I_{23}	BA 1.0	75.00	88.80	1.95	173.16
I_{24}	BA 1.25	66.67	75.00	2.15	161.25
I_{25}	BA 1.5	83.00	70.00	1.75	122.50
I_{26}	BA 1.75	66.67	75.00	2.25	168.75
I_{27}	BA 2.0	83.00	69.00	1.56	107.64
I_{28}	2,4-D 0.5, BA 0.5	91.67	72.70	1.73	125.77
I_{29}	2,4-D 1.0, BA 0.5	83.00	70.00	1.85	129.50
I_{30}	2,4-D 1.5, BA 0.5	83.00	80.00	2.05	164.00
I_{31}	2,4-D 2.0, BA 0.5	100.00	80.00	2.04	169.32
I_{32}	2,4-D 0.5, BA 1.0	91.67	90.00	2.32	208.80
I_{33}	2,4-D 1.0, BA 1.0	83.00	80.00	2.10	168.00
I_{34}	2,4-D 1.5, BA 1.0	91.67	72.70	2.36	171.57
I_{35}	2,4-D 2.0, BA 1.0	83.00	80.00	2.13	170.40
I_{36}	2,4-D 0.5, BA 1.5	100.00	83.00	2.46	204.18
I_{37}	2,4-D 1.0, BA 1.5	91.67	72.70	2.07	150.49
I_{38}	2,4-D 1.5, BA 1.5	100.00	75.00	2.54	190.50
I_{39}	2,4-D 2.0, BA 1.5	91.67	72.72	2.55	185.44
I_{40}	2,4-D 0.5, BA 2.0	83.00	70.00	2.78	194.60
I_{41}	2,4-D 1.0, BA 2.0	100.0	83.00	2.44	202.52
I_{42}	2,4-D 1.5, BA 2.0	91.67	90.00	2.93	263.99
I_{43}	2,4-D 2.0, BA 2.0	100.00	91.60	2.38	218.00
I_{44}	2,4-D 0.1, BA 0.5, ABA 0.5	83.00	60.00	1.25	75.00
I_{45}	2,4-D 0.5, BA 0.5 ABA 0.5.	91.67	72.70	2.50	181.75

Table 5a Continued

I_{46}	2,4-D 1.0, BA 0.5, ABA 0.5	83.00	80.00	2.63	210.40
I_{47}	2,4-D 0.1, BA 1.0, ABA 0.5	91.67	90.10	1.84	165.78
I_{48}	2,4-D 0.1, BA 1.5, ABA 0.5	91.67	81.80	1.65	174.97
I_{49}	2,4-D 0.5, BA 0.5, ABA 1.0	91.67	63.60	1.25	79.50
I_{50}	2,4-D 1.0, BA 0.5, ABA 1.0	83.00	70.00	2.03	142.10
I_{51}	2,4-D 1.5, BA 0.5, ABA 1.0	100.00	75.00	1.23	92.25
I_{52}	2,4-D 2.0 BA 0.5, ABA 1.0	75.00	77.78	2.03	157.89
I_{53}	2,4-D 0.5, BA 1.0, ABA 1.0	83.00	80.00	2.25	180.00
I_{54}	2,4-D 0.5, BA 0.5 ABA 1.5	91.67	81.81	2.25	184.07
I_{55}	2,4-D 1.0, BA 0.5, ABA 1.5	83.00	70.00	1.58	110.60
I_{66}	2,4-D 1.5, BA 0.5, ABA 1.5	91.67	72.70	2.32	168.66
I_{57}	2,4-D 0.1	83.00	80.00	1.19	95.20
I_{58}	2,4-D 0. 2	75.00	66.67	2.00	133.34
I_{59}	2,4-D 0.3	83.00	70.00	1.55	108.50
I_{60}	2,4-D 0.4	50.00	66.67	1.55	103.34
I_{61}	2,4-D 0.5	83.00	80.00	2.80	224.00
I_{62}	2,4-D 1.0	75.00	77.78	2.25	175.05
I_{63}	2,4-D 1.0, NAA 1.0 BA 1.0	83.00	70.00	2.60	182.00
I_{64}	BA 1.0, GA_3 1.0	83.00	80.00	1.60	128
I_{65}	NAA 0.5,kinetin 2.0	91.67	81.81	2.42	197.98
I_{66}	2,4-D 4.0, NAA 4.0, kinetin 4.0	75.00	66.67	2.43	162.01
I_{67}	2,4-D 3.0, NAA 3.0	83.00	70.00	2.52	176.40

The data represents the average value of 12 replications
Culture medium: MS+ CW 200.0 ml l^{-1}, Sucrose 40.0g l^{-1} and Agar 6.0g l^{-1}

All the cultures in treatments I_{31} (2,4-D 2.0 mg l^{-1}, BA 0.5 mg l^{-1}), I_{36} (2,4-D 0.5 mg l^{-1}, BA 1.5 mg l^{-1}), I_{38} (2,4-D 1.5 mg l^{-1}, BA 1.5 mg l^{-1}), I_{41} (2,4-D 1.0 mg l^{-1}, BA 2.0 mg l^{-1}), I_{43} (2,4-D 2.0 mg l^{-1}, BA 2.0 mg l^{-1}) and I_{51} (2,4-D 1.5 mg l^{-1}, BA 0.5 mg l^{-1}, ABA 1.0 mg l^{-1}) survived without any contamination. On the other hand only 41.60 per cent cultures survived when they were inoculated in I_6 medium (2,4-D 0.1 mg l^{-1}, BA 0.3 mg l^{-1}). I_{60} medium (2,4-D 0.4 mg l^{-1}) retained only 50.00 per cent cultures. Twenty treatments had a survival rate of 91.67 (I_1, I_2, I_4, I_9, I_{11}, I_{13}, I_{18}, I_{28}, I_{32}, I_{34}, I_{37}, I_{39}, I_{42}, I_{45}, I_{47}, I_{48}, I_{49}, I_{54}, I_{56}, I_{65}) (Table 5 a). Survival rate was 66.67 per cent in I_{19} (BA 1.0 mg l^{-1}, IAA 1.0 mg l^{-1}), I_{24} (BA 1.25 mg l^{-1}) and I_{26} (BA 1.75 mg l^{-1}) media.

Explants cultured in I_8 medium (2,4-D 0.2 mg l^{-1}, BA 0.1 mg l^{-1}) alone induced embryogenic calli. The calli formed were uniform, compact and nodular (Plate 2).

Treatment I_{43} (2,4-D 2.0 mg l^{-1}, BA 2.0 mg l^{-1}) recorded the highest per cent (91.60) of cultures initiating callus (Plate 3). A growth score of 2.38 and a callus index of 218 were recorded by this treatment. Cultures in I_3 treatment (2,4-D 0.3 mg l^{-1}, BA 0.3 mg l^{-1}) which initiated 88.89 per cent callus, recorded the highest growth score of 3.30 and callus index of 293.94. A high callus initiation of 90.90 per cent was recorded by the cultures in I_2 medium (2,4-D 0.2 mg l^{-1}, BA 0.2 mg l^{-1}) having a growth score and callus index of 2.80 and 254.52 respectively. A lowest per cent (60.00 per cent) of callus initiation was recorded by cultures in I_{44} medium (2,4-D 0.1 mg l^{-1}, BA 0.5 mg l^{-1} and ABA 0.5 mg l^{-1}) with a growth score and callus index of 1.25 and 75.00 respectively. The lowest growth score of 1.19 was recorded by the cultures in I_{57} medium (2,4-D 0.1 mg l^{-1}). Even though the growth score of I_{44} (1.25) was greater than I_{57} (1.19) it gave a lower callus index value (75.00) than I_{57} (92.20).

Plate 2. Induction of embryogenic callus in MS basal medium supplemented with 2,4-D 0.2 mg l^{-1}, BA 0.1 mg l^{-1}, sucrose 40.0 g l^{-1}, CW 200 ml l^{-1} and agar 6.0 g l^{-1}

Plate 3. Callus generated in MS basal medium supplemented with 2,4-D 2.0 mg l^{-1}, BA 2.0 mg l^{-1}, sucrose 40.0 g l^{-1}, CW 200 ml l^{-1} and agar 6.0 g l^{-1}

Cultures initiated in I_1 media (2,4-D 0.1 mg l^{-1}, BA 0.1 mg l^{-1}) recorded a fairly good growth score of 2.98 with a callus index of 216.74. A growth score of 2.93 and 2.89 was recorded by the cultures in the media I_{42} (2,4-D 1.5 mg l^{-1}, BA 2.0 mg l^{-1}) and I_6 (2,4-D 0.1 mg l^{-1}, BA 0.3 mg l^{-1}) respectively. The callus indices of cultures in this media were 263.99 and 231.20 respectively.

Cultures initiated in I_{61} media which contained 2,4-D (0.5 mg l^{-1}) recorded a growth score of 2.80 and callus index of 224.0. At the same time, cultures in I_{57} supplemented with 2,4-D (0.1 mg l^{-1}) gave a growth score of 1.19 and a callus index value of 95.20.

4.2.2.2 Half Strength MS Basal Medium

Twenty three treatments supplemented with different plant growth substances were tried in half strength MS media for inducing somatic embryogenesis (Table 5 b).

A maximum survival rate of 91.60 per cent was noted by cultures inoculated in I_{82} medium (2,4-D 1.0 mg l^{-1}, BA 1.0 mg l^{-1}, GA_3 5.0 mg l^{-1}). 83.00 per cent survival rate was observed in many treatments which included I_{68} (2,4-D 0.1 mg l^{-1}, BA 0.1 mg l^{-1}), I_{70} (2,4-D 0.5 mg l^{-1}, BA 0.1 mg l^{-1}), I_{72} (2,4-D 1.0 mg l^{-1}, BA 0.1 mg l^{-1}), I_{74} (2,4-D 2.0mg l^{-1}, BA 0.1 mg l^{-1}), I_{76} (2,4-D 1.0 mg l^{-1}, BA 2.0 mg l^{-1}), I_{77} (2,4-D 0.5 mg l^{-1}, BA 1.0 mg l^{-1}), I_{79} (2,4-D 0.5 mg l^{-1}, BA 0.5 mg l^{-1}, NAA 1.0 mg l^{-1}), I_{81} (2,4-D 1.0 mg l^{-1}, BA 1.0 mg l^{-1}, NAA 1.0 mg l^{-1}), I_{85} (2,4-D 1.0 mg l^{-1}, BA 1.0 mg l^{-1}, NAA 1.0 mg l^{-1}), I_{86} (GA_3 1.5 mg l^{-1}) and I_{89} (GA_3 0.5 mg l^{-1}). Only 58.30 per cent cultures survived in I_{83} (2,4-D 5.0 mg l^{-1}, BA 1.0 mg l^{-1}, NAA 1.0 mg l^{-1}) and I_{87} (IBA 0.5 mg l^{-1}) media. In treatments I_{71} (2,4-D 0.5 mg l^{-1}, BA 1.0 mg l^{-1}), I_{88} (IBA 1.0 mg l^{-1}), I_{90} (GA_3 1.0 mg l^{-1}) 75.00 per cent cultures survived. In the remaining treatments the cultures showed a survival rate of 67.00 per cent.

