STANDARDISATION OF *IN VITRO* TECHNIQUES FOR THE RAPID CLONAL PROPAGATION OF BAEL [*Aegle marmelos* (L.) Corr.]

BY

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THESIS
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DECLARATION

I hereby declare that this thesis entitled "**Standardisation of *in vitro* techniques for the rapid clonal propagation of 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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CERTIFICATE

Certified that this thesis entitled **"Standardisation of *in vitro* techniques for the rapid clonal propagation of bael [*Aegle marmelos* (L.) Corr.]"** is a record of research work done independently by Mrs. Hazeena. M.S. (98-12-02) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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LIST OF ABBREVIATIONS

BA	-	6- benzyl amino purine
CD	-	Critical difference
GA_3	-	Gibberellic acid
IAA	-	Indole-3-acetic acid
IBA	-	Indole-3-butyric acid
MS	-	Murashige and Skoog
NAA	-	Naphthalene acetic acid
NS	-	Not significant
SH	-	Schenk and Hildebrandt
WPM	-	Woody plant medium
B5	-	Gamborg *et al.*
1/2 MS	-	Half strength Murashige and Skoog
2,4-D	-	2,4- dichloro phenoxy acetic acid

Introduction

1. INTRODUCTION

Aegle marmelos (L.) Corr. commonly known as bael, is an important indigenous fruit tree of India belonging to the family Rutaceae. The importance of bael lies in its curative properties, making it one of the most useful medicinal plant of India.

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. 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.

Ayurvedic medicine manufacturers are facing a serious problem of adulteration of bael roots, mainly due to its scarcity. The unscientific and destructive extraction methods also have lead to this scarcity (Nambiar *et al.*,

2000). Since the supply 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 methods and to ensure high rate of multiplication. There are several reports on *in vitro* propagation of bael using different explants (Arya *et al.*, 1981 ; Bhati *et al.*, 1992 ; Varghese *et al.*, 1993; Hossain *et al.*, 1994a ; Islam *et al.*, 1996a ; Arumugam *et al.*, 1997 ; Ajithkumar and Seeni, 1998 ; Arumugam and Rao, 2000). The present study was undertaken with the objective of evolving protocols for the *in vitro* propagation of bael for high frequency plant regeneration system. The study also aims at the refinement of media for the *in vitro* propagation of bael with respect to the ecotypes relevant to Kerala conditions. The results may serve as useful guidelines for standardising *in vitro* techniques for rapid propagation of this endangered plant species.

Review of Literature

2. REVIEW OF LITERATURE

Aegle marmelos (L.) Corr. is an important medicinal fruit tree with considerable traditional and socio-cultural values. It belongs to the family Rutaceae (Maheswari and Dwivedi, 1988).

Bael is propagated either through seeds or rarely by root suckers. Propagation by methods such as grafting and cutting are also not commercially feasible (Hossain *et al.*, 1993). Bael is a cross pollinated species which leads to highly heterozygous population (Hossain *et al.*, 1994b). There is great demand for bael in Indian systems of medicine, such as Ayurveda. Hence *in vitro* clonal propagation offers a valuable and reliable procedure for the large scale propagation of *Aegle marmelos* .

The main commercial application of tissue culture technology so far has been in the production of clonal plants at a very rapid rate compared to conventional methods. These plants are reported to grow faster and mature earlier than seed propagated plants (Vasil and Vasil, 1980). The use of tissue culture techniques for clonal propagation has become the most widely used application of tissue culture technology in horticulture in the recent years (Thorpe, 1990).

According to Murashige (1974) there are three possible routes available for *in vitro* propagule multiplication (a) enhanced release of axillary buds (b) production of adventitious shoots through organogenesis and (c) somatic embryogenesis. In shoot tip culture, genetic uniformity is favoured. Callus mediated somatic organogenesis is not recommended for clonal propagation,

but is ideal for recovery of useful variant lines. Somatic embryogenesis is limited to a few species but results in the most rapid mode of plant regeneration.

2.1. *In vitro* propagation

2.1.1. *via* Enhanced release of axillary buds

Micropropagation by axillary bud proliferation has proved to be the most reliable method for large scale production of many crop plants (Satyakala *et al.*, 1995).

Morel (1960) was the pioneer in applying shoot tip culture as a clonal multiplication tool. Axillary and apical shoots contain quiescent or active meristems depending on the physiological state of the plant. Due to apical dominance only a limited number of axillary meristems can develop into shoots under *in vivo* conditions. Since the mechanism of apical dominance has been demonstrated to be under the control of various growth regulators, the proportion of these substances in the media can be so manipulated as to induce each meristem to regenerate shoots in culture. In axillary shoot proliferation, cytokinin is utilized to overcome apical dominance of shoots and to enhance the branching of lateral buds from leaf axils (Wickson and Thimann, 1958 ; Phillips, 1975).

In general, the technique of proliferation by axillary shoots is applicable to any plant that produces regular axillary shoots and responds to an available cytokinin (Hussey, 1986).

In this route, primary meristems like shoot tips and axillary buds are cultured which assure genetic uniformity of the progeny to a great extent (Rao and Lee, 1986).

2.1.1.1. Factors affecting enhanced release of axillary buds

The key factors that influence *in vitro* clonal propagation include explant, culture medium, plant growth substances, organic media supplements, mode of culture and culture conditions.

2.1.1.1.1. Explant

Success of *in vitro* propagation always depends on the proper selection of explants. The response varies according to the type, stage and physiological age of the explants.

Stem segments of mature trees of *Aegle marmelos* was used as explant (Varghese *et al.*, 1993), but there was formation of a limited number of shoots preceded by extensive callus formation. Successful callus-free release of axillary buds was reported from single node segments of *Aegle marmelos* by Ajithkumar and Seeni (1998).

Direct shoot proliferation of axillary buds from nodal explants of adult plant has been reported in many medicinal plants like *Trichopus zeylanicus* (Krishnan *et al.*, 1995), *Vitex negundo* (Sahoo and Chand, 1998), *Withania somnifera* (Teli *et al.*, 1999), *Naregamia alata* (Daniel *et al.*, 1999) and *Murraya koenigii* (Babu *et al.*, 2000).

High frequency axillary shoot proliferation from cotyledonary nodes is also possible. The cotyledons have been shown to possess high morphogenetic potential (Fazekas *et al.*, 1986). Tissue culture methodology for plant regeneration from cotyledon explants is well established for herbaceous ornamentals and vegetable crops. However, investigators have

made relatively slow progress in developing regeneration protocols for tree species (Hossain *et al.*, 1994a).

Hossain *et al.* (1994a) induced regeneration of plantlets from *in vitro* cultured cotyledons of *Aegle marmelos*. The proximal part of the cotyledon had the highest regeneration potential. Cotyledonary node explants of *Aegle marmelos* differentiated to multiple shoots on MS medium supplemented with different concentrations of BAP, kinetin and NAA (Arumugam and Rao, 1996). Axillary shoot proliferation was reported in *Murraya koenigii* by using intact axenic seedlings (Bhuyan *et al.*, 1997). The shoot buds originated from the region adjacent to the base of primary shoot and the epicotyledonary node of intact seedling. Saini and Jaiswal (2000) found that among the various seedling explants, cotyledonary node exhibited maximum shoot regeneration from axillary region in *Peganum harmala*, a perennial medicinal plant of Rutaceae. Direct shoot regeneration with high frequency from cotyledonary node of one month old seedlings was reported in *Citrus sinensis* by Daming *et al.*(2000).

2.1.1.1.2. Culture medium

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

2.1.1.1.2.1. Basal media

Selection of the culture medium depends upon the plant species and the purpose of cell, tissue or organ culture resorted to (Wang and Charles, 1991).

A wide variety of media have been reported to be used. The earliest and widely used were White's (1943) and Heller's (1953) basal media. Since 1960, most researchers have been using MS (Murashige and Skoog, 1962), B5 (Gamborg et al., 1968) or SH (Schenk and Hildebrant, 1972) media. As MS medium is characterised by high concentration of mineral salts, some workers found it beneficial to reduce its strength by half (Griffis *et al.*, 1981). After 1980, the most popular media are WPM (Lloyd and Mc Cown, 1980) and DCR (Gupta and Durzan, 1985), especially for woody plants.

Murashige and Skoog medium is the most commonly used media for *in vitro* propagation of medicinal plants. In vitro production of plantlets from cotyledonary node cultures of *Aegle marmelos* was also reported in MS medium (Hossain *et al.*, 1994a ; Arumugam and Rao, 1996). Ajithkumar and Seeni (1998) achieved rapid clonal propagation of *Aegle marmelos* by enhanced axillary bud proliferation from young single node segments when cultured in MS nutrient medium. Axillary bud release of many medicinal plants like *Vitex negundo* (Kannan and Jasrai, 1998), *Naregamia alata* (Daniel *et al.*, 1999), *Peganum harmala* (Saini and Jaiwal, 2000) and *Withania somnifera* (Kulkarni *et al.*, 2000) was obtained on MS medium. Komalavalli and Rao (2000) reported that MS medium was best for shoot sprouting followed by B5, SH and WPM in *Gymnema sylvestre.*

Axillary bud release in *Adhatoda beddomei* was induced on SH medium by Sudha and Seeni (1994). Krishnan *et al.* (1995) obtained plantlet regeneration of *Trichopus zeylanicus* in WPM. Nodal cuttings from mature

Murraya koenigii cultured in WPM produced 12-30 multiple shoots per node (Babu *et al.*, 2000).

2.1.1.1.2.2. Plant growth substances

The growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators supplied in the medium and the growth substance produced endogenously by the cultured cell. Selection and addition of growth regulators at the optimum level are crucial for successful plant tissue culture (Krikorian, 1982).

Commonly used growth regulators in tissue culture include auxins, cytokinins, gibberellins and abscissins. BAP has been the most effective cytokinin for meristem, shoot tip and bud cultures followed by kinetin (Murashige, 1974). Cytokinin has been utilised to overcome the apical dominance of shoot to enhance the branching of lateral buds from leaf axils.

In *Aegle marmelos,* shoot regeneration efficiency of BA was found to be superior to other cytokinins (Hossain *et al.*, 1994a). The highest number of shoots per explant was observed on MS medium supplemented with 3.00 mgl^{-1} BAP in *Aegle marmelos* (Arumugam and Rao, 1996). Murashige and Skoog medium with BA at 0.10 mgl^{-1} proved to be the best with respect to the establishment percentage, early release of buds, length of shoot and number of nodes in *Holostemma annulare* (John, 1996). High frequency direct shoot proliferation was induced in intact seedlings of *Murraya koenigii* on modified MS medium suplemented with BA 5.00 mgl^{-1} (Bhuyan *et al.*, 1997). Multiple shoot formation induced by BA has been reported in *Gymnema sylvestre* (Reddy *et al.*, 1998), *Holarrhena pubescens* (Sumana *et al.*,

1999) and *Withania somnifera* (Kulkarni *et al.*, 2000). BA and KIN were found to induce multiple shoots from nodal segments in *Murraya koenigii* (Babu *et al.*, 2000). When BA and kinetin were used in combination, per cent response doubled.

Daniel *et al.* (1999) reported that in *Naregamia alata*, MS medium with BA and GA_3 was good for shoot proliferation from nodes. With BA alone, the axillary shoots did not show elongation and with GA_3 alone a single elongated shoot developed. Sahoo and Chand (1998) observed that although callus-free multiple shoot formation was a function of cytokinin activity alone, faster bud break coupled with internode elongation was dependent on synergistic influence of gibberellic acid in *Vitex negundo*.