Table 5b. Effect of plant growth substances in inducing somatic embryogenesis from cotyledon explants of *Aegle marmelos*

	Plant growth substances (mgl^{-1})	Survival rate (%)	Cultures initiating callus (%)	Growth score	Callus index
I_{68}	2,4-D 0.1, BA 0.1	83.00	80.00	1.70	136.00
I_{69}	2,4-D 0.1 , BA1.0	67.00	75.00	2.25	168.75
I_{70}	2,4-D 0.5 , BA 0.1	83.00	70.00	2.70	189.00
I_{71}	2,4-D 0.5 , BA 1.0	75.00	77.78	2.29	178.12
I_{72}	2,4-D 1.0 , BA 0.1	83.00	80.00	2.75	220.00
I_{73}	2,4-D 1.0 , BA 1.0	67.00	87.50	2.81	245.88
I_{74}	2,4-D 2.0 , BA 0.1	83.00	80.00	1.95	156.00
I_{75}	2,4-D 2.0 , BA 1.0	67.00	62.50	2.15	134.87
I_{76}	2,4-D 1.0 , BA 2.0	83.00	80.00	2.42	193.60
I_{77}	2,4-D 0.5 , BA 1.5	83.00	80.00	2.53	202.40
I_{78}	2,4-D 1.0 , BA 0.5, GA_3 5.0	67.00	75.00	2.40	180.00
I_{79}	2,4-D 0.5 , BA 0.5 NAA 1.0	83.00	70.00	1.50	105.00
I_{80}	2,4-D 1.0 , BA 1.0 ABA 1.0	67.00	87.50	2.40	210.00
I_{81}	2,4-D 1.0 , BA 1.0 NAA 0.1	83.00	80.00	1.55	124.00
I_{82}	2,4-D 1.0 , BA 1.0 GA_3 5.0	91.60	72.72	1.99	144.71
I_{83}	2,4-D 5.0 BA 1.0 NAA 1.0	58.30	71.40	2.05	146.37
I_{84}	BA 2.0 GA_3 10.0	67.00	87.50	2.25	196.88
I_{85}	2,4-D 1.0 , BA 1.0 NAA 1.0	83.00	80.00	2.03	162.40
I_{86}	GA_3 1.5	83.00	80.00	1.75	140.00
I_{87}	IBA 0.5	58.30	71.42	1.95	139.27
I_{88}	IBA 1.0	75.00	55.56	1.25	69.45
I_{89}	GA_3 0.5	83.00	60.00	1.51	90.60
I_{90}	GA_3 1.0	75.00	77.78	1.70	132.26

The data represents the average value of 12 replications
Culture medium: Half MS+CW 200.0 ml l^{-1}, Sucrose 40.0g l^{-1} and Agar 6.0g l^{-1}

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87.50 per cent cultures in treatment I_{73} (2,4-D 1.0 mg l^{-1}, BA 1.0mg l^{-1}) I_{80} (2,4-D 1.0 mg l^{-1}. BA 1.0 mg l^{-1}, ABA 1.0 mg l^{-1}), I_{84} (BA 2.0 mg l^{-1}, GA_3 10.0 mg l^{-1}) initiated callus. The lowest percentage initiation of callus (55.56) was recorded by cultures in I_{88} medium containing IBA 1.0 mg l^{-1}. Cultures in treatment supplemented with GA_3 0.5 mg l^{-1} (I_{89} medium) initiated 60.00 per cent callus whereas cultures in I_{75} medium (2,4-D 2.0 mg l^{-1}, BA 1.0 mg l^{-1}) initiated 62.50 per cent callus.

Cultures in treatment I_{73} (2,4-D 1.0 mg l^{-1}, BA 1.0 mg l^{-1}) gave a growth score of 2.81 and a callus index value of 245.88. Cultures in I_{72} medium which initiated 80.00 per cent callus recorded a growth score of 2.75 and callus index of 220. The lowest growth score of 1.25 was recorded by the cultures in treatment I_{88} supplemented with IBA 1.0 mg l^{-1}. Cultures in GA_3 (0.5 mg l^{-1}) containing medium (I_{89}) also recorded a low growth score of 1.51 compared to other treatments. The callus index in I_{89} medium was 90.60.

4.2.2.3 MT Basal Medium

Nine treatments involving various combinations of 2,4-D and BA were used for analysing the response of explants in MT basal medium (Table 5 c).

The survival rate of cultures in the treatment varied between 58.30 and 100.00 per cent. Cent per cent survival of cultures were noted in I_{92} medium supplemented with 2,4-D 0.2 mg l^{-1}, BA 0.1 mg l^{-1}. But only 50 per cent of the cultures in I_{92} medium produced callus with a growth score of 1.42 and callus index of 71.00. 91.60 per cent of cultures in I_{94} medium (2,4-D 0.4 mg l^{-1}. BA 0.1 mg l^{-1}) survived and only 27.27 per cent of cultures initiated callus. The growth score and callus index of this culture were 1.33 and 36.27 respectively.

The lowest survival rate of 58.30 per cent was noted in the cultures in treatment I_{96} (2,4-D 0.1 mg l^{-1}) and BA 0.2 mg l^{-1}). 42.80 per cent of cultures in I_{96} medium produced callus. The growth score and callus index recorded were 1.25 and 53.50 respectively.

Callus initiation of 33.33 per cent was recorded by cultures in I_{99} medium (2,4-D 0.1 mg l^{-1}, BA 0.5 mg l^{-1}). The growth score and callus index were 1.48 and 49.33 respectively. The lowest growth score of 1.16 was recorded by cultures in I_{91} medium (2,4-D 0.1 mg l^{-1}, BA 0.1 mg l^{-1}). In I_{91} 44.40.0 per cent of cultures initiated callus, recording callus index value of 51.50. The highest growth score of 1.5 was recorded by cultures in I_{95} medium with a callus index of 83.34. The lowest callus index value of 36.27 was noted in cultures of I_{94} medium (2,4-D 0.4 mg l^{-1}. BA 0.1 mg l^{-1}) where the callus initiation was also low (27.27 per cent).

4.2.2.4 SH Basal medium

The plant growth substances used in this media were 2,4-D and BA in various combinations. Totally nine treatments were selected for study (Table 5 d).

Survival rate of cultures in this media was high, ranging between 66.67 to 100 per cent. Eventhough survival rate was high, the cultures initiating embryogenic callus was low. The value ranged between 0 to 37.5. In I_{100} media (2,4-D 0.1 mg l^{-1}, BA 0.1 mg l^{-1}) cent per cent survival of cultures was noted but none of them initiated embryogenic callus. Similarly in I_{101} medium (2,4-D 0.2 mg l^{-1}, BA 0.1 mg l^{-1}) no callus initiation was noted.

The highest callus initiation (37.50 per cent) was noted by cultures in I_{106} (2,4-D 0.1 mg l^{-1}, BA 0.3 mg l^{-1}) with a growth score of 1.25 and callus index of 46.88. Survival rate in I_{103} medium (2,4-D 0.4 mg l^{-1}, BA

Table 5c. Effect of plant growth substances in inducing somatic embryogenesis from cotyledon explants of *Aegle marmelos*

Plant growth substances (mgl^{-1})		Survival rate (%)	Cultures initiating callus (%)	Growth score	Callus index
I_{91}	2,4-D 0.1, BA 0.1	75.00	44.40	1.16	51.50
I_{92}	2.4-D 0.2. BA 0.1	100.00	50.00	1.42	71.00
I_{93}	2,4-D 0.3 BA 0.1	83.00	30.00	1.25	37.50
I_{94}	2,4-D 0.4 BA 0.1	91.60	27.27	1.33	36.27
I_{95}	2,4-D 0.5, BA 0.1	75.00	55.56	1.50	83.34
I_{96}	2,4-D 0.1, BA 0.2	58.30	42.80	1.25	53.50
I_{97}	2,4-D 0.1 BA 0.3	66.67	50.00	1.33	76.50
I_{98}	2,4-D 0.1, BA 0.4	83.00	40.00	1.30	52.00
I_{99}	2,4-D 0.1 ', BA 0.5	75.00	33.33	1.48	49.33

The data represents the average value of 12 replications
Culture medium : MT+ CW 200.0 ml l^{-1}, Sucrose 40.0g l^{-1} and Agar 6.0g l^{-1}

Table 5d. Effect of plant growth substances in inducing somatic embryogenesis from cotyledon explants of *Aegle marmelos*

Plant growth substances (mgl^{-1})		Survival rate (%)	Cultures initiating callus (%)	Growth score	Callus index
I_{100}	2,4-D 0.1, BA 0.1	100.0	0.00	0.00	0.00
I_{101}	2,4-D 0.2, BA 0.1	91.60	0.00	0.00	0.00
I_{102}	2,4-D 0.3 BA 0.1	83.00	20.00	1.50	30.00
I_{103}	2,4-D 0.4 BA 0.1	100.00	25.00	1.00	25.00
I_{104}	2,4-D 0.5, BA 0.1	75.00	22.20	1.33	29.53
I_{105}	2,4-D 0.1, BA 0.2	83.00	10.00	1.00	10.00
I_{106}	2,4-D 0.1 BA 0.3	66.67	37.50	1.25	46.88
I_{107}	2,4-D 0.1, BA 0.4	83.00	20.00	1.25	25.00
I_{108}	2,4-D 0.1, BA 0.5	91.60	18.00	1.00	18.00

The data represents the average value of 12 replications
Culture medium: SH+CW 200.0 ml l^{-1}, Sucrose 40.0g l^{-1}, Agar 6.0g l^{-1}

0.1 mg l^{-1}) was 100.00 per cent but only 25.00 per cent of cultures initiated embryogenic callus with a growth score of 1.0 and callus index of 25.00. Growth score was the highest (1.5) in cultures of I_{102} (2,4-D 0.3 mg l^{-1}, BA 0.1 mg l^{-1}) medium which produced a callus initiation of 20.00 per cent and callus index of 30.