For axillary bud proliferation, exogenous auxin was not always needed. Although exogenous auxins do not induce axillary shoot proliferation, culture growth has been improved by their presence (Wang and Hu, 1980). In *Raulwolfia micrantha* eventhough BA released axillary bud, it alone was insufficient to sustain the growth of buds in to shoots. NAA was used in combination to get accelerated growth of shoots (Sudha and Seeni, 1996).

Bud break in single node segments of *Aegle marmelos* was dependent on cytokinin supply, but the synergistic combination of BAP and IAA induced the formation of maximum number of shoots (Ajithkumar and Seeni, 1998). One of the possible roles of auxin at the elongation stage is to nullify the suppressive effect of high cytokinin concentration, thereby restoring normal shoot growth (Lundergan and Janick, 1980). Hossain *et al.* (1994a) reported that in *Aegle marmelos*, BA at higher level of 5.00 mgl^{-1}

inhibited shoot elongation. The highest shoot regeneration could be attained in medium supplemented with BA 2.00 mgl^{-1} and IAA 0.20 mgl^{-1}.

2.1.1.1.2.3. Carbon source

Plant cells and tissues in the culture medium lack autotrophic ability and therefore need external carbon for energy. Sucrose is the main carbon energy source in most tissue culture media. The concentration of sucrose varied from 2.00 to 30.00 gl^{-1} (Oka and Ohyama, 1982). Sucrose also acts as an osmoticum that can stimulate and regulate morphogenesis (Wethrell, 1984). Many other carbon sources are also found used, instead of sucrose. First work in this line was reported by Gaurtheret (1945). The use of alternative carbon sources like glucose, maltose, raffinose, fructose and galactose were found to be less effective and mannose and lactose being the least effective.

Hossain *et al.* (1994a) observed that media supplemented with 40.00 gl^{-1} sucrose was best for shoot induction and elongation in *Aegle marmelos*. Regeneration of *Poncirus trifoliata* through *in vitro* culture of apical bud was studied at varying sucrose levels by Nagao *et al.* (1994). The best response in terms of number of buds produced and length of buds were obtained on media supplemented with 30.00-45.00 gl^{-1} of sucrose. Sumana *et al.* (1999) reported that sucrose at three per cent was the sugar of choice for shoot regeneration when compared to glucose in *Holarrhena pubescens*. Multiple shoots could be induced from nodal explants when cultured in basal media containing two per cent sucrose in *Withania somnifera* (Kulkarni *et al.*, 2000).

2.1.1.1.2.4. Amino acid supplements

Amino acid supplements are often incorporated in to the tissue culture media for obtaining better proliferation of shoots.

2.1.1.1.2.4.1. Casein hydrolysate

In cases where nutritional requirements have not been established, mixtures of amino acids such as casein hydrolysate may be added between 0.05 and 0.10 per cent (Huang and Murashige, 1977).

In *Eucalyptus camaludensis*, addition of casein hydrolysate was found to be superior to any other organic supplement (Kumar, 1993). Incorporation of casein hydrolysate enhanced the frequency of cultures with adventitious shoots up to 68.00 per cent in *Phyllantus fraternus* (Rajasubramaniam and Saradhi, 1994). In contrast, casein hydrolysate did not significantly improve shoot sprouting frequency in *Gymnema sylvestre* (Komalavalli and Rao, 2000).

2.1.1.1.2.4.2. Adenine sulphate

Adenine sulphate when added to the medium, often enhance growth and shoot formation (Skoog and Tsui, 1948).

Multiple shoot induction in *Dioscorea floribunda* was reported by Sinha and Chathurvedi (1979) in a medium supplemented with adenine sulphate along with BAP and NAA. In *Aegle marmelos*, shoot proliferation was considerably increased in adenine sulphate supplemented medium (Arumugam and Rao, 1996).

2.1.1.1.2.5. Solidifying agent

Gelling or solidifying agents are commonly used for preparing semi-solid and solid tissue culture media. They support the tissues growing in static conditions. A change in agar concentration affect the overall nutrient concentration in the experiment (Razdan, 1993).

Media were gelled with 0.70 per cent agar for regeneration of plantlets from *in vitro* cultured cotyledons of *Aegle marmelos* (Hossain *et al.*, 1994a). Arumugam and Rao (1996) reported use of medium containing 0.80 per cent agar for production of plantlets from cotyledonary node cultures of *Aegle marmelos.*

2.1.1.1.3. Culture conditions

Murashige (1977) observed that light intensity, quality and duration affect the growth of *in vitro* grown cultures. The optimum day length period was considered to be 16 hours for a wide range of plants. Yeoman (1986) reported that the usual environment temperature of species should be taken in to account for it's better performance under *in vitro* conditions. However, most of *in vitro* cultures are grown successfully at temperatures around 25 $\pm$ 2 $^{\circ}$C.

Air humidity under culture room condition is most frequently set at 70.00 per cent (Hu and Wang, 1983). Relative humidity is an important factor in hardening and planting out of *in vitro* raised plants.

Maintanence of cultures in 16 hour light and 8 hour dark cycle was reported by Mumtaz *et al.* (1990) in *Catharanthus roseus*. Cultures were maintained at 25 $\pm$ 2 °C with 60 per cent relative humidity under flourescent

light intensity at 2000 lux for 16/8 hour light/dark cycles in *Aegle marmelos* (Arumugam and Rao, 1996). Daniel *et al.* (1999) reported incubation of cultures at 25 $\pm$1 °C under 12 hour photoperiod in *Naregamia alata.*

2.1.2. Somatic organogenesis

Somatic organogenesis may be direct or callus mediated (Evans *et al.*, 1981). In direct somatic organogenesis, adventitious shoots arise directly from tissues of the explant and not from previously formed callus. Indirect somatic organogenesis requires the re-determination of the differentiated cells leading to callus formation. Separate shoot and root initials are characteristically formed in callus cultures (George and Sherrington, 1984).

Levels of plant growth regulating substances in the culture medium, particularly high auxin and low cytokinins, often lead to callus formation. On the other hand if the auxin level is reduced in the medium, it may lead to adventitious shoot formation (Skoog and Miller, 1957 ; Hussey, 1986).

Though callus may be obtained from virtually any species only in some plants it can be regenerated. The reason for this inability may be due to the higher proportion of polyploid or aneuploid cells in those callus (Smith and Street, 1974). The main disadvantage in callus mediated organogenesis compared to clonal propagation, is genetic variation developing in many of it's component cells. However, the regenerated variants can be used to complement the existing natural variability.

2.1.2.1. Direct somatic organogenesis

2.1.2.1.1. Explant

The induction of direct shoot regeneration depends on the plant organ from which the explant is derived and above all, on the plant species (George and Sherrington, 1984).

Hypocotyl was used for inducing direct organogenesis in *Aegle marmelos* (Hossain *et al.*, 1995). Direct organogenesis from hypocotyl surface was related to the juvenile nature of the explant in *Citrus halimii* (Normah *et al.*, 1997). Kulkarni *et al.* (2000) reported direct shoot regeneration from hypocotyl explant in *Withania somnifera.*

Sim *et al.* (1989) could obtain highest yield of direct bud formation from intact roots of whole seedlings in *Citrus mitis.* Bud formation from cultured roots has also been reported in *Citrus aurantifolia* (Bhat *et al.*, 1992). Adventitious shoot regeneration was induced from root segments by Bhati *et al.* (1992) and root tip of intact seedlings by Islam *et al.* (1996a) in *Aegle marmelos.*

2.1.2.1.2. Plant growth substances

Generally, low auxin and high cytokinin in the medium result in the induction of shoot morphogenesis. Auxin alone or in combination with a very low concentration of cytokinin is important in the induction of root primordia (Skoog and Miller, 1957).

Incorporation of BAP and kinetin each at 0.50 mgl^{-1} was found to be the optimal concentration for enhanced production of shoot from proximal end of root in *Aegle marmelos* (Bhati *et al.*, 1992). Direct organogenesis

from hypocotyl explants of *Aegle marmelos* was obtained on MS medium supplemented with BAP 0.10 mgl^{-1} (Hossain *et al.*, 1995). Islam *et al.* (1996b) reported that a medium containing 1.00 mgl^{-1} and 0.20 mgl^{-1} NAA produced high frequency adventitious plant regeneration from radicle explants of *Aegle marmelos*. Normah *et al.* (1997) observed that BA at 11.1 μM concentration produced maximal shoot proliferation in *Citrus halimii*.

2.1.2.2. Indirect somatic organogenesis

2.1.2.2.1. Explant

Multiple shoot regeneration from nodal explant callus was achieved in *Ocimum sanctum* L. (Shahzad and Siddiqui, 2000).

Kumar and Bhavanandan (1989) could induce shoots from leaf callus of *Plumbago rosea*. Organogenesis from leaf callus was reported in *Raulwolfia caffra* by Upadhyay *et al.* (1992).

Adventitious shoots were differentiated from callus developed from hypocotyl segments in *Phyllantus fraternus* (Rajasubramaniam and Saradhi, 1994). Callus induction was reported from hypocotyl explant in *Citrus microcarpa* by Hong *et al.* (1997).

Callus induction from stem (Varghese *et al.*, 1993), nucellus (Hossain *et al.*, 1993), embryonic axis (Islam *et al.*, 1995), hypocotyl (Arya *et al.*, 1981 ; Arumugam *et al.*, 1997) and leaf (Islam *et al.*, 1993; Arumugam and Rao, 1998) has been reported in *Aegle marmelos*.

2.1.2.2.2. Plant growth substances

Generally, a high concentration of auxin and a low concentration of cytokinin in the medium promote abundant cell proliferation with the formation of callus (Skoog and Miller, 1957).

Callus was initiated from stem explants of *Aegle marmelos* on medium supplemented with 1.00 mgl^{-1} kinetin and 5.00 mgl^{-1} NAA. The callus with meristemoids showed maximum number of sprouting buds when transferred to medium augmented with 1.00 mgl^{-1} BAP (Varghese *et al.*, 1993). Hossain *et al.* (1993) reported callogenesis and adventitious bud initiation from nucellus in medium containing BA and NAA. It was observed that a higher concentration of NAA or a lower concentration of NAA and BA together yielded more callus.

Hard, green and compact irregularly shaped calli were induced from mature and immature seeds of *Aegle marmelos* on media with 10.00 μM 2,4-D and 0.50 μM BA. The best results for shoot formation were obtained when kinetin and IAA were used (Islam *et al.*, 1995). Arumugam *et al.* (1997) observed that a combination of 2,4-D, NAA and kinetin was best for callus initiation and proliferation from cotyledon explants of *Aegle marmelos.*

Callus initiation and shoot bud proliferation from immature leaflets of *Aegle marmelos* were obtained in MS medium fortified with NAA 0.10 mgl^{-1} and BAP 0.01 mgl^{-1}. Regeneration of plantlets was maximum when transferred to media with higher concentration of cytokinins and lowered concentration of auxin (Arumugam and Rao, 1998).

2.1.3. Somatic embryogenesis

Somatic embryogenesis was first reported by Reinert (1958) and Steward (1958) in carrot. Somatic embryogenesis is the development of embryos from somatic cells (Mascarenhas, 1989).

Embryogenesis generally proceeds from globular to the heart, torpedo, cotyledonary and mature somatic embryo stages of development (Tulecke, 1987).

According to Sharp *et al.* (1982) somatic embryogenesis can be direct or indirect as initiated from pre embryogenic cells (PEDCs) or induced embryogenic determined cells (IEDCs). In PEDCs the embryogenic pathway is predetermined and the cells need only the synthesis of an inducer to express it's potential. IEDCs on the other hand, require an induction treatment to the embryogenic state by exposure to specific auxins. Once the embryogenic state has been reached, both the cell types proliferate in the same manner.

The most important application of somatic embryogenesis is in the large scale clonal propagation of plants (Janick, 1993). Production of artificial seeds and direct regeneration from protoplast are favoured by somatic embryogenesis (Razdan, 1993). Other uses include crop improvement, metabolite production, disease elimination (Al-Abta *et al.*, 1979), germplasm conservation (Ammirato, 1983) and generating somaclonal variants in the species (Razdan, 1993).

Induction of somatic embryogenesis was observed in leaf disc cultures of many medicinal plants like *Raulwolfia caffra* (Upadhyay *et al.*, 1992), *Aconitum* spp. (Giri *et al.*, 1993), *Plantago ovata* (Jasrai *et al.*, 1993), *Azadirachta indica* (Su *et al.*, 1997) and *Tylophora indica* (Manjula *et al.*, 2000).

Plant regeneration *via* somatic embryogenesis in *Citrus* sp. has been reported by several scientists (Das *et al.*, 1995 ; Jumin and Nito, 1996; Perez *et al.*, 1998 ; Carimi *et al.*, 1999).

In *Aegle marmelos*, somatic embryogenesis and plant regeneration using zygotic embryos was reported by Islam *et al.* (1996c). The highest response (18.00 per cent) was obtained when 2,4-D and BA was used. Arumugam and Rao (2000) reported somatic embryogenesis in *Aegle marmelos* in solid medium containing 2,4-D, BA and ABA in combination.

2.1.4. Rooting

2.1.4.1. *In vitro* rooting

The plantlets produced in vitro should have a strong and functional root system.Hu and Wang (1983) observed that three phases are involved in rhizogenesis, *viz.* induction , initiation and elongation.

Auxin alone or in combination with a very low concentration of cytokinin is important in the induction of root primordia (Skoog and Miller, 1957). Among the auxins, IBA and NAA have been most effective for root induction (Ancora *et al.*, 1981). IBA was found to be best for inducing rooting in *Aegle marmelos* in several reports(Hossain *et al.*, 1993 ; Islam *et al.*, 1993 ; Arumugam and Rao, 1996 ; Islam *et al.*,1996a). Rhizogenesis of

shoots was achieved in the presence of IAA by Bhati *et al.* (1992) and Varghese *et al.* (1993).

The root elongation phase is also very sensitive to auxin concentration. High concentrations of auxin inhibited root elongation (Thimann, 1977). Hossain *et al.* (1994a) observed that in *Aegle marmelos* at higher IBA concentration there was reduction in root length.

All cytokinins inhibit root induction and BA which is widely used for shoot multiplication does so particularly strongly that roots are delayed even after transferring to cytokinin free medium (Yeoman, 1986). Islam *et al.* (1996a) reported that no rooting occurred on microshoots of *Aegle marmelos* cultured on auxin-free media containing BA alone.

Several researchers have shown that *in vitro* rooting can successfully be achieved by reducing salt concentration in the media. Abundant rooting was observed when the salt concentration in the medium was reduced to one-half, one-third or one-fourth of standard strength (Lane, 1979 ; Skirvin and Chu, 1979). In *Aegle marmelos,* rooting of regenerated shoots were obtained on half-strength MS containing auxin (Ajithkumar and Seeni, 1998 ; Arumugam and Rao, 1998).

2.1.4.2. *Ex vitro* rooting

The major cost of producing *in vitro* plants lies in the rooting and hardening stages (Rajeevan and Pandey, 1986). For rooting under *ex vitro* conditions, the shoots for rooting could be handled as microcuttings without using aseptic conditions. *Ex vitro* rooting is preferred in many crops with a

view to save time and resources (Meane and Debergh,1983 ; Preece and Sutter, 1991).

John (1996) observed *ex vitro* rooting to be better than *in vitro* rooting in *Holostemma annulare*. Maximum rooting was obtained by giving pulse treatment with IBA 1000.00 mgl^{-1}. Survival of regenerated plantlets under *ex vitro* conditions was found to be 83.33 per cent while that of *in vitro* shoots were only 31.6 per cent in *Citrus halimii* (Normah *et al.*, 1997). Babu *et al.* (2000) reported that *in vitro* and *ex vitro* rooting treatments did not produce significantly different rooting results in *Murraya koenigii*.

2.1.5. Hardening and planting out

Acclimitization is important in the case of micropropagated plants because *in vitro* plant matured is not adapted for *ex vitro* conditions. Langford and Wainwright (1987) observed that physiologically, the leaves grown *in vitro* are incapable of significant photosynthesis. The stomata are unable to close and as cuticular wax on the leaf surface is minimal it is unable to control water loss.

Light, temperature and relative humidity are the major factors to be controlled during acclimatization. A period of humidity acclimatization is required for newly transferred plantlets (Hu and Wang, 1983). Standardised rhizosphere environment is necessary for getting better growth of plants (Zimmerman and Fordham, 1985).

In *Adhatoda beddomei*, Sudha and Seeni (1994) observed that more than 70.00 per cent of 120 plantlets transferred directly to the nursery were lost within 6-8 days. The plants hardened for atleast four weeks in the

humidity chamber showed 95.00 per cent rate of subsequent establishment in pots.

Daniel *et al.* (1999) reported that a proper hardening method is a major factor, which determines plantlet survival and establishment in the field. In *Naregamia alata*, initial conditioning on a sugar-free MS basal medium and later in vermiculite substantially increased the survival rates of the plants in the field compared to those transferred to the field without the vermiculite stage. Nourishing the plantlets while in vermiculite with a dilute nutrient solution was also mandatory for satisfactory shoot growth and root establishment.

Rooted plantlets of *Aegle marmelos* were individually transferred to soil mixed with compost (2:1) in small plastic pots and during first two weeks they were kept covered using inverted beakers to maintain high humidity (Islam *et al.*, 1996a).

Materials and Methods

3. MATERIALS AND METHODS

Investigations were carried out at the Plant Molecular Biology and Biotechnology Centre, College of Agriculture, Vellayani with the objective of standardising *in vitro* techniques for the rapid clonal propagation of bael (*Aegle marmelos* L. Corr.) during 1999 to 2001.

The materials used and methods tried for the various routes of *in vitro* propagation, namely, enhanced release of axillary buds, somatic organogenesis and somatic embryogenesis are described in this chapter.

3.1 Explants

Nodal segments from new flushes and cotyledon from immature and mature seeds of a 60 year old bael tree were used as explant.

3.1.1 Nodal segments

Shoot nodal segments with axillary buds from fresh sprouts of mature bael tree were used for inoculation.

3.1.2 Cotyledon

A mature embryo generally possesses an embryonic root (radicle), an embryonic shoot (plumule) and one or two cotyledons. Thus cotyledons are the first formed leaves, with a store of energy for the developing plant. Cotyledon from the ovules of tender bael fruits (about 90-120 days after pollination) were used as explants for induction of somatic embryogenesis, whereas cotyledon from ovules of ripe fruits were used for enhanced release of axillary buds and somatic organogenesis (Plate 1).

Plate 1. Mature ripe fruit of *Aegle marmelos*


5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
2 3 4 5 6 7 8 9

3.2 Collection and preparation

A 60 year old tree with a large trunk having 50 cm diameter and regular flowering and fruiting characters, growing in a homestead area in Thiruvananthapuram city served as the source of explant.

Cuttings from new flushes with youngest five-six leaves were collected from the crown of the mature tree and brought to the laboratory. After leaf excision, the cuttings were washed thoroughly in tap water with a few drops of the wetting agent, Labolene, followed by washing with double glass distilled water.

Seeds were obtained from freshly harvested fruits. The hard shell of the fruit was broken and seeds taken out. They were then washed thoroughly in running tap water for 15 min to remove the mucilagenous sheaths. The seeds were then washed in tap water with a few drops of the wetting agent, labolene, followed by washing with double glass distilled water.

3.3 Surface sterilisation

Surface sterilisation of the plant materials was carried out inside a laminar air flow chamber. After initial cleaning, they were transferred to a sterile beaker. The shoot apices and nodal segments were treated with mercuric chloride (0.08 per cent) for eight min. The seeds were treated with mercuric chloride (0.10 per cent) for 10 min. This was followed by washing four to five times with sterile double glass distilled water.

3.4 Inoculation and incubation

The glassware and tools (beakers, petri plates, blades, forceps etc.) required for inoculation were washed thoroughly, rinsed with double glass distilled water, covered with aluminium foil and autoclaved at 121 ^{0}C and 1.06 kg/cm^{2} pressure for 45 min.

All the inoculation operations were carried out in a laminar air flow chamber (Klenzaids, model 1104).

The surface sterilized shoot explants were cut using sterile blade, to get 1.00 – 1.50 cm single nodal cuttings. The cotton plugs of the test tubes were removed and the rim was flamed. The nodal explants were vertically inoculated on the media. The rim of the test tube was flamed again and cotton plugs were replaced.

The surface sterilized seeds were dissected using sterile blade so that the cotyledon inside the seed coat was cut longitudinally into two equal halves. The cotyledon was then separated and taken out from seed coat carefully. Cotton plugs of the test tubes were removed and the rim was flamed. The cotyledon with or without embryo axis was inoculated on to the media. The rim of the culture vessels were flamed again and cotton plugs were replaced.

The cultures were then transferred to the culture rooms and incubated either in light or in darkness as per treatment. Light (photo period 16 h) was provided by white fluorescent tubes, giving an intensity of 3000 lux. Darkness was provided by placing the cultures in culture racks covered with

black polythene sheets and by covering the culture vessels with aluminium foil.

3.5 Media

The basal media used for the study were MS (Murashige and Skoog, 1962), B5 (Gamborg *et al.*, 1968), SH (Schenk and Hildebrandt, 1972) and WPM (Lloyd and Mc Cown, 1980). The chemicals used for the preparation of the culture media were of analytical grade from Sisco Research Laboratory (Bombay), Merck (Bombay) and British Drug House (Bombay).

Standard procedures were followed for the preparation of the basal media (Thorpe, 1980). Stock solution of major and minor nutrients, organics and plant growth substances were prepared by dissolving the required quantity of chemicals in specific volume of double glass distilled water and were stored under refrigerated conditions (4^0C).

The glass wares used for the preparation of the media were washed with dilute Labolene and rinsed with double glass distilled water. Specific quantities of the stock solutions were pipetted out into 1000 ml beaker. Sucrose, casein hydrolysate, adenine sulphate and inositol were added fresh and dissolved. The pH of the medium was adjusted between 5.6 and 5.8 using 0.1 N NaOH or 0.1 N HCl with the aid of an electronic pH meter (Philips make, model PP9046). Agar (in the case of solid medium) was added to the medium and final volume made upto 1000 ml. Agar was not added in the case of liquid medium.