4.2.3 Coconut Water

The data pertaining to the results of the trial conducted to study the effect of three different levels of coconut water (0.0, 100.0, 200.0 ml l^{-1}) on induction of somatic embryogenesis are presented in Table 6 and Fig. 3.

Among the three different levels of coconut water tried, survival rate of cultures was high (83 per cent) in medium without coconut water (C_1) and medium containing 100.0 ml coconut water (C_2). It was also observed that 75.00 per cent cultures survived in medium containing 200.0 ml coconut water (C_3). 70.00 per cent of cultures initiated callus in C_1 (0.0 ml l^{-1}) and C_2 (100.0 ml l^{-1}) whereas it was 77.78 in C_3 medium.

On evaluating the growth score and callus index the results showed that growth score was the highest in C_3 medium (2.30) followed by C_2 (1.95) and C_1 (1.78). C_3 also recorded the highest callus index (178.89) whereas it was 124.60 and 136.50 in C_1 and C_2 respectively.

4.2.4 Malt extract

Two different concentrations of malt extract 250.0 mg l^{-1} (M_1) and 500.0 mg l^{-1} (M_2) were studied to assess its impact on induction of somatic embryogenesis (Table 7).

In M_1 (MS+2, 4-D 0.5 mgl^{-1}, kinetin 0.5 mgl^{-1}+malt extract 250.0 mgl^{-1}, sucrose 40.0 gl^{-1}, agar 6.0 gl^{-1}), 91.67 percent cultures survived with out any contamination. In the case of M_2 (MS + 2, 4-D 0.5 mg l^{-1}, kinetin 0.5 mg l^{-1} + malt extract 500.0 mgl^{-1}, Sucrose 40.0 g l^{-1}, agar 6.0 gl^{-1}), 83.00 per cent

Table 6. Effect of coconut water in inducing somatic embryogenesis from cotyledon explants of *Aegle marmelos*

Coconut water(ml l^{-1})	Survival rate (%)	Cultures initiating callus (%)	Growth score	Callus index
C_1 No CW	83.00	70.00	1.78	124.60
C_2 100.00	83.00	70.00	1.95	136.50
C_3 200.00	75.00	77.78	2.30	178.89

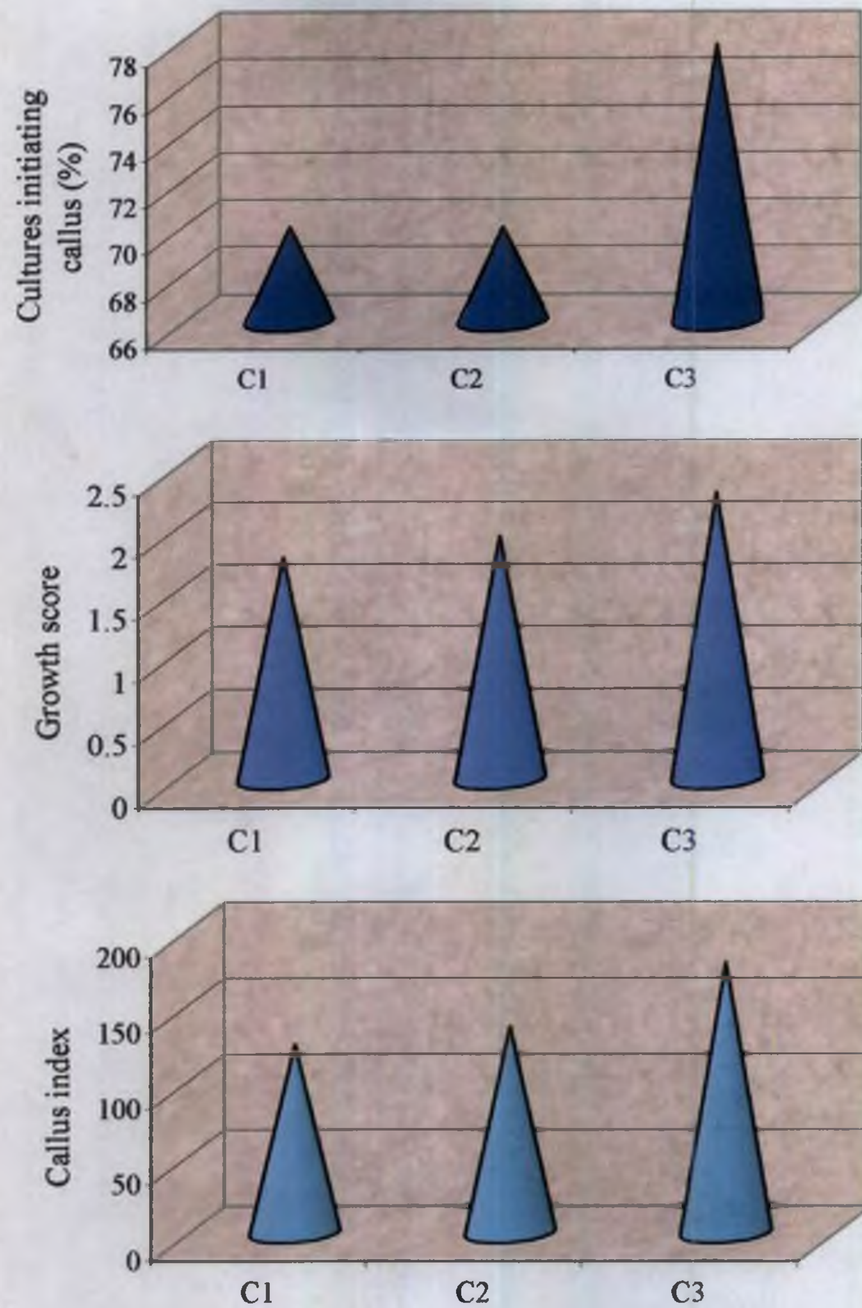
The data represents the average value of 12 replications
Culture medium : MS + 2,4-D 0.2 mg l^{-1}, BA 0.1 mg l^{-1}, Sucrose 40.0 g l^{-1} and Agar 6.0 g l^{-1}.

Table 7. Effect of malt extract in inducing somatic embryogenesis from cotyledon explants of *Aegle marmelos*

Malt extract (mg l^{-1})	Survival rate (%)	Cultures initiating callus (%)	Growth score	Callus index
M_1 (250.00)	91.67	83.30	2.12	176.80
M_2 (500.00)	83.00	80.00	2.42	193.60

The data represents the average value of 12 replications

Culture medium – MS + 2, 4-D 0.5 mg l^{-1}, kinetin 0.2 mg l^{-1}, Sucrose 40.0 g l^{-1} and Agar 6.0 g l^{-1}



C1 – MS + 2,4-D 0.2 mg l^{-1}, BA 0.1 mg l^{-1}, Sucrose 40.0 g l^{-1} Agar 6.0 g l^{-1} and CW 0.0 ml l^{-1}

C2 – MS + 2,4-D 0.2 mg l-1, BA 0.1 mg l^{-1}, Sucrose 40.0 g l^{-1}. Agar 6.0 g l^{-1} and CW 100.0 ml l^{-1}

C3 – MS + 2,4-D 0.2 mg l^{-1}, BA 0.1 mg l^{-1}, Sucrose 40.0 g l^{-1}, Agar 6.0 g l^{-1} and CW 200.0 ml l^{-1}

Fig. 3 Effect of coconut water in inducing somatic embryogenesis from cotyledon explants of *Aegle marmelos*

survived. In M_1 medium 83.30 per cent cultures initiated callus, where as in M_2 it was 80.00 per cent

The cultures obtained in M_1 medium recorded a growth score of 2.12 and a callus index value of 176.80. The corresponding values recorded in M_2 were 2.42 and 193.60 respectively.

4.2.5 Sucrose

The effect of sucrose in inducing somatic embryogenesis was analysed by comparing the response of explants at four different levels (20.0, 30.0, 40.0 and 60.0 g l^{-1}). The culture media was MS supplemented with 2,4-D 1.0 mg l^{-1}, BA 0.5 mg l^{-1}, CW 200.0 ml l^{-1} and AC 0.5 g l^{-1} (Table 8).

The data revealed that survival rate ranged between 75.00 to 91.60 per cent. S_2 (30.0 g l^{-1}) retained 91.60 per cent cultures without contamination, whereas in S_1 (20.0 g l^{-1}) and S_3 (40.0 g l^{-1}) only 75.00 per cent cultures survived. Survival rate was 83.00 per cent in S_4 medium (60.00 g l^{-1}).

Per cent cultures initiating embryogenic callus was high in S_3 medium (88.80 per cent) whereas cultures in S_1 medium initiated only 75.00 per cent callus.

A comparison of growth score and callus index in different cultures showed that the values were high in S_3 medium (2.90 and 257.52 respectively) and least in S_1 medium (1.48 and 111.00 respectively). The corresponding values obtained in cultures of S_2 and S_4 were 2.78, 227.4 and 1.98, 158.4 respectively.

4.2.6 Activated Charcoal

Three levels of activated charcoal along with zero level were supplemented to the media to study the effect on induction of somatic

Table 8. Effect of sucrose in inducing somatic embryogenesis from cotyledon explants of *Aegle marmelos*

Sucrose ($g\ l^{-1}$)	Survival rate (%)	Cultures initiating callus (%)	Growth score	Callus index
S_1 20.0	75.00	75.00	1.48	111.00
S_2 30.0	91.60	81.80	2.78	227.40
S_3 40.0	75.00	88.80	2.90	257.52
S_4 60.0	83.00	80.00	1.98	158.40

The data represents the average value of 12 replications
Culture medium : MS + 2.4-D 0.2 mg l^{-1}, BA 0.1 mg l^{-1}, CW 200.0 ml l^{-1} and Agar 6.0 g l^{-1}

Table 9. Effect of activated charcoal in inducing somatic embryogenesis from cotyledon explants of *Aegle marmelos*

AC ($g\ l^{-1}$)	Survival rate (%)	Cultures initiating callus (%)	Growth score	Callus index
A_1 0.0	91.60	81.80	2.65	216.77
A_2 0.5	83.00	80.00	2.79	223.20
A_3 1.0	83.00	80.00	2.43	194.47
A_4 1.5	75.00	75.00	1.29	96.75

The data represents the average value of 12 replications
Culture medium : MS + 2,4-D 1.0 mg l^{-1}, BA 0.5 mg l^{-1}, sucrose, 40.0 g l^{-1}, CW 200.0 ml l^{-1} and Agar 6.0 g l^{-1}

embryogenesis. The concentration of activated charcoal used for the study were 0.0 g l^{-1} (A_1), 0.5 g l^{-1} (A_2), 1.0 g l^{-1} (A_3) and 1.5 g l^{-1} (A_4). The culture media MS supplemented with 2,4-D 1.0 mg l^{-1}, BA 0.5 mg l^{-1}, CW 200.0 ml l^{-1}, sucrose 40.0 g l^{-1}, agar 6g l^{-1} (Table 9).