The solution was then heated by placing the beaker on a heating mantle and stirring thoroughly for uniform mixing, till agar melted. Activated

charcoal, when used in the medium, was added at this stage. The medium was poured to pre-sterilized culture vessels which were rinsed with double glass distilled water. Corning brand test tubes (25 x 150 mm) and Erlenmeyer flasks (100 ml) were used as culture vessels. The test tubes and flasks were filled with 15 ml and 30 ml of medium, respectively. The culture vessels containing the medium were plugged tightly with cotton. They were then autoclaved at 121 ^{0}C and 1.06 kg/cm^2 pressure for 20 min.

3.6 *In vitro* multiplication procedures

The following routes of *in vitro* propagation were tried.

Sl. No.	Method of multiplication	Explants
1.	Enhanced release of axillary buds	Shoot nodal segments, Cotyledon
2.	Direct somatic organogenesis	Cotyledon
3.	Indirect somatic organogenesis	Cotyledon without embryoaxis
4.	Somatic embryogenesis	Cotyledon without embryoaxis

3.7 Enhanced release of axillary buds

3.7.1 Shoot proliferation

3.7.1.1 Plant growth substances

Shoot nodal segments and cotyledon were subjected to treatments with different combination of plant growth substances. MS was used as the basal medium. Different combinations of BA (0.50 – 2.50 mgl^{-1}) and IAA (1.00 – 4.00 mgl^{-1}) were tried (Table 1). The treatments were replicated six times. The cultures were provided with a light intensity of 3000 lux and an

illumination for six hours. The best treatment was later tried on half strength MS, SH, B5 and WPM to assess its effect on shoot proliferation.

In the case of nodal segments, observations were recorded on the number of days taken for bud initiation, number of shoots and length of shoots. Observations were taken in the case of cotyledon cultures on the number of shoots, length of the longest shoots, average length of the shoots and number of leaves per shoot eight weeks from inoculation.

3.7.1.1.1 Gibberellic acid

The best plant growth substance treatment other than GA_3 was used to study the effect of GA_3 on shoot proliferation. Varying levels of GA_3 (1.00, 2.00, 3.00 mgl^{-1}) were tried (Table 2).

3.7.1.2 Carbon source

3.7.1.2.1 Sucrose

Varying levels of sucrose (20.00, 30.00, 40.00, 50.00 gl^{-1}) were tried to study their effect on shoot proliferation (Table 3).

3.7.1.2.2 Glucose

The effect of glucose at different levels (20.00, 30.00, 40.00, 50.00 gl^{-1}) on shoot proliferation was studied (Table 4).

3.7.1.3 Amino acids

3.7.1.3.1 Casein hydrolysate

The cultures were transferred to media with varying levels of casein hydrolysate (100.00, 200.00, 300.00 mgl^{-1}) to study the effect on shoot proliferation (Table 5).

Table 1. Plant growth substances tried for shoot proliferation *via* enhanced release of axillary buds from shoot nodal and cotyledon explants of *Aegle marmelos*

Medium : MS + Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 gl^{-1})

Treatment No.	Plant growth substances (mgl^{-1})
EAB 1	BA 0.50
EAB 2	BA 0.50 + IAA 1.00
EAB 3	BA 0.50 + IAA 2.00
EAB 4	BA 0.50 + IAA 4.00
EAB 5	BA 1.50
EAB 6	BA 1.50 + IAA 1.00
EAB 7	BA 1.50 + IAA 2.00
EAB 8	BA 1.50 + IAA 4.00
EAB 9	BA 2.50
EAB 10	BA 2.50 + IAA 1.00
EAB 11	BA 2.50 + IAA 2.00
EAB 12	BA 2.50 + IAA 4.00
EAB 13	IAA 1.00
EAB 14	IAA 2.00
EAB 15	IAA 4.00

Table 2. Gibberellic acid levels tried for shoot proliferation *via* enhanced release of axillary buds from cotyledon explants of *Aegle marmelos*

Medium : MS + Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 gl^{-1}) + BA (0.50 mgl^{-1})

Treatment No.	GA_3 (gl^{-1})
T 1	1.00
T 2	2.00
T 3	3.00

Table 3. Sucrose levels tried for shoot proliferation *via* enhanced release of axillary buds from cotyledon explants of *Aegle marmelos*

Medium : MS +Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 gl^{-1}) + BA (0.50 mgl^{-1})

Treatment No.	Sucrose (gl^{-1})
S 1	20.00
S 2	30.00
S 3	40.00
S 4	50.00

Table 4. Glucose levels tried for shoot proliferation *via* enhanced release of axillary buds from cotyledon explants of *Aegle marmelos*

Medium : MS + Inositol (100.00 mgl^{-1}) + Sucrose (300.00 gl^{-1}) + Agar (8.00 gl^{-1}) + BA (0.50 mgl^{-1})

Treatment No.	Glucose (gl^{-1})
G 1	20.00
G 2	30.00
G 3	40.00
G 4	50.00

Table 5. Casein hydrolysate levels tried for shoot proliferation *via* enhanced release of axillary buds from cotyledon explants of *Aegle marmelos*

Medium : MS + Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 gl^{-1}) + BA (0.50 mgl^{-1})

Treatment No.	Casein hydrolysate (mgl^{-1})
CH 1	100.00
CH 2	200.00
CH 3	300.00

3.7.1.3.2 Adenine sulphate

Varying levels of adenine sulphate (10.00, 20.00, 30.00, 40.00, 50.00 mgl^{-1}) were tried to assess the effect on shoot proliferation (Table 6).

3.7.1.4 Ethylene inhibitors

The effect of ethylene inhibitor, cobaltous chloride at two different levels (10.00 and 15.00 mgl^{-1}) on shoot proliferation was studied (Table 7).

3.7.1.5 Gelling agent

Different levels of agar (5.00 to 8.00 gl^{-1}) were tried to study their effect on shoot proliferation (Table 8).

3.7.1.6 Mode of culture

Liquid medium as well as solid medium were tried in order to assess the effect of mode of culture on shoot proliferation.

3.7.1.7 Culture conditions

The cultures 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 shoot proliferation.

3.8 Somatic organogenesis

3.8.1 Direct organogenesis

3.8.1.1 Initiation

Cotyledonary explant was subjected to the initiation treatments. MS was used as the basal media. Different combinations of BA (0.10 to 2.50 mgl^{-1}) and IAA (2.00, 4.00 mgl^{-1}) were tried. The treatments were replicated

Table 6.Adenine sulphate levels tried for shoot proliferation *via* enhanced release of axillary buds from cotyledon explants of *Aegle marmelos*

Medium : MS + Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 gl^{-1}) + BA (0.50 mgl^{-1})

Treatment No.	Adenine sulphate (mgl^{-1})
Ad S 1	10.00
Ad S 2	20.00
Ad S 3	30.00
Ad S 4	40.00
Ad S 5	50.00

Table 7.Cobaltous chloride levels tried for shoot proliferation *via* enhanced release of axillary buds from cotyledon explants of *Aegle marmelos*

Medium : MS + Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 gl^{-1}) + BA (0.50 mgl^{-1})

Treatment No.	Cobaltous chloride (mgl^{-1})
CC 1	10.00
CC 2	15.00

Table 8. Agar levels tried for shoot proliferation *via* enhanced release of axillary buds from cotyledon explants of *Aegle marmelos*

Medium : MS +Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 gl^{-1}) + BA (0.50 mgl^{-1})

Treatment No.	Agar (mgl^{-1})
AG 1	5.00
AG 2	6.00
AG 3	7.00
AG 4	8.00

six times. Observations were taken on the number of cultures showing direct organogenesis and number of shoots per culture three weeks from inoculation (Table 9).

3.8.1.2 Shoot proliferation

The shoots obtained by direct organogenesis were then transferred to shoot proliferation medium. The treatments tried were same as that for initiation. The treatments were replicated six times. Observations were taken on the number of shoots, length of the longest shoot, average length of shoots and number of leaves per shoot six weeks after subculutre.

3.8.2 Indirect somatic organogenesis

3.8.2.1 Callus initiation

Explants (cotyledons) were subjected to different treatments for callus initiation. The treatments involved different levels of BA (0.10, 0.50 mgl^{-1}), 2,4-D (0.10, 0.50 mgl^{-1}) and NAA (1.00, 2.00 mgl^{-1}). The treatments were replicated six times (Table 10).

Observations were recorded on the number of cultures initiating callus from cotyledons. Callus index (CI) was computed by multiplying per cent cultures initiating callus with growth score (G). Growth of the callus was assessed based on the visual rating (with score 1.00 to the smallest and score 4.00 to the largest). The mean score was expressed as growth score, 'G' (Poor – 1, Medium – 2, Good – 3, Profuse – 4).

3.8.2.2 Shoot proliferation

The callus was cultured into treatments within varying levels of cytokinins and auxins. Different concentrations of BA (0.10 to 2.00 mgl^{-1}),

Table 9.Plant growth substances tried for initiation of direct somatic organogenesis from cotyledon explants of *Aegle marmelos*

Medium : MS + Inositol (100.00 gl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 gl^{-1})

Treatment No.	Plant growth substances (mgl^{-1})
DSO 1	BA 0.10
DSO 2	BA 0.10 + IAA 2.00
DSO 3	BA 0.10 + IAA 4.00
DSO 4	BA 0.20
DSO 5	BA 0.20 + IAA 2.00
DSO 6	BA 0.20 + IAA 4.00
DSO 7	BA 0.40
DSO 8	BA 0.40 + IAA 2.00
DSO 9	BA 0.40 + IAA 4.00
DSO 10	BA 0.80
DSO 11	BA 0.80 + IAA 2.00
DSO 12	BA 0.80 + IAA 4.00
DSO 13	BA 1.60
DSO 14	BA 1.60 + IAA 2.00
DSO 15	BA 1.60 + IAA 4.00
DSO 16	BA 2.50
DSO 17	BA 2.50 + IAA 2.00
DSO 18	BA 2.50 + IAA 4.00

Table 10. Plant growth substances tried for callus initiation from cotyledon explants of *Aegle marmelos*

Medium: MS + Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) +Agar (8.00 gl^{-1})

Treatment No.	Plant growth substances (mgl^{-1})
C 1	BA 0.10
C 2	BA 0.10 + 2,4-D 0.10
C 3	BA 0.10 + 2,4-D 0.50
C 4	BA 0.50
C 5	BA 0.50 + 2,4-D 0.10
C 6	BA 0.50 + 2,4-D 0.50
C 7	2,4-D 0.10
C 8	2,4-D 0.50
C 9	NAA 1.00
C 10	NAA 1.00 + BA 0.10
C 11	NAA 1.00 + BA 0.50
C 12	NAA 2.00
C 13	NAA 2.00 + BA 0.10
C 14	NAA 2.00 + BA 0.50

Kinetin (1.00, 2.00 mgl^{-1}), 2,4-D (0.50, 0.10 mgl^{-1}), NAA (1.00, 2.00 mgl^{-1}) and IAA (0.50, 1.00 mgl^{-1}) were tried to assess the effect of these plant growth substances on regeneration of shoots from callus. The treatments were replicated six times (Table 11). Observations were taken on the number of cultures developing shoots, number of shoots, length of the longest shoot and average length of shoots.

3.9 Somatic embryogenesis

3.9.1 Induction

The cotyledon obtained from the seeds of immature fruits were subjected to different treatments for induction of somatic embryogenesis. The basal media used was MS. The combinations of plant growth substance tried were the same as those tried for callus initiation in the case of *in vitro* propagation *via* indirect somatic organogenesis (Table 10).

3.10 Rooting

3.10.1 *In vitro* rooting

3.10.1.1 Plant growth substances

Well developed shoots having 3.00 – 5.00 cm length were separated and subjected to different rooting treatments.

Trials were conducted initially on MS medium. The shoots were then cultured into treatments with varying levels of IBA (0.50 to 2.50 mgl^{-1}), IAA (1.00 to 2.50 mgl^{-1}) and NAA (1.00 mgl^{-1}). Each treatment was replicated six times (Table 12).

Table 11. Plant growth substances tried for shoot proliferation *via* indirect somatic organogenesis from cotyledon explants of *Aegle marmelos*

Medium : MS +Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 gl^{-1})

Treatment No.	Plant growth substances (mgl^{-1})
ISO 1	BA 0.10 + 2,4-D 0.100
ISO 2	BA 0.50 + 2,4-D 0.10
ISO 3	BA 0.10 + NAA 1.00
ISO 4	BA 0.10 + NAA 2.00
ISO 5	BA 0.50 + NAA 1.00
ISO 6	BA 0.50 + NAA 2.00
ISO 7	BA 0.10
ISO 8	BA 0.50
ISO 9	BA 1.00 + IAA 0.50
ISO 10	BA 1.00 + IAA 1.00
ISO 11	BA 2.00 + IAA 0.50
ISO 12	BA 2.00 + IAA 1.00
ISO 13	Kinetin 1.00
ISO 14	Kinetin 1.00 + NAA 1.00
ISO 15	Kinetin 1.00 + NAA 2.00
ISO 16	Kinetin 2.00
ISO 17	Kinetin 2.00 + NAA 1.00
ISO 18	Kinetin 2.00 + NAA 2.00
ISO 19	NAA 1.00
ISO 20	NAA 2.00

Observations were taken on the number of cultures initiating roots, number of days for root initiation, number of roots, length of roots and nature of roots.

3.10.1.2 Basal media

Comparison on the effect of different basal media such as full and half strength MS, B5, SH and WPM on *in vitro* rooting was made.

3.10.1.3 Sucrose

Varying levels of sucrose (10.00, 20.00, 30.00, 40.00 gl^{-1}) were tried to assess the effect of sucrose on rooting (Table 13).

3.10.2 *Ex vitro* rooting

Well developed shoots having 3.00 – 8.00 cm length were separated and the unrooted plantlets were given different combinations of hormone treatment. Both quick dip and slow dip methods were tried. In the case of quick dip, 1000.00 ppm IBA and 500.00 ppm IBA were tried for 20 seconds. For slow dip, plantlets were kept in IBA 100.00 ppm solution overnight. The treated plantlets were planted out on a suitable potting medium. Observations were taken on per cent survival of plantlets after two weeks and four weeks.

3.11 Planting out and acclimatization

A few plantlets that showed normal growth were planted out in plastic pots. The cotton plug of the culture vessels were removed, sterile water added to the vessels and kept as such for 10 to 15 min. Then rooted plantlets were taken out from culture vessels with the help of forceps. The agar adhering to

Table 12. Plant growth substances tried for *in vitro* rooting of *Aegle marmelos*

Medium : MS + Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 gl^{-1})

Treatment No.	Plant growth substances (mgl^{-1})
R 1	IBA 0.50
R 2	IBA 1.00
R 3	IBA 1.50
R 4	IBA 2.00
R 5	IBA 2.50
R 6	IAA 1.00
R 7	IAA 1.50
R 8	IAA 2.00
R 9	IAA 2.50
R 10	NAA 1.00

Table 13. Sucrose levels tried for *in vitro* rooting of *Aegle marmelos*

Medium : MS + Inositol (100.00 mgl^{-1}) + Agar (8.00 gl^{-1}) + IBA (2.50 mgl^{-1})

Treatment No.	Sucrose (gl^{-1})
SR 1	10.00
SR 2	20.00
SR 3	30.00
SR 4	40.00

the roots was completely removed by thorough washing with running tap water.

The plantlets were treated with Indofil (0.30 %) solution for 30 minutes before planting out. The effect of potting media on the *ex vitro* establishment of the plantlets were tried by providing different media (sand, soilrite and soilrite : sand in the ratio 2 : 1) (Table 14).

Observations were taken on the per cent survival of plantlets after two weeks and four weeks.

3.12 Statistical analysis

Completely randomised design was followed for statistical analysis wherever necessary as per Panse and Sukhatme (1985).

Table 14 Potting media tried for *ex vitro* establishment of *Aegle marmelos* plantlets

Container : Plastic pots

Treatment No.	Media
PO 1	Sand
PO 2	Soilrite
PO 3	Soilrite : Sand (2 : 1)

Results

4. RESULTS

Investigations were carried out for standardising *in vitro* techniques for the rapid clonal propagation of *Aegle marmelos* (L.) Corr., at the Plant Molecular Biology and Biotechnology Centre, College of Agriculture, Vellayani during 1999-2001. The results of the studies are presented in this chapter.

4.1 Enhanced release of axillary buds

Shoot nodal segments and cotyledons were used as explants.

4.1.1 *Shoot proliferation*

4.1.1.1 Plant growth substances

Fifteen treatments involving various combination of plant growth substances (BA and IAA) were tried to study their effect on shoot proliferation from nodal segments and cotyledons(Table 1).

4.1.1.1.1 Nodal segments

The survival rate of cultures varied from 16.66 per cent to 50.00 per cent(Table 15). EAB 7 (BA 1.50 mgl^{-1} and IAA 2.00 mgl^{-1}), EAB 9 (BA 2.50 mgl^{-1}), EAB10 (BA 2.50 mgl^{-1} and IAA 1.00 mgl^{-1}) and EAB12 (BA 2.50 mgl^{-1} and IAA 4.00 mgl^{-1}) recorded the highest percentage of survival. The least survival rate of 16.66 per cent was observed in EAB 4 (BA 0.50 mgl^{-1} and IAA 4.00 mgl^{-1}), EAB 6 (BA 1.50 mgl^{-1} and IAA 1.00 mgl^{-1}), EAB 11 (BA 2.50 mgl^{-1} and IAA 2.00 mgl^{-1}) and EAB 13 (IAA 1.00 mgl^{-1}).

With respect to the number of days taken for bud initiation, there was not much difference between the various treatments (Table 15). Bud initiation was earliest (3.50 days) when cultured in EAB 13 (IAA 1.00 mgl^{-1}) and late (6.33 days) in EAB 7 (BA 1.50 mgl^{-1} and IAA 2.00 mgl^{-1}). Swelling of the dormant axillary buds took place within a week in all the treatments and was followed by differentiation into shoots (Plate 2).

Regarding the number of shoots, treatment EAB 10 (BA 2.50 mgl^{-1} and IAA 1.00 mgl^{-1}) registered the maximum value (7.33) (Plate 3). The least number of shoots (2.00) was observed in the treatments EAB 3 (BA 0.50 mgl^{-1} and IAA 2.00 mgl^{-1}), EAB 8 (BA 1.50 mgl^{-1} and IAA 4.00 mgl^{-1}), EAB 12 (BA 2.50 mgl^{-1} and IAA 4.00 mgl^{-1}) and EAB 13 (IAA 1.00 mgl^{-1}) (Table 15, Fig. 1).

The length of the shoots was also maximum (2.26 cm) in the treatment EAB 10 while the cultures in EAB 1 (BA 0.50 mgl^{-1}) produced the shortest shoots (0.60 cm).

But in the *in vitro* shoots developed from nodal explants, there were serious problems of precocious leaf fall and tip necrosis because of which further maintenance of cultures and elongation of regenerated shoots could not be resorted to (Plate 4).

4.1.1.1.2 Cotyledons

All the treatments with plant growth substances and control registered cent percent survival rate showing growth and multiple shoot production from the cotyledonary node (Plate 5).

Table 15. Effect of plant growth substances on shoot proliferation *via* enhanced release of axillary buds from shoot nodal explants of *Aegle marmelos*

* Treatments	Survival rate (%)	No. of days for bud initiation	No. of shoots per culture	Length of the shoots (cm)
EAB 1	50.00	4.00	3.00	0.60
EAB 2	33.00	4.17	4.00	1.30
EAB 3	33.00	5.00	2.00	1.35
EAB 4	16.66	4.67	3.00	1.60
EAB 5	33.33	4.17	3.50	1.00
EAB 6	16.66	4.17	4.00	0.70
EAB 7	50.00	6.33	2.33	1.56
EAB 8	16.66	4.33	2.00	1.90
EAB 9	50.00	3.83	4.33	1.26
EAB 10	50.00	4.67	7.33	2.26
EAB 11	16.66	4.33	3.00	1.70
EAB 12	50.00	5.33	2.00	0.90
EAB 13	16.66	3.50	2.00	1.20
EAB 14	33.33	4.33	2.50	1.50
EAB 15	33.33	4.50	2.50	1.00
Control	33.33	4.00	3.00	0.80

The data represent mean value of six replications
*Treatment combinations are given in Table 1.

Culture medium : MS + Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 gl^{-1})

Plate 2. Bud initiation *via* enhanced release of axillary buds from nodal segments

Plate 3. Shoot proliferation *via* enhanced release of axillary buds from nodal segments in MS basal medium supplemented with BA 2.50 mgl^{-1} and IAA 1.00 mgl^{-1}

Fig. 1 Effect of plant growth substances on shoot proliferation *via* enhanced release of axillary buds from shoot nodal explants of *Aegle marmelos*