It was observed that the survival rate of cultures was maximum in A_1 medium with no activated charcoal (91.6 per cent). The percentage survival was 83.00 per cent in A_2 (0.5 g l^{-1}) and A_3 media (1.0 g l^{-1}) and 75.00 per cent in A_4 media (1.5 g l^{-1}). 81.80 per cent cultures initiated callus in A_1 which was the highest value. Callus initiation was 80.00 per cent in A_2 and A_3 media whereas it was 75.00 per cent in A_4 medium.

The growth score and callus indices analysis showed that the value was highest in A_2 medium (2.79 and 223.20 respectively). The treatment without activated charcoal also showed a good growth score of 2.65 and callus index of 216.77. The least value of growth score and callus index was observed in A_4 medium (1.29 and 96.75 respectively).

4.2.7 Culture Conditions

Effect of culture condition was also studied to induce somatic embryogenesis form cotyledon explants (Table 10). Darkness combined with low temperature was congenial for the production of embryogenic callus. 75.00 per cent of the cultures initiated callus when inoculated in MS basal medium supplemented with 2.4-D 0.3 mg l^{-1} and BA 0.1 mg l^{-1}, sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1}, activated charcoal 0.5 g l^{-1}. Agar 6g l^{-1}. Only 25.00 per cent cultures produce callus when they were kept under light and low temperature.

4.2.8 Frequency of Subculture

Effect of frequency of subculture was studied by subculturing at an interval of 10 days, 15 days and 30 days into the medium (Table 11).

Table 10. Effect of culture conditions in inducing somatic embryogenesis from cotyledon explants of *Aegle marmelos*

Treatment	Cultures initiating callus (%)
Light (3000 lux, 16 h photo period) + low temperature (26 ± 2°C)	25.00
Dark + low temperature (26 ± 2°C)	75.00

The data represents the average value of 12 replications

Culture medium : MS + 2,4-D 0.3 mg l^{-1}, BA 0.1 mg l^{-1} + Sucrose 40.0 g l^{-1} CW 200.0 ml l^{-1}, AC 0.5 g l^{-1} and Agar 6.0 g l^{-1}

Table 11. Effect of frequency of subculture in inducing somatic embryogenesis from cotyledon explants of *Aegle marmelos*

Subculturing intervals (days)	Survival rate (%)	Cultures initiating callus (%)
10	83.00	70.00
15	92.00	90.90
30	92.00	81.80

The data represents the average value of 12 replications

Culture medium – MS + 2,4-D 1.5 mg l^{-1}, BA 0.5 mg l^{-1}, Sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1} and Agar 6.0 g l^{-1}

The survival rate of the cultures, which were subcultured after 10 days were 83.00 percent. But the survival rate was 92.00 per cent, when subcultured after 15 and 30 days. 70.00 per cent cultures produced callus when subcultured after 10 days. However, the per cent initiation of callus was 90.90 and 81.10 when subcultured after 15 and 30 days, respectively.

4.2.9 Mode of culture

4.2.9.1 Liquid Culture

Liquid culture with filter paper bridges were tried to assess its effect on induction of somatic embryogenesis in bael. Various combinations of 2, 4-D, BA and ABA were utilized for the study (Table 12). The treatments which produced good callus formation in solid medium were selected for analysing its effect on liquid medium.

While comparing the survival rate of cultures it was noticed that the solid medium retained the highest percentage of cultures (91.67 to 100.00 per cent). On the other hand, it was low in liquid medium where the value ranged between 66.67 to 83.00 per cent. The lowest value of survival rate (66.67) was noted in cultures of L_2 treatment (MS + 2,4-D 2.0 mg l^{-1}, BA 0.5 mg l^{-1}, sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1}) in liquid culture. Cent per cent survival of cultures in the same treatment was noted in solid medium. Cultures in treatment L_1 (MS + 2,4-D 0.1 mg l^{-1}, BA 0.1 mg l^{-1}, sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1}) and L_4 (MS + 2,4-D 1.5 mg l^{-1}, BA 2.0 mg l^{-1}, sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1}) recorded a survival rate of 75.00 per cent in liquid medium. The corresponding value in solid medium was 91.67 per cent. The highest value (83.00 per cent) of survival rate in liquid medium was noticed by cultures inoculated in L_3 medium (MS + 2,4-D 0.5 mg l^{-1}, BA 0.5 mg l^{-1}, ABA 0.5 mg l^{-1}, sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1}). But 91.67 per cent of cultures in L_3 medium survived without contamination in solid culture.

Table 12. Effect of mode of cultures in inducing somatic embryogenesis from cotyledon explants of *Aegle marmelos*

Treatments	Survival rate (%)		Cultures initiating callus (%)		Growth score		Callus index	
	Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid
L_1	91.67	75.00	72.73	66.67	2.98	2.98	216.74	152.00
L_2	100.00	66.67	80.00	87.50	2.04	2.83	163.20	247.63
L_3	91.67	83.00	72.73	70.00	2.50	2.38	181.75	166.60
L_4	91.67	75.00	90.00	77.78	2.93	2.00	263.99	155.56

The data represents the average value of 12 replications

L_1 – MS + 2,4-D 0.1 mg l^{-1}, BA 0.1 mg l^{-1}, Sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1}
L_2 – MS + 2,4-D 2.0 mg l^{-1}, BA 0.5 mg l^{-1}, Sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1}
L_3 – MS + 2,4-D 0.5 mg l^{-1}, BA 0.5 mg l^{-1}, Sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1}
L_4 – MS + 2,4-D 1.5 mg l^{-1}, BA 2.0 mg l^{-1}, Sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1}

The highest per cent cultures initiating callus was 87.50 in L_2 liquid medium with filter paper bridge, whereas it was 90.00 per cent in L_2 solid medium (Plate 4). However the cultures in solid medium initiated only 80.00 per cent callus. The cultures in solid medium recorded a growth score of 2.04 and a callus index of 163.20, which were low when compared with liquid medium. The growth score and callus index recorded in L_2 liquid medium was the highest (2.83 and 247.62 respectively). Except L_2 all other treatments showed a higher response in solid medium.

The cultures in L_3 medium recorded a callus initiation per cent of 72.73 in solid medium and 70.00 in liquid medium with a growth score of 2.50 and 2.38 respectively. The callus indices in the above cases were 181.75 and 166.60 respectively.

In solid medium the highest callus initiation value (90.00 per cent) was given by cultures in L_4 medium. In the liquid media the cultures in the same treatment initiated only 77.78 per cent callus. In the case of L_1 treatments the cultures initiating callus were 72.73 and 66.67 in solid and liquid medium respectively.

Evaluating the response of cultures in L_1 and L_4 media, a high growth score values 2.98 and 2.93 were noted in solid cultures. But the response of cultures in the same treatment in liquid medium were not satisfactory, where the growth score values were only 2.28 and 2.00 respectively. The callus index value for L_1 and L_4 were 216.74 and 263.99 in solid media, whereas the values obtained in liquid medium were 152 and 155.56 respectively.

4.2.9.2 Nurse Culture

Two different concentrations of 2,4-D and BA in MS basal medium along with nurse tissues were tried for inducing somatic embryogenesis in bael (Table 13). The nurse tissues used were cotyledons.

Plate 4. Callus initiation in liquid medium with filter paper bridge

Table 13. Effect of nurse culture on induction of somatic embryogenesis in *Aegle marmelos*

Treatments	Survival rate (%)	Cultures initiating callus (%)	Growth score	Callus index
N_1	91.67	72.70	1.15	83.61
N_2	100.00	83.00	1.75	145.25

The data represents the average value of 12 replications

N_1 – MS + 2,4-D 0.2 mg l^{-1}, BA 0.2 mg l^{-1}, Sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1} and Agar 6.0 g l^{-1}

N_2 – MS + 2,4-D 0.1 mg l^{-1}, BA 0.1 mg l^{-1}, Sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1} and Agar 6.0 g l^{-1}

Table 14. Effect of nurse culture using plant extract in inducing somatic embryogenesis from cotyledon explants of *Aegle marmelos*

Treatments	Survival rate (%)	Cultures initiating callus (%)	Growth score	Callus index
P_1	91.67	72.79	2.06	149.95
P_2	91.67	63.63	1.96	124.71

The data represents the average value of 12 replications

P_1 – MS + 2,4-D 0.2 mg l^{-1}, BA 0.2 mg l^{-1}, Sucrose 40.0 g l^{-1}, CW 200.0 ml l^{-1} and Agar 6.0 g l^{-1}

P_2 – MS + 2,4-D 0.1 mg l^{-1}, BA 0.1 mg l^{-1}, Sucrose 40.0 g l^{-1}, CW 200.0ml l^{-1} and Agar 6.0 g l^{-1}

The survival rate was high in both the treatments. N_1 (MS + 2,4-D 0.2 mg l^{-1}, BA 0.2 mg l^{-1}, sucrose 40.0 g l^{-1}, AC 0.5 g l^{-1}, CW 200.0 ml l^{-1}) treatment recorded a survival rate of 91.67 per cent whereas the value in N_2 (MS + 2,4-D 0.1 mg l^{-1}, BA 0.1 mg l^{-1}, sucrose 40.0 g l^{-1}, AC 0.5 g l^{-1}, CW 200.0 ml l^{-1}) was 100.00 per cent. The cultures initiating callus was 72.70 per cent in N_1 whereas it was 83.30 per cent in N_2. The growth score recorded by the cultures were 1.15 and 1.75 in N_1 and N_2 respectively. The cultures in N_1 media recorded a callus index value of 83.61 whereas it was 145.25 in N_2 medium.