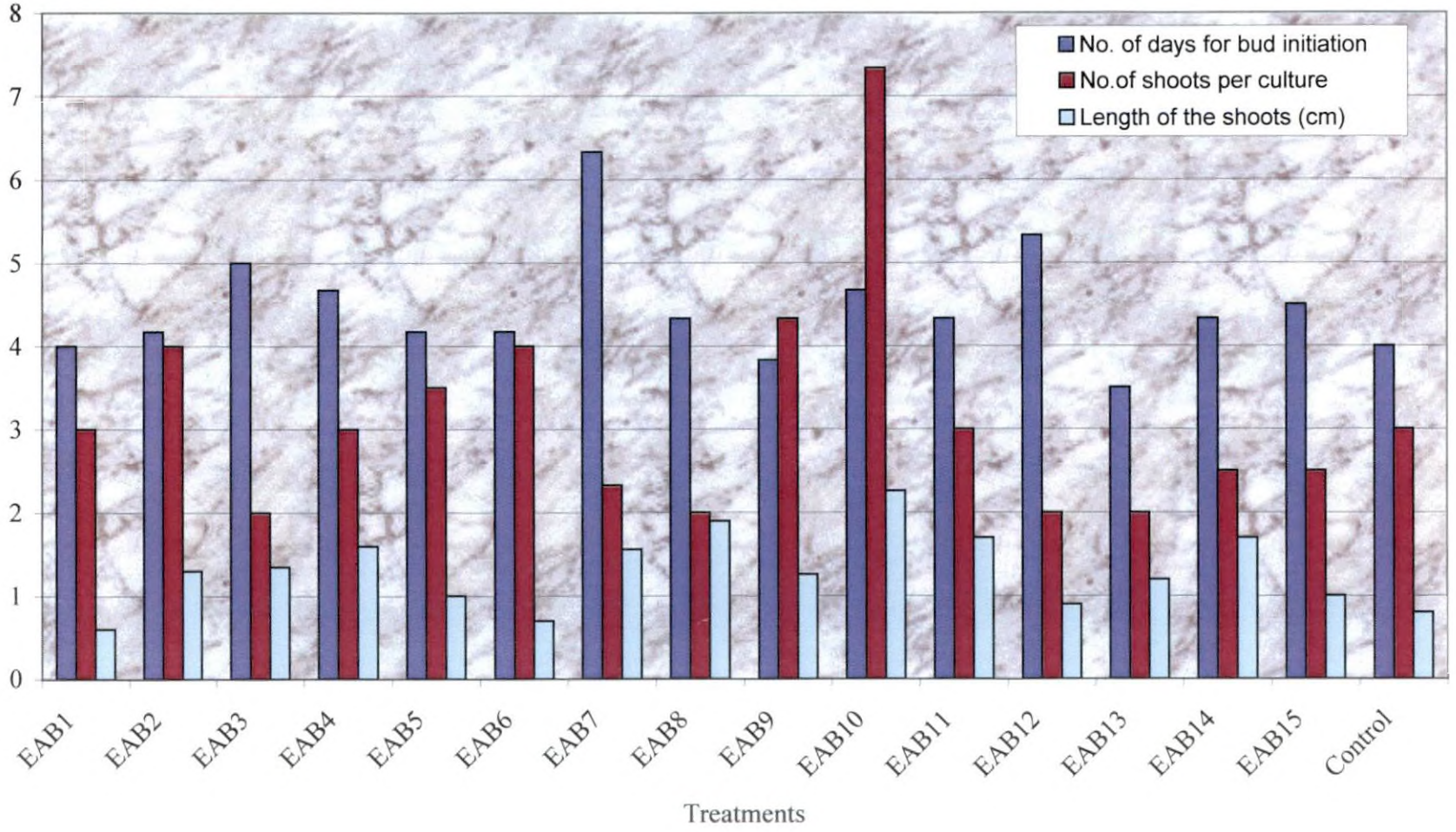
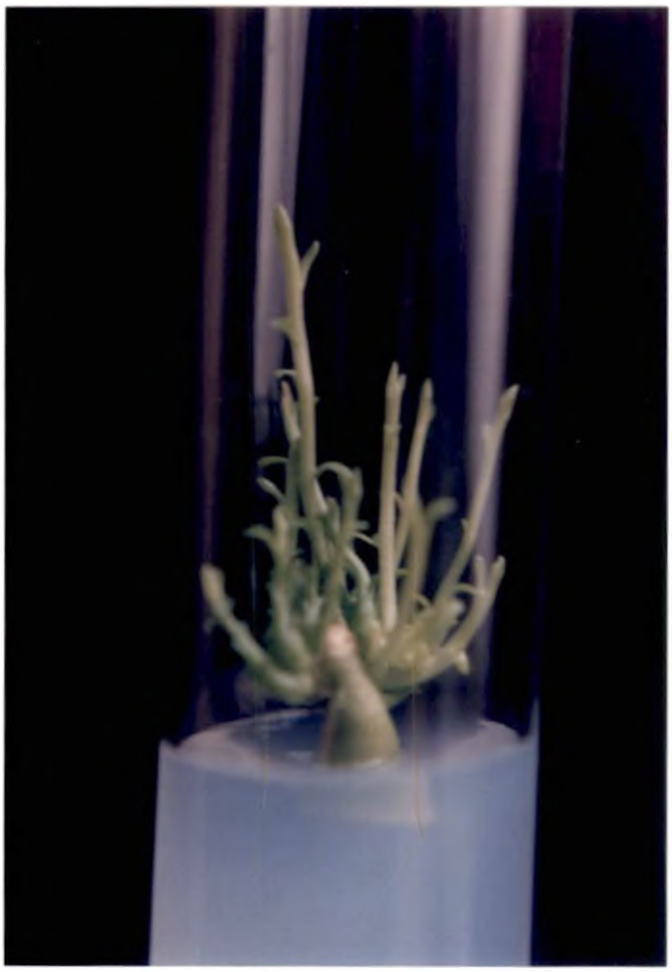


Plate 4. Precocious leaf fall and tip necrosis in culture kept for shoot proliferation *via* enhanced release of axillary buds from nodal explant

Plate 5. Multiple shoot production from cotyledon



With regard to the number of shoots, treatment EAB 1 (BA 0.50 mgl^{-1}) produced maximum number of shoots per culture (49.00) (Plate 6) which was on par with EAB 2 (BA 0.50 mgl^{-1} and IAA 1.00 mgl^{-1}) (45.17). These two treatments were significantly superior to all other treatments. Minimum number of shoots per culture was noticed in the treatments with auxin alone in the medium. The least number of shoots per culture (1.33) was recorded by the treatment EAB 14 (IAA 2.00 mgl^{-1}) which was on par with EAB 13 and EAB 15 (Fig. 2). But the cultures in the medium with no plant growth substances (control), produced only one shoot per culture in all the replications (Table 16).

Treatments with auxin alone exhibited maximum elongation of shoots and were significantly different from other treatments. Maximum length of the longest shoot (8.25 cm) was recorded by EAB 14 (IAA 2.00 mgl^{-1}) which was on par with EAB 13, EAB 15 and control. The length of the longest shoot was minimum (1.73) in EAB 12 (BA 2.50 mgl^{-1} and IAA 4.00 mgl^{-1}).

Average length of the shoots was also highest (7.20 cm) in the treatment EAB 14 which was on par with EAB 13, EAB 15 and control. The least recorded value (0.45 cm) for average length of shoot was in the treatment EAB 9 (BA 2.50 mgl^{-1}).

In the treatments with BA alone, there was significant reduction in the length of the longest shoot and average length of the shoots as the concentration of BA increased from 0.50 mgl^{-1} to 2.50 mgl^{-1} (Plate 7). The length of the longest shoot recorded in EAB 1 (BA 0.50 mgl^{-1}) was 4.93 cm while that in EAB 9 (BA 2.50 mgl^{-1}) was 1.83 cm. Average length of the

Plate 6. Shoot proliferation *via* enhanced release of axillary buds from cotyledon on MS basal medium supplemented with BA 0.50 mgl^{-1}

Plate 7. Comparison of growth of shoots regenerated on MS basal medium supplemented with BA 2.50 mgl^{-1} and BA 0.50 mgl^{-1}

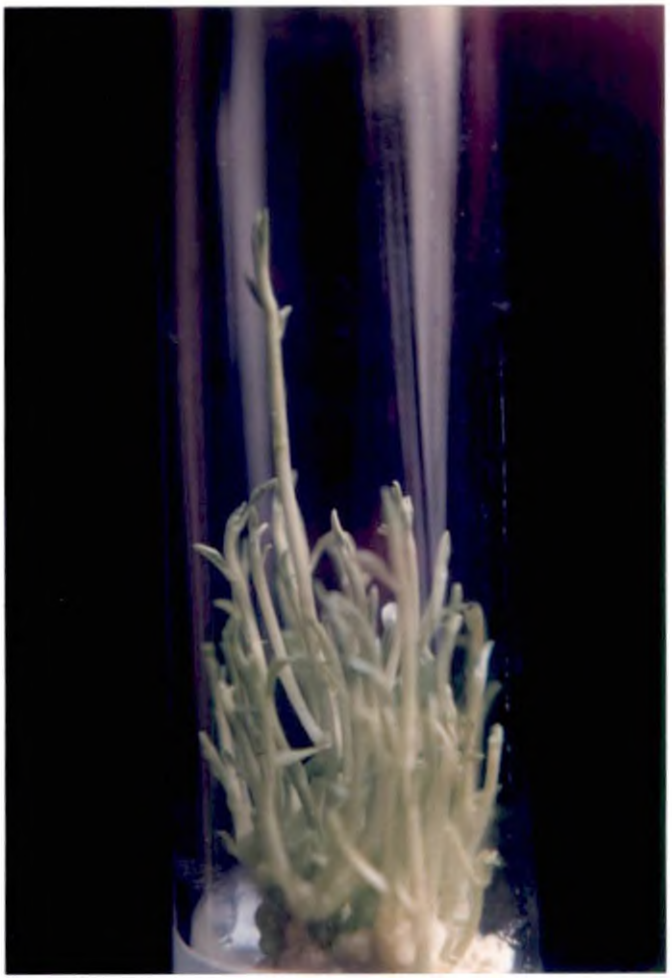
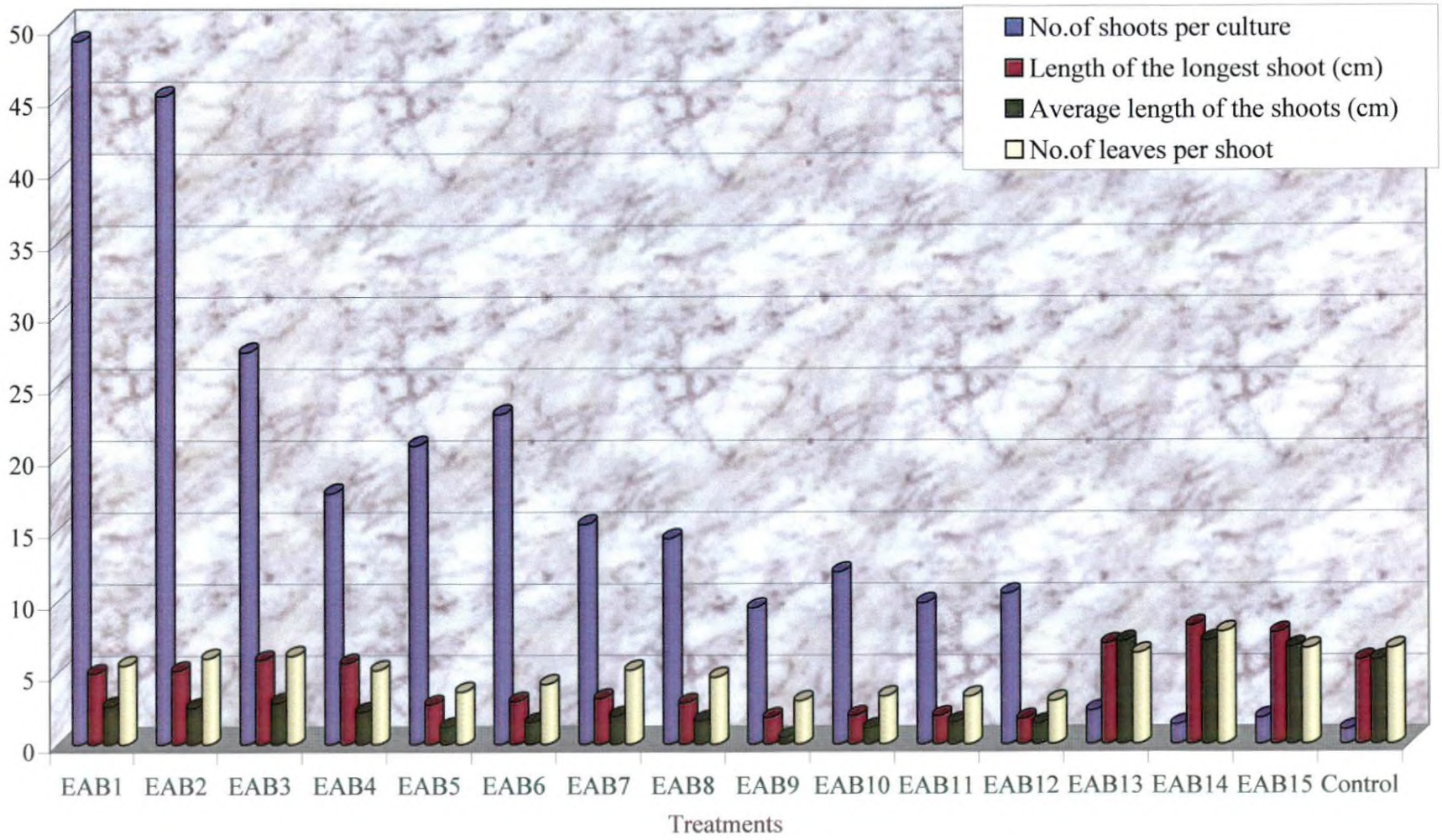


Fig. 2 Effect of plant growth substances on shoot proliferation *via* enhanced release of axillary buds from cotyledon explants of *Aegle marmelos*



shoots registered by EAB1 was 2.67 cm while that by EAB 9 was only 0.45 cm.

Treatment EAB 14 (IAA 2.00 mgl^{-1}) produced maximum number of leaves per shoot (7.83) and was on par with EAB 13, EAB 15 and control. Least number of leaves per shoot (3.00) was recorded in EAB 9 (BA 2.50 mgl^{-1}) and EAB 12 (BA 2.50 mgl^{-1} and IAA 4.00 mgl^{-1}). EAB 5, EAB 6, EAB 10 and EAB11 were on par with these two treatments (Table 16).

Micro cuttings taken from these *in vitro* regenerated shoots when sub cultured, also responded well and produced new shoots not only from axils but also from the basal portion (Plate 8).

4.1.1.2 Basal media

Basal media such as MS (full and half strength), SH, B5 and WPM were compared to assess their effect on shoot proliferation (Fig. 3).

Full strength MS was found to be significantly superior to others with respect to number of shoots, length of the longest shoot and average length of the shoots (Plate 9).

Maximum number of shoots (49.00) were produced by cultures in full strength MS. WPM produced least number of shoots (12.17) which was on par with SH and significantly different from others. The effects of B5 and half strength MS were on par producing 30.50 and 21.50 shoots respectively, but also were significantly inferior to full strength MS (Table 17).

Highest value for the length of the longest shoot (4.93) and average length of the shoots (2.67 cm) were recorded by the cultures grown in MS

Table 16. Effect of plant growth substances on shoot proliferation *via* enhanced release of axillary buds from cotyledon explants of *Aegle marmelos*

*Treatments	Survival rate (%)	No. of shoots per culture	Length of the longest shoot (cm)	Average length of the shoots (cm)	No. of leaves per shoot
EAB 1	100.00	49.00	4.93	2.67	5.50
EAB 2	100.00	45.17	5.13	2.55	6.00
EAB 3	100.00	27.33	5.87	2.90	6.17
EAB 4	100.00	17.50	5.63	2.25	5.17
EAB 5	100.00	20.83	2.75	1.18	3.67
EAB 6	100.00	23.00	2.97	1.50	4.17
EAB 7	100.00	15.33	3.18	1.95	5.17
EAB 8	100.00	14.33	2.85	1.63	4.67
EAB 9	100.00	9.50	1.83	0.45	3.00
EAB 10	100.00	12.00	1.95	1.10	3.33
EAB 11	100.00	9.83	1.95	1.52	3.33
EAB 12	100.00	10.50	1.73	1.42	3.00
EAB 13	100.00	2.33	7.00	7.15	6.33
EAB 14	100.00	1.33	8.25	7.20	7.83
EAB 15	100.00	1.83	7.75	6.77	6.67
Control	100.00	1.00	5.87	5.87	6.67
CD at 5% level	-	7.58	1.34	1.23	1.49

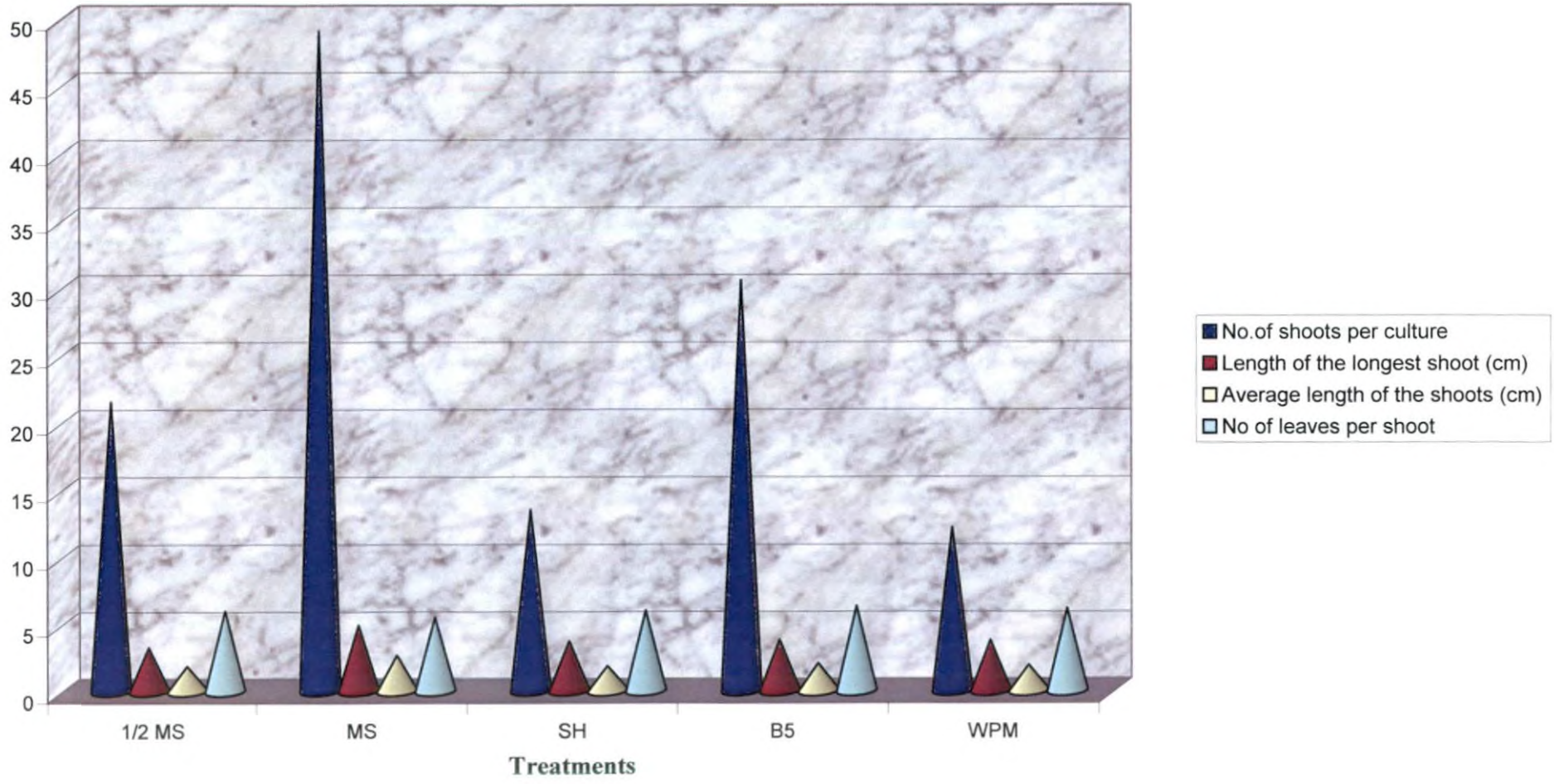
The data represents mean value of six replications
*Treatment details are given in Table 1.

Culture medium : MS + Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 gl^{-1})

Plate 8. Shoot proliferation from axils and basal portion of *in vitro* microcuttings on MS basal medium supplemented with BA 0.50 mgl^{-1}

Plate 9. Shoot proliferation *via* enhanced release of axillary buds from cotyledon on MS basal medium

Fig. 3 Effect of Basal Media on Shoot Proliferation *via* Enhanced Release of Axillary Buds From Cotyledon Explants of *Aegle marmelos*



whereas half strength MS, SH, B5 and WPM were significantly inferior to MS but on par with each other.

No significant difference was observed among the treatments for the number of leaves per shoot.

4.1.1.3 Gibberellic acid

Study on the effect of three different levels of gibberellic acid was conducted.

It was observed that gibberellic acid did not have any significant influence on the number of shoots, length of the longest shoot and number of leaves per shoot when compared to the control.

Average height of the shoots was observed to increase at higher levels of gibberellic acid (2.00 mgl^{-1} and 3.00 mgl^{-1}). Average height of the shoots was maximum (4.33 cm) at 3.00 mgl^{-1} of gibberellic acid (GA 3) and was significantly superior to control. Gibberellic acid at 2.00 mgl^{-1} (GA 2) and 3.00 mgl^{-1} (GA 3) were found to be on par with regard to their effect on the average height of the shoots (Table 18).

4.1.1.4 Carbon Source

4.1.1.4.1 Sucrose

Data pertaining to the results of the trail conducted to study the effect of four different levels of sucrose (20.00, 30.00, 40.00 and 50.00 gl^{-1}) on shoot proliferation are presented in Table 19.

Among the four different levels of sucrose tried, S 4 (50.00 gl^{-1}) produced maximum number of shoots per culture (54.67) (Plate 10) and was

Table 17. Effect of basal media on shoot proliferation *via* enhanced release of axillary buds from cotyledon explants of *Aegle marmelos*

* Treatments	No. of shoots per culture	Length of the longest shoot (cm)	Average length of the shoots (cm)	No. of leaves per shoot
1/2 MS	21.50	3.27	1.88	6.00
MS	49.00	4.93	2.67	5.50
SH	13.50	3.73	1.87	6.00
B5	30.50	3.83	2.07	6.33
WPM	12.17	3.80	1.93	6.17
CD at 5% level	9.41	1.05	0.54	NS

The data represents mean value of six replications

NS – Not Significant

Supplements : BA (0.50 mgl^{-1}) + Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 mgl^{-1})

Table 18. Effect of gibberellic acid on shoot proliferation *via* enhanced release of axillary buds from cotyledon explants of *Aegle marmelos*

* Treatments	No. of shoots per culture	Length of the longest shoot (cm)	Average length of the shoots (cm)	No. of leaves per shoot
T 1	47.67	5.52	3.08	5.66
T 2	49.67	5.73	3.98	6.17
T 3	48.00	5.88	4.33	6.33
Control	49.00	4.93	2.67	5.50
CD at 5% level	NS	NS	0.67	NS

The data represents mean value of the six replications
*Treatment details are given in Table 2.

NS – Not Significant

Culture medium : MS + BA (0.50 mgl^{-1}) + Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 gl^{-1})

on par with S 3 (40.00 gl^{-1}) and S2 (30.00 gl^{-1}). Sucrose at 20.00 gl^{-1} (S 1) was significantly inferior to all others and produced the least number of shoots per culture (22.17).

However, the different levels of sucrose did not evoke any significant difference with regard to length of the longest shoot, average length of the shoots and number of leaves per shoot.

4.1.1.4.2 Glucose

Glucose was tried at four different levels (20.00, 30.00, 40.00 and 50.00 gl^{-1}) to assess its effect on shoot proliferation.

With regard to the number of shoots per culture, glucose at the various levels were found to be significantly inferior to the control having sucrose at 30.00 gl^{-1} in the medium. Among the different levels of glucose, G 3 (40.00 gl^{-1}) produced maximum number of shoots per culture (36.00). Treatment G 1 (20.00 gl^{-1}) produced the least number of shoots per culture (17.67) and was on par with G 2 and G 4 (Table 20).