Original plant extract obtained from germinated seedlings *in vitro* were supplemented in MS basal medium along with other supplements for inducing somatic embryogenesis (Table 14). In treatments tried *viz.*, P_1 (MS + 2,4-D 0.2 mg l^{-1}, BA 0.2 mg l^{-1}, sucrose 40.0 g l^{-1}, AC 0.5 g l^{-1}, CW 200.0 ml l^{-1}) and P_2 (MS + 2,4-D 0.5 mg l^{-1}, BA 0.1 mg l^{-1}, sucrose 40.0 g l^{-1}, AC 0.5 g l^{-1}, CW 200.0 ml l^{-1}), 91.67 per cent cultures survived. In P_1 72.70 per cent cultures initiated callus and in P_2 only 63.63 per cent responded.

The growth score of cultures in P_1 medium was also high (2.06) when compared with that of P_2 (1.96). The same trend was noted with respect to callus indices where values were 149.95 and 124.71 respectively.

4.3 INITIATION OF SOMATIC EMBRYOS

4.3.1 Plant Growth Substances

The embryogenic calli from the induction medium were transferred to initiation medium of 16 treatments for observing the growth and development of somatic embryos (Table 15).

Among the various treatments tried embryogenic calli subcultured in T_6 medium (BA 0.2 mg l^{-1}) initiated globular somatic embryos (Plates 5 and 6). In this treatment about 40-50 somatic embryos were initiated which were cream in colour and can be easily separated from one another.

Table 15. Effect of plant growth substances on the initiation of somatic embryos in bael

Plant growth substances (mg l^{-1})	Cultures survived (%)	Cultures initiating somatic embryos	Other features noted
T_1 GA_3 0.5	75.00	0.0	Creamy white friable callus with linear protuberances which later turned as the shoot primordium
T_2 GA_3 1.0	66.67	0.0	Pale green friable callus producing embryoid like structures
T_3 GA_3 1.5	58.30	0.0	Callus with atypical structures with malformed shoots and roots
T_4 GA_3 2.0	83.30	0.0	Cream coloured callus which later turned brown and perished
T_5 BA 0.1	58.30	0.0	Light green coloured callus producing distinct shoots with small leaves
T_6 BA 0.2	100.00	100	Embryogenic callus with 40-50 globular somatic embryos which are kept in the same media for further growth
T_7 BA 0.3	91.60	0.0	Pale yellow callus producing root like structures in a few replications
T_8 BA 0.4	83.30	0.0	Profuse growth of callus with multiple shoots
T_9 BA 0.5	75.00	0.0	Embryoid like structures showing unequel development of shoot and root structures in few replications.
T_{10} BA 0.6	83.30	0.0	Compact callus, embryiod like structures with disproportionate shoot and root structures in a few cases.
T_{11} BA 0.7	66.67	0.0	Light green fragile callus with linear protuberences.
T_{12} BA 0.8	66.67	0.0	Pale yellow callus with small shoot like structures
T_{13} BA 0.9	58.30	0.0	Callus with plumby shoot growth only.
T_{14} BA 1.0	66.67	0.0	Callus with atypical structures with dis proportionate root and shoot ratio
T_{15} BA 2.0	91.60	0.0	Yellow callus which later changed to pale brown
T_{16} No PGS	75.00	0.0	Soft whitish loose calli having poor growth which later degenerated

The data represents the average value of six replications.

Culture medium: Half strength MS + Sucrose 40.0g $l^{-1}$and Agar 6.0 g l^{-1}.

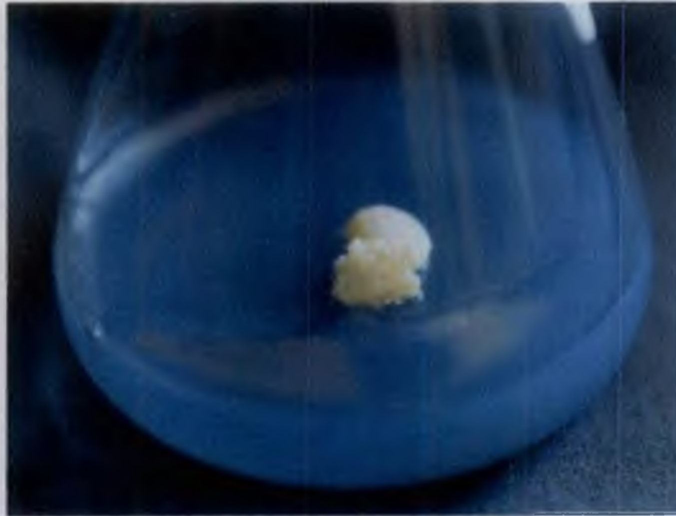


Plate 5. Somatic embryos initiated in half strength MS basal medium supplemented with BA 0.2 mg l^{-1}, sucrose 40.0 g l^{-1} and agar 6.0 g l^{-1}

Plate 6. Enlarged view of the somatic embryos

They are subcultured in the same media for further growth. In treatment T_1 (GA_3 0.5 mgl^{-1}), even though no somatic embryo was formed in any of the replications, the survival rate of the callus was 75.00 per cent. The creamy white friable callus produced linear protuberances which later transformed into shoot primordium (Plate 7). 66.67 per cent cultures survived in T_2 (GA_3 1.0 mgl^{-1}). Here the callus initially produced embryoids like structures and eventually transformed into shoots. Atypical structures with malformed roots and shoots were noted in T_3 medium (GA_3 1.5 mgl^{-1}). A high survival rate of 83.30 per cent was noticed in T_4 (GA_3 2.0 mgl^{-1}) medium. In this medium, callus which was creamy white initially later turned brown and perished (Plate 8). In T_5 (BA_3 0.1 mgl^{-1}) medium, 58.30 per cent cultures survived producing distinct shoots with small leaves. A high survival rate (91.60 per cent) was noted by cultures in T_7 medium (BA 0.3 mgl^{-1}). Few replications in the treatment produced root like structures with out the formation of shoots (Plates 9 and 10). The per cent survival was 83.30 per cent in T_8 (BA 0.4 mgl^{-1}). Instead of forming somatic embryos, multiple shoot formation was noted in this treatment. The cultures inoculated in T_9 medium (BA 0.5 mg l^{-1}) produced embryoid like structures initially, later differentiated into shoots or roots (Plate 11). Disproportionate shoot and root growth was observed in T_{10} medium (BA 0.6 mg l^{-1}) where 83.30 per cent cultures remained without contaimination. In T_{11} (BA 0.7 mg l^{-1}) medium, the cultures formed light green fragile callus with linear protuberences which later transformed to shoot initials (Plate 12). T_{12} (BA 0.8 mg l^{-1}) medium also produced small shoot like structures without forming somatic embryos. In T_{13} medium, 58.30 per cent cultures survived and the callus showed organogenesis and produced plumby shoots (Plate 13). Atypical structures with disproportionate root and shoot ratio were noted in T_{14} medium ((BA 1.0 mg l^{-1}), whereas callus formed in T_{15} medium (BA 2.0 mg l^{-1}) turned to pale brown and degenerated. No plant growth substance was added in T_{16} medium and here

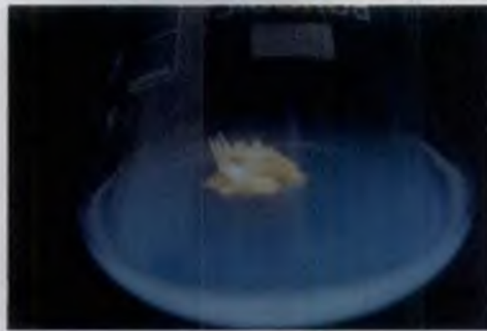


Plate 7. Creamy white friable callus produced in half strength MS basal medium supplemented with GA_3 0.5 mg l^{-1}, sucrose 40.0 g l^{-1} and agar 6.0 g l^{-1} which later transformed into shoot primordia

Plate 8. Callus initiated in half strength MS basal medium supplemented with GA_3 2.0 mg l^{-1}, sucrose 40.0 g l^{-1} and agar 6.0 g l^{-1} which was creamy white initially and later turned brown and perished

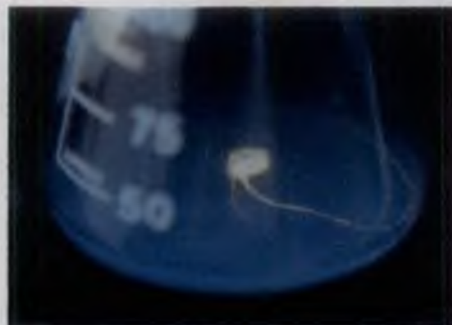


Plate 9 & 10. Callus initiated in half strength MS basal medium supplemented with BA 0.3 mg l^{-1}, sucrose 40.0 g l^{-1} and agar 6.0 g l^{-1} producing root like structures

Plate 11. Embryoid like structures initiated in half strength MS basal medium supplemented with BA 0.5 mg l^{-1}, sucrose 40.0 g l^{-1} and agar 6.0 g l^{-1} which later differentiated into shoots and roots

Plate 12. Callus with shoot initials formed in half strength MS basal medium supplemented with BA 0.7 mg l^{-1}, sucrose 40.0 g l^{-1} and agar 6.0 g l^{-1}

Plate 13. Callus initiated in half strength MS basal medium supplemented with BA 0.8 mg l^{-1}, sucrose 40.0 g l^{-1} and agar 6.0 g l^{-1} showing organogenesis with plumby shoots

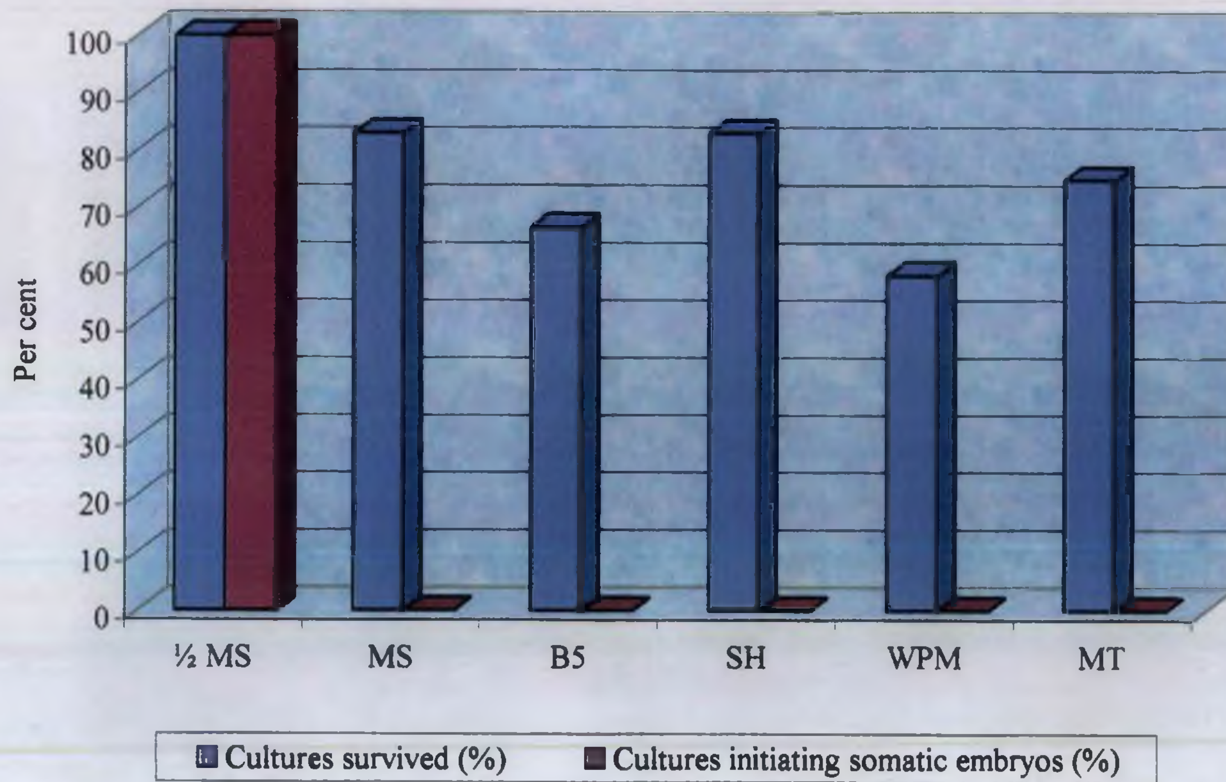


Fig. 4 Effect of basal media in initiating somatic embryos from cotyledon explants of *Aegle marmelos*

the cultures recorded a survival rate of 75.00 per cent. In this treatment soft white loose callus was formed which later got degenerated (Plate 14).

4.3.2 Basal Media

To study the effect of basal media on the initiation of somatic embryos the cultures from the induction media were transferred to six different basal media such as MS (full and half strength), MT, SH, B_5 and WPM (Table 16, Fig. 4).

The study revealed that survival rate was high in half strength MS basal medium (100 per cent). Survival rate of 83.00 per cent was noted in MS and SH basal medium, whereas 75.00, 66.67 and 58.30 per cent of cultures survived in MT, B_5 and WPM basal medium respectively.

Presence of globular somatic embryos were noted only in half strength MS basal media (Plates 5 and 6). In full strength MS basal medium small shoot like protuberences were formed on the callus (Plate 15). In B_5 and MT media callus turned brown and perished. Poor callus growth was observed in SH and WPM basal media (Plate 16).

4.3.3 Sucrose

Four different concentrations of sucrose (20.0, 30.0, 40.0 and 60.0g l^{-1}) were tried for the initiation of somatic embryogenesis in bael (Table 17). The survival rate of cultures varied between 60.00 to 100.00 percent. The highest survival rate of 100.00 per cent was observed in cultures when they were inoculated in medium containing 40.0 g l^{-1} sucrose whereas only 41.60 per cent cultures survived in media containing 30.0 g l^{-1} sucrose. The survival rate was 66.67 and 58.30 per cent in cultures containing 20.0 and 60.0 g l^{-1} sucrose respectively. Somatic embryos were formed in media containing 40.0 g l^{-1} sucrose (Fig. 5). Initiation of somatic embryos took place in all the replications of this treatment. But in medium

Plate 14. Soft white loose callus formed in half strength MS basal medium without plant growth substances

Plate 15. Callus initiated in MS basal medium supplemented with BA 0.2 mg l^{-1}, sucrose 40.0 g l^{-1} and agar 6.0 g l^{-1} with small shoot like protuberances

Plate 16. Poor callus growth in SH basal medium supplemented with BA 0.2 mg l^{-1}, sucrose 40.0 g l^{-1} and agar 6.0 g l^{-1}

Table 16. Effect of basal media on the initiation of somatic embryos in bael

Basal media	Cultures survived (%)	Cultures initiating somatic embryos (%)	Other features noted
½ MS	100.00	100.0	Presence of globular somatic embryos, some of them showed slight elongation and growth
MS	83.00	0.0	Embryoid like yellow callus with small white shoot like protuberences
B_5	66.67	0.0	Pale brown callus which in the roots, later turned brown and degenerated
SH	83.00	0.0	White loose callus with no proper growth
WPM	58.30	0.0	Callus light green and fragile with no potuberence
MT	75.00	0.0	Callus white intially but later turned brown and perished

The data represents the average value of six replications

Supplements: BA 0.2mg l^{-1}, Sucrose 40.0 g l^{-1} and Agar 6.0g l^{-1}.

Table 17. Effect of sucrose on the initiation of somatic embryos in bael

Sucrose (gl^{-1})	Cultures survived (%)	Cultures initiating somatic embryos (%)	Other features noted
20.0	66.67	0.0	Friable callus with shoot formation
30.0	41.60	0.0	Friable callus, no embryo formation, leaf like structures noticed
40.0	100.00	100.0	Cream coloured globular embryos
60.0	58.30	0.0	Callus produced plumby shoots with malformed leaves

The data represents the average value of six replications

Culture medium : Half strength MS + BA 0.2mg l^{-1} and Agar 6.0g l^{-1}

containing 20.0 and 30.0 g l^{-1} sucrose, non embryogenic friable callus were formed. Plumby shoots with malformed structures were noticed in medium supplemented with 60.0 g l^{-1} of sucrose.

The somatic embryos were subcultured in the same media in which they initiated and kept for making observations on germination. Even after two months, no symptoms of development were noted.

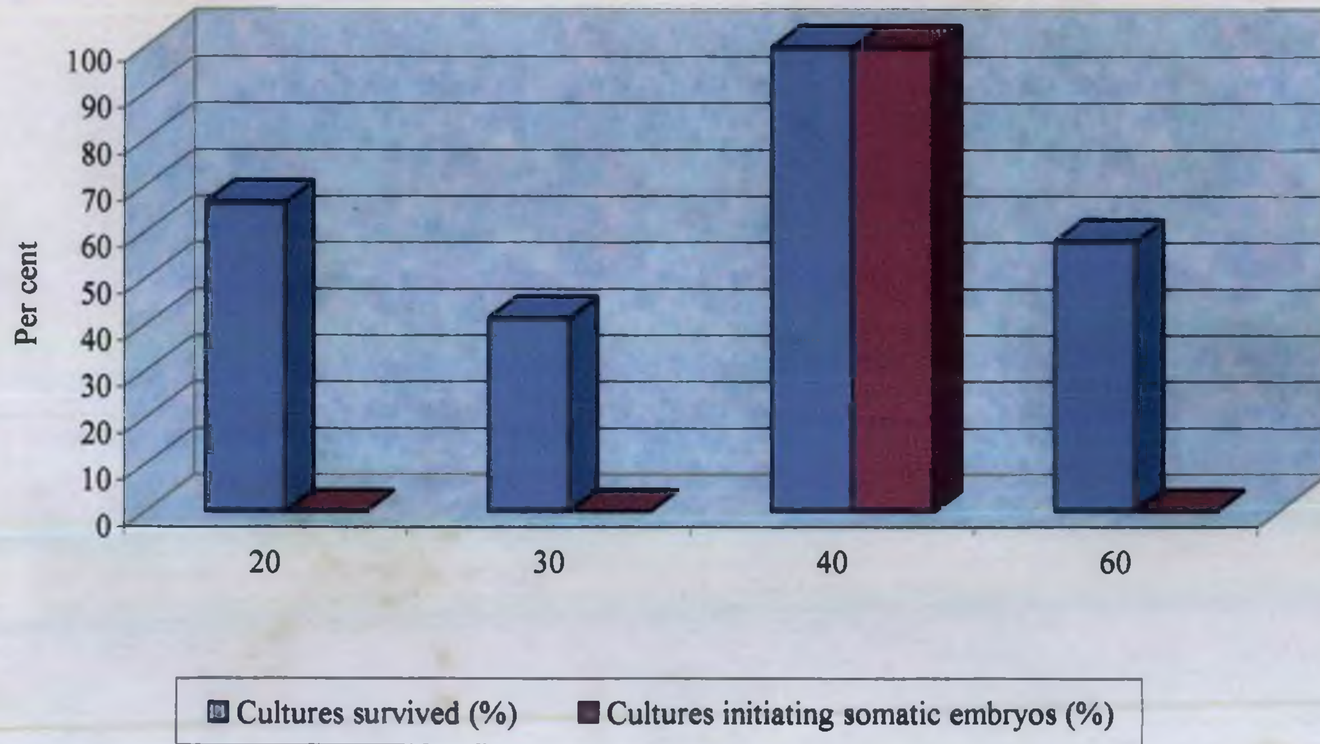


Fig. 5 Effect of sucrose in initiating somatic embryos from cotyledon explants of *Aegle marmelos*

DISCUSSION

5. DISCUSSION

Bael is an important medicinal fruit tree distributed throughout the plains and hilly tracts of India. The different plant parts and its extract possess a number of pharmacological properties. The officinal parts are roots, leaves and fruits. They have anti-helminthic, anti-microbial, anti-diarrhoeal and cardiotonic properties. Leaves have anti-asthmatic properties while fruit pulp can be used in the treatment of leucoderma. The medicinal property of the plant is due to the presence of the active principle 'marmelosin'.

Bael is usually propagated by seeds. The seedlings are not true to type and exhibit large variability. Since bael produces recalcitrant seeds, it limits the distant distribution and storage of propagules. Even though vegetative propagation methods like patch budding is practiced in North India, the rate of multiplication is not sufficient to meet the increasing demand in herbal drug industry. Over exploitation of plant as well as habitat destruction have significantly reduced the population of bael in natural habitat. Hence a reliable clonal propagation technique would undoubtedly aid in the multiplication of bael and thereby help in conserving the germplasm.

Plant tissue culture has been successfully used to micropropagate different medicinal plants. Rare and endangered cultivars can be multiplied and prevented from becoming extinct. Clonal propagation techniques will be useful in the rapid multiplication of bael. Among the clonal propagation methods, somatic embryogenesis is a versatile technique for rapid multiplication of plants and offers a superior option for developing elite plants. Somatic embryogenesis is a process in which somatic cells develop into plants through a series of stages which is similar to that of zygotic embryo development (Williams, 1987). It has gained attention as an important plant propagation method for large scale propagation of plants having uniform size within a short time.

The present studies were conducted for standardizing *in vitro* somatic embryogenesis in bael. The results of the investigation are discussed.

The various steps involved in somatic embryogenesis are induction of embryogenic callus and initiation, maturation and germination of embryoids. The influence of culture medium, culture conditions and frequency of subculture on *in vitro* response of explants were observed in the present study.

Embryogenic potential is largely a function of explant, its stage of development and interaction of the explant with the growth medium (Litz and Grey, 1992). In many systems, the developmental stage of explant is crucial for the expression of somatic embryos and often involves partial differentiation rather than uniformity (Maheshwaran and Williams, 1986). For somatic embryogenesis certain tissue explants give a better yield of embryogenic calli and embryoids (Novak and Konecna, 1982). The internal state of explant is of prime importance in expression of somatic embryogenesis, with other conditions such as exogenous growth substances being simply permissive for expression of intrinsically determined pattern of development (Tisserat *et al.*, 1979). Previous reports by scientists showed that cotyledons, hypocotyls, immature and mature leaves from *in vitro* raised seedlings were ideal for somatic embryogenesis in bael (Islam *et al.*, 1996; Arumugam and Rao, 2000). In the present study, out of eight explants tried for inducing somatic embryogenesis, cotyledon responded better than others. Cotyledonary explant initiated 90.00 per cent callus when cultured in MS basal medium supplemented with 2,4-D 0.3 mg l^{-1}, BA 0.3 mg l^{-1}, sucrose 40.0 g l^{-1} and CW 200.0 ml l^{-1} (Table 6). The response of internodes, hypocotyls, *ex vitro* leaves were 66.67, 62.50 and 66.67 per cent, respectively, in the same medium. At the same time, initiation of callus in the same medium by explants like nucellus, *in vitro* leaf and integument were only 30.00, 20.00 and 10.00 per cent, respectively. A high growth score of 3.3 was recorded by cotyledon in this treatment. Among the other explants used, hypocotyls recorded a growth score of 1.33 in the same

treatment with a callus index of 80.00, whereas the callus index value of cotyledon was 297.00. Cotyledon also recorded a high growth score of 2.98 when inoculated in MS basal medium supplemented with 2,4-D 0.1 mg l^{-1}, BA 0.1 mg l^{-1}, sucrose 40.0 g l^{-1} and CW 200.0 ml l^{-1}. In the same treatment *in vitro* leaf and integument produced no callus. This showed the superiority of cotyledon over other explants in initiating callus formation in bael. Cotyledons are the first formed leaves with a store of energy for the developing plant. In perennial crops, somatic embryogenesis was reported from cotyledonary explants of mandarins (Gill *et al.*, 1995), *Juglans regia* (Tulecke and Mc Granahan, 1985) and *Melia azaderach* (Deb, 2001). These studies confirmed the morphogenic potential of cotyledons in woody plant species. In *Aegle marmelos* also, cotyledons has been identified as the best explant for inducing somatic embryogenesis (Islam *et al.*, 1996). The present study revealed that hypocotyls and internodes can also be used as explant for inducing callus. Somatic embryogenesis has been reported from hypocotyls in satsuma mandarins (Ling *et al.*, 1990) and troyer citrange (Belkoura *et al.*, 1995) whereas, investigations by Arumugam and Rao (2000) proved that cotyledons and hypocotyls were ideal for embryogenesis in *A. marmelos*.

Choice of basal medium can influence the induction of embryogenic callus in the cultures. It has been observed that different species in the same genera requires different conditions for somatic embryogenesis. In *Citrus reticulata* (Vijayakumari and Singh, 2001), *C. unshiu* (Nito and Iwamasa, 1990), somatic embryogenesis occurred in MS medium, whereas in *C. limon* (Carimi *et al.*,1994) and *C. sinensis* (Fiore *et al.*, 2002), MT medium favoured somatic embryogenesis. In the current study, different basal media were tried for induction treatment such as MS, half strength MS, MT, SH, B_5 and WPM. Among these, MS basal medium was found to be ideal for induction in bael. In this medium callus formation by cotyledons was 90.00 per cent. In half strength MS medium also, the response was fairly good (88.80 per cent). But in MT and SH medium the cultures initiated 27.00 and 12.50 per cent calli only. Growth score of cultures were also high in MS (2.80) closely followed by half strength MS (2.50). Growth

and morphogenesis of plant tissues *in vitro* are largely governed by the composition of culture media. Although the basic requirements of cultured plant tissues are similar to those of whole plants, in practice, nutritional components promoting optimal growth of tissues may vary with respect to the particular species. Media compositions are therefore, formulated considering specific requirements of particular culture system. In a survey of different studies conducted on somatic embryogenesis in various crop plants, Evans *et al.* (1981) noted that 70.00 per cent of explants initiated somatic embryogenesis when cultured on MS medium or modified MS medium. The key element of the MS medium is the presence of high levels of nitrogen in the form of ammonium nitrate. A substantial amount of nitrogen, usually in reduced form, such as ammonium salts is required for somatic embryogenesis. Induction was noticed in MS basal media in many plants in Rutaceae family. In satsuma mandarins (Nito and Iwamasa, 1990) and mandarin oranges (Gill *et al.*, 1995) and yooza (Song *et al.*, 1991a), MS basal medium proved to be ideal for induction. In *Aegle marmelos* also induction of somatic embryogenesis occurred in MS basal medium (Islam *et al.*, 1996). This medium was also found to be superior in many perennial crops like mango (Muralidharan *et al.*, 1994), jack (Rao *et al.*, 1981) and date palm (Sharon and Shankar, 1998).

Half strength MS basal medium was also good in inducing somatic embryogenesis due to its low ionic strength. Induction of somatic embryogenesis in half strength basal media was reported in mango (Litz *et al.*, 1982; Litz, 1984; Bindu, 1995; Sulekha, 1996) and cashew (Nair *et al.*, 1993; Rekha, 1999). Arumugam and Rao (2000) could also induce somatic embryogenesis of *Aegle marmelos* in half strength MS basal medium.

Growth regulator concentration in the culture medium is critical to the control of growth and morphogenesis (Skoog and Miller, 1957). Somatic embryogenesis is found to be auxin dependent. The presence of auxin mostly 2,4-D in the medium is generally essential for inducing somatic embryogenesis (Litz and Conover, 1982). The requirement of growth

regulator for different species in the same genera may vary. For induction, 2, 4-D alone induced somatic embryogenesis in *C. junos* (Song *et al.*, 1991 a) and *C. grandis* (Song *et al.*1991 b), but in *C. limon* (Carimi *et al.*, 1994) and *C. unshiu* (Nito and Iwamasa, 1990) combinations of 2, 4-D and BA induced embryogenesis. The ability to form somatic embryogenesis, in most cases is not merely an intrinsic property of a species. Instead, it is a property under genetic control such that individual genotypes within a species can differ in their ability to undergo somatic embryogenesis (Litz and Gray, 1992). In the present study, among the various treatments tried in MS basal medium, combination of 2,4-D (0.2mg l^{-1}) and BA (0.1mg l^{-1}) induced embryogenic callus in 16.67 per cent cultures (Table 8, Plate 2). Of the 67 treatments tried in MS medium, 2,4-D 2.0 mg l^{-1} and BA 2.0 mg l^{-1} supplemented, recorded the highest per cent (91.60) of cultures initiating callus. The treatments supplemented with 2,4-D 0.2 mg l^{-1} and BA 0.2 mg l^{-1} also recorded 90.90 per cent callus initiation. When BA and 2,4-D were supplemented alone, the callus initiation was low compared to that of combination treatment. High growth score value (3.30) was recorded by cultures inoculated in medium containing 2,4-D (0.3 mg l^{-1}) and BA (0.3 mg l^{-1}). It was noticed that the response of callus initiation in media supplemented with 2,4-D, BA and ABA was low when compared with the treatments supplemented with 2,4-D and BA. These results are in conformity with the findings of Islam *et al.* (1996). The same trend was observed by other scientists also. Ling *et al.* (1990) reported that in satsuma, induction occurred in medium containing 2,4-D and BA. Similar findings were also reported in *Citrus grandis* (Song *et al.*, 1991 b), orange jessamine (Jumin and Nito, 1995) and lemon (Carimi *et al.*, 1994).

For somatic embryogenesis the optimal concentration and form of nitrogen appears to be critical (Sharp *et al.*, 1980). The benefit of reduced nitrogen in addition to nitrate nitrogen for induction of somatic embryogenesis has already been established (Evans *et al.*, 1981). In the present studies, reduced nitrogen was provided in the form of coconut water. The earliest success in somatic embryogenesis was achieved in media

supplemented with coconut milk or coconut water (Steward *et al.*, 1958). Coconut water is extremely useful both in embryo induction and maturation (Vasil and Vasil, 1980). In the present study, CW 100.0 ml l^{-1} when added in the medium, 70.00 per cent cultures initiated callus. The same trend could be observed in medium without coconut water also. On the other hand, when CW 200.0 ml l^{-1} was added in the medium an increase of 7.78 per cent callus induction could be obtained. This showed that coconut water improved the callus induction. Coconut water acts as a source of reduced nitrogen in the medium (Tulecke *et al.*, 1961). Steward *et al.* (1964) claimed that coconut water in the medium was required for cell division and embryo formation in carrot cell cultures. In perennial fruit crops like mango, coconut water is essential for induction. Jana *et al.* (1994) and Bindu (1995) reported that coconut water 200.0 ml l^{-1} was essential in inducing somatic embryogenesis in mango. According to Mathew and Litz (1992) coconut water can even replace plant growth substance in inducing somatic embryogenesis in pre-embryogenic determined cells (PEDCS). Islam *et al.* (1996) could also induce somatic embryogenesis of *Aegle marmelos* in culture medium containing 200.0 ml l^{-1} of coconut water.

According to Song *et al.* (1991 b) malt extract proved to be a better supplement than yeast and coconut milk in *Citrus* sp. In the present study it was found that malt extract 250.0 mg l^{-1} was ideal for inducing callus. In many *Citrus* sp. *viz.*, Nagpur mandarins (Parthasarathy and Nagaraju, 2000; Vijayakumari and Singh, 2001), Mexican lime (Ghazvini and Shirani, 2002) and yooza (Song *et al.*, 1991 a), malt extract induced higher percentage of cotyledonary embryos.

The presence of activated charcoal in the medium has proven useful for somatic embryo development. In mango, activated charcoal hastened the differentiation of somatic embryos (Litz, 1986; Sulekha 1996). In the present study, activated charcoal in the medium has no pronounced effect in inducing callus.

Along with culture medium, culture conditions also influence somatic embryogenesis. In the current study, when incubated in induction medium and kept under darkness, 75.00 per cent of cultures initiated callus. Keeping the cultures under light (3000 lux, 16 h photo period) initiated only 25.00 per cent callus. Similar results were obtained in perennial crops like mango and citrus. Jana *et al.* (1994), Bindu (1995) and Sulekha (1996) observed that darkness favoured induction in mango. In sweet orange also induction occurred in darkness (Carimi *et al.*, 1998).

Frequency of subculture also influenced the effectiveness in embryogenesis. In the current study, it was observed that subculturing at 15 days interval proved to be ideal for inducing callus formation (90.90 per cent) where as only 70.00 per cent cultures initiated callus when subcultured after 10 days. The effectiveness of subculturing in perennial crops was noticed by various scientists in mango (Litz, 1984; Sulekha, 1996), sweet orange (Carimi *et al.*, 1998) and apple (Paul *et al.*, 1994).

The results of this study revealed that solid culture was ideal for inducing somatic embryogenesis. Callus initiation percentage ranged from 72.00 to 90.00 per cent in solid culture and 66.67 to 87.50 per cent in liquid culture with filter paper bridges (Plate 4). These findings agree with the reports of Sulekha (1996) and Ramesh (1998). Liquid culture containing the filter paper bridges will prevent the direct contact of explant with the medium, which also helps in the rapid growth of the explant (Goodwin, 1966). The explant will absorb essential nutrients from the liquid medium through filter paper.

In nurse culture technique, the metabolites present in the nurse tissues helps the cultures to grow further. This technique was also tried in inducing somatic embryogenesis in bael, using cotyledon as nurse tissue. However, in this study no positive results could be obtained. Successful reports on nurse culture technique was reported in mango somatic embryogenesis (Litz *et al.*, 1998).

The calli obtained in various induction treatments were transferred to initiation media supplemented with plant growth substances which included different concentrations of GA_3 and BA with varying levels of sucrose. Different basal media like MS (half and full strength), MT, B_5, SH and WPM were also tried for initiating somatic embryos. Among the various treatments tried, embryogenic callus was formed only in one treatment (BA 0.2 mg l^{-1}). The callus was uniform, compact and nodular with 40-50 globular somatic embryos (Plate 5). The somatic embryos were cream in colour and could be easily separated from one another (Plate 6). However, the percentage initiation of embryogenic callus was only 16.67 per cent. Islam *et al.* (1996) could also initiate somatic embryos in a medium containing BA (0.2 mg l^{-1}) with only 18.00 per cent somatic embryo formation. In all other treatments in the present study, the calli formed were friable and did not produce any globular protuberances. Moreover, the callus growth was not uniform or there was early embryoid differentiation. In certain cases embryoid like structures showing unequal development of root and shoot were observed (Plate 11). In few instances, the calli were redifferentiated into some leafy structures without roots, whereas in certain treatments, only root development was noted (Plates 9 and 10). In almost all cases, development of atypical structures were noted. Hazeena (2001) also observed that somatic embryos were not formed in cultures of bael even after two months of inoculation.

The somatic embryos initiated in the present study in half strength MS basal medium supplemented with BA 0.2 mg l^{-1} and sucrose 40.0 g l^{-1} were kept in the same culture medium for germination. However, germination was not observed even after two months.

The treatment already known to favour somatic embryogenic pathway in other crops, involving various components of culture medium as well as culture conditions have been tried for the induction of somatic embryogenesis in bael. However, such treatments were found to be ineffective. Though somatic embryos could be induced and initiated by

culturing the cotyledon explant on MS basal medium supplemented with 2,4-D (0.2 mg l^{-1}) and BA (0.1 mg l^{-1}), growth and germination could not be obtained even after two months. Sharp *et al.* (1980) pointed out that natural chemical suppressors of somatic embryogenesis were present in crops like citrus. Seeds of bael also contain a powerful germination inhibitor, 'psoralin' (Nambiar *et al.*, 2000). These growth suppressors perhaps 'side-tracked' the growth of somatic embryos. In addition, proliferation of somatic embryos in culture is often associated with poor growth or suppression of main embryonic axis (Maheswaran and Williams, 1986). Wann (1990) opined that the response of explants in specific culture conditions also depends on physiological status due to climate, site and genotype. These factors might also have contributed to the low frequency of embryo formation in the present study.

Further studies on refinement of culture media and culture conditions based on the results of present study are necessary for evolving a reliable protocol for somatic embryogenesis in *Aegle marmelos*.

SUMMARY

6. SUMMARY

Investigations for standardizing *in vitro* propagation techniques *via* somatic embryogenesis in *Aegle marmelos* were carried out at the Plant Molecular Biology and Biotechnology Centre, College of Agriculture. Vellayani, during 2001-2003. Different explants such as cotyledons, nucellus, hypocotyls, integuments, *in vitro* leaves, roots, internodes and *ex vitro* leaves were used. The effect of different basal media, plant growth substances, sucrose, coconut water and activated charcoal on the *in vitro* response of various stages of somatic embryogenesis like induction and initiation were studied. The *in vitro* response as influenced by culture conditions and frequency of subculture were also studied.

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

1. Different explants *viz.*, cotyledons, nucellus, hypocotyl, integuments, *in vitro* leaves, roots, internodes and *ex vitro* leaves were tried for inducing somatic embryogenesis. Among these, cotyledons responded better with 77.78 to 90.00 percentage callus initiation

2. Among the various basal media tried in inducing somatic embryogenesis, full strength MS media was found to be the best.

3. Out of the various plant growth substances tried for induction of somatic embryogenesis, a combination of 2,4-D 0.2 mg l^{-1} and BA 0.1 mg l^{-1} induced embryogenic callus. However, the callus initiation per cent was only 16.67.

4. Among the different levels of coconut water tried, the maximum callus induction (77.78 per cent) was registered in the medium containing 200.00 ml l^{-1} of coconut water.

5. Malt extract 250.0 mg l^{-1} in the culture medium was found to be ideal for inducing callus when compared to malt extract 500.0 mg l^{-1}
6. Among the various levels of sucrose incorporated in the medium, sucrose 40.0 g l^{-1} initiated maximum callus induction (88.80 per cent).
7. Addition of activated charcoal did not evoke any significant effect on callus induction from any of the explants tried.
8. Darkness favoured maximum callus induction compared to light
9. Subculturing in the medium of same composition at an interval of 15 days increased the percentage induction of callus by 20.00 than when subcultured at 10 days interval.
10. Solid medium was found to be superior to liquid medium. Liquid medium with filter paper bridges favoured organogenesis rather than embryogenesis.
11. Nurse culture technique had no beneficial effect on callus induction
12. Out of the different plant growth substances tried to initiate embryogenesis, formation of globular somatic embryos was noted in medium supplemented with BA 0.2 mg l^{-1}
13. Half strength MS basal medium was found to be superior over full strength MS, MT, SH and WPM in initiating somatic embryos.
14. Sucrose 40.0 g l^{-1} in the culture medium initiated somatic embryos when compared to 20.0, 30.0 and 60.0 g l^{-1}.
15. 40 to 50 globular cream coloured somatic embryos were initiated in half strength MS basal medium supplemented with BA 0.2 mg l^{-1}, sucrose 40.0 g $l^{-1}$and agar 6.0 g l^{-1}

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*Original not seen

IN VITRO SOMATIC EMBRYOGENESIS IN BAEL [*Aegle marmelos* (L.) Corr.]

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ABSTRACT

Standardization of techniques for the *in vitro* propagation of bael [*Aegle marmelos* (L.) Corr.] *via* somatic embryogenesis was attempted. The studies were carried out at the Plant Molecular Biology and Biotechnology Centre, College of Agriculture, Vellayani, during 2001-2003. Attempts were made to induce somatic embryogenesis using explants such as cotyledons, nucellus, hypocotyls, integuments, *in vitro* leaves, roots, internode and *ex-vitro* leaves. The effects of culture medium (basal media, major and minor nutrients, plant growth substances, sucrose, coconut water, activated charcoal and malt extract), culture conditions and frequency of subculture on various stages of somatic embryogenesis were studied.

Among the various explants tried, somatic embryogenesis could be induced only from cotyledons. Induction of embryogenic calli from cotyledonary explants occurred in MS basal medium supplemented with 2,4 D 0.2 mg l^{-1}, BA 0.1 mg l^{-1}, CW 200.0 ml l^{-1}, sucrose 40.0 g l^{-1} and agar 6.0 g l^{-1}. But the percentage initiation was low (16.67 per cent). Dark culture condition was found to favour callus initiation. Sub culturing in the medium of same composition at an interval of 15 days increased the percentage induction of callus by 20.00 than when subcultured at 10 days interval.

Liquid culture with filter paper bridges induced organogenesis than embryogenesis.

The embryogenic calli obtained in the induction medium, when subcultured in half strength MS basal medium supplemented with BA 0.2 mg l^{-1}, sucrose 40.0 g l^{-1}, and agar 6.0 g l^{-1} initiated 40-50 globular somatic embryos. Even after two months, the somatic embryos did not germinate. However, they are kept in the same medium for observing further growth and development.

Further studies on refinement of culture media and culture conditions are necessary for evolving a reliable protocol for somatic embryogenesis in *Aegle marmelos*.