Regarding the length of the longest shoot, G 3 (40 gl^{-1}) recorded the highest value (5.20 cm) which was on par with G 2 and control. Minimum length for the longest shoot (3.4 cm) was recorded by G 1 (20.00 gl^{-1}).

Average length of the shoots was highest (2.67 cm) in control which was on par with G 3, whereas G 1 registered the least value (1.58 cm).

In the case of number of leaves per shoot also, the highest value was observed in control (5.50) which was on par with G 3. Minimum number of leaves per shoot (3.33) was recorded by G 1 which was on par with G 4.

Table 19.Effect of sucrose on shoot proliferation *via* enhanced release of axillary buds from cotyledon explants of *Aegle marmelos*

* Treatments	No. of shoots per culture	Length of the longest shoot (cm)	Average length of the shoots (cm)	No. of leaves per shoot
S 1	22.17	4.50	2.16	4.83
S 2	49.00	4.93	2.67	5.50
S 3	50.50	5.12	2.63	5.50
S 4	54.67	5.37	2.70	5.83
CD at 5% level	11.31	NS	NS	NS

The data represents mean value of the six replications
*Treatment details are given in Table 3.

NS – Not Significant

Culture medium : MS + BA (0.50 mgl^{-1}) + Inositol (100.00 mgl^{-1}) + Agar (8.00 gl^{-1})

Table 20.Effect of glucose on shoot proliferation *via* enhanced release of axillary buds from cotyledon explants of *Aegle marmelos*

* Treatments	No. of shoots per culture	Length of the longest shoot (cm)	Average length of the shoots (cm)	No. of leaves per shoot
G 1	17.67	3.40	1.58	3.33
G 2	23.50	4.40	2.00	3.83
G 3	36.00	5.20	2.55	4.33
G 4	26.17	4.23	1.85	3.50
Control	49.00	4.93	2.67	5.50
CD at 5% level	9.78	0.86	0.54	1.27

The data represents mean value of the six replications
*Treatment details are given in Table 4.

Culture medium : MS + BA (0.50 mgl^{-1}) + Inositol (100.00 mgl^{-1}) + Agar (8.00 gl^{-1})

4.1.1.5 Amino acids

4.1.1.5.1 Casein hydrolysate

Three levels of casein hydrolysate (100.00, 200.00, 300.00 mgl^{-1}) were supplemented to the media to study the effect on shoot proliferation.

There was no significant difference in the number of shoots produced per culture among the different levels of casein hydrolysate as well as control (Table 21).

Length of the longest shoot was maximum (4.93 cm) in control which was on par with CH 1. The minimum length (3.80 cm) was recorded for the treatment CH 3 which was on par with CH 2 (3.83 cm).

With regard to average length of the shoots, control (2.66 cm) was found to be significantly superior to the different levels of casein hydrolysate tried.

Highest number of leaves per shoot (5.50) was produced by the cultures kept in media without casein hydrolysate and was significantly superior to all other treatments supplemented with casein hydrolysate in the medium. Least number of leaves (3.17) were produced by cultures kept in treatments CH 2 and CH 3.

4.1.1.5.2 Adenine sulphate

Significant difference was observed among the five different levels of adenine sulphate (Table 22, Fig. 4).

Highest number of shoots per culture (58.67) was obtained in AdS 2 (20.00 mgl^{-1}) (Plate 11) which was significantly superior to all other

Table 21. Effect of casein hydrolysate on shoot proliferation *via* enhanced release of axillary buds from cotyledon explants of *Aegle marmelos*

* Treatments	No. of shoots per culture	Length of the longest shoot (cm)	Average length of the shoots (cm)	No. of leaves per shoot
CH 1	47.67	4.13	1.41	3.50
CH 2	48.83	3.83	1.45	3.17
CH 3	48.50	3.80	1.43	3.17
Control	49.00	4.93	2.66	5.50
CD at 5% level	NS	0.96	0.96	1.34

The data represents mean value of the six replications
*Treatment details are given in Table 5.

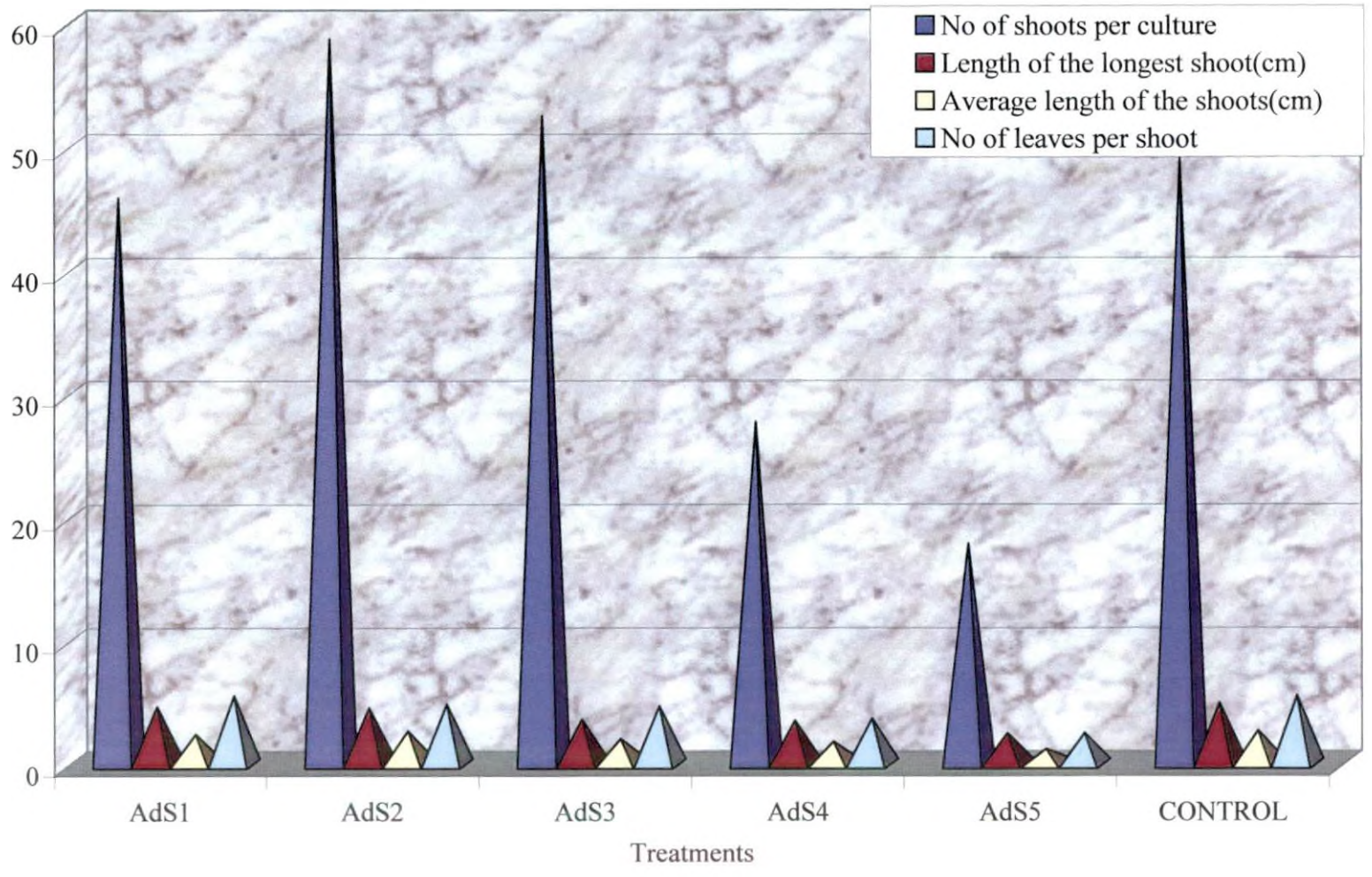
NS – Not Significant

Culture medium : MS + BA (0.50 mgl^{-1}) + Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 gl^{-1})

Plate 10. Shoot proliferation *via* enhanced release of axillary buds from cotyledon on MS basal medium supplemented with sucrose 50.00 mgl^{-1}

Plate 11. Shoot proliferation *via* enhanced release of axillary buds from cotyledon on MS basal medium supplemented with adenine sulphate

Fig. 4 Effect of Adenine Sulphate on shoot prolifertaion *via* enhanced release of axillary buds from cotyledon explants of *Aegle marmelos*



treatments and control, but was on par with AdS 3. It was observed that with the increasing levels of adenine sulphate there was reduction in the number of shoots. The least number of shoots (17.83) was recorded in the treatment AdS 5 (50.00 mgl^{-1}).

Control recorded the highest value for the length of the longest shoot (4.93 cm) and average length of the shoots (2.67 cm). The treatments AdS 1 and AdS 2 were on par with control. Higher levels of adenine sulphate exhibited inhibitory action on the length of the shoots. Treatment AdS 5 (50.00 mgl^{-1}) recorded the least value for the length of the longest shoot (2.47 cm) as well as for the average length of the shoots (1.20 cm).

Treatment AdS1 (10.00 mgl^{-1}) produced maximum number of leaves per shoot (5.50) and was on par with control. Least number of leaves per shoot (2.50) was recorded in the treatment AdS 5.

4.1.1.6 Ethylene inhibitors

Cobaltous chloride tried at the two levels 10.00 mgl^{-1} and 15.00 mgl^{-1} did not produce any significant difference with regard to number of shoots, length of the longest shoot, average length of the shoots and number of leaves per shoot (Table 23).

4.1.1.7 Gelling agent

Data recorded on the effect of four different levels of agar (5.00, 6.00, 7.00 and 8.00 gl^{-1}) on shoot proliferation is presented in Table 24.

No significant variation was noticed among the treatments for the number of shoots per culture, the length of the longest shoot, average length

Table 22. Effect of adenine sulphate on shoot proliferation *via* enhanced release of axillary buds from cotyledon explants of *Aegle marmelos*

* Treatments	No. of shoots per culture	Length of the longest shoot (cm)	Average length of the shoots (cm)	No. of leaves per shoot
AdS 1	45.83	4.63	2.40	5.50
AdS 2	58.67	4.53	2.67	4.83
AdS 3	52.50	3.60	2.03	4.67
AdS 4	27.67	3.48	1.80	3.67
AdS 5	17.83	2.47	1.20	2.50
Control	49.00	4.93	2.67	5.50
CD at 5% level	9.66	0.95	0.51	1.01

The data represents mean value of the six replications
*Treatment details are given in Table 6.

Culture medium : MS + BA (0.50 mgl^{-1}) + Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 gl^{-1})

of the shoots and number of leaves per shoot. However, the maximum number of shoots per culture (60.33) was produced by the treatment AG 1 (5.00 gl^{-1}). It was also noted that a progressive increase in the number of shoots per culture occurred with lower levels of agar.

4.1.1.8 Mode of culture

The effect of solid and liquid media on shoot proliferation was found to be significantly different (Table 25).

With respect to the number of shoots produced per culture, solid media was found to be significantly superior to liquid media. The number of shoots per culture, length of the longest shoot, average length of the shoots and number of leaves per shoot were higher in solid media (49.00, 4.93 cm, 2.67 cm, and 5.50) than in liquid media (13.50, 2.83 cm,1.52 cm and 4.00), respectively.

4.1.1.9 Culture conditions

Light had significant influence on the shoot proliferation. Cultures under lighted condition produced an average of 49.00 shoots, while under dark produced only 20.33 shoots (Table 26).

There was no significant difference in the length of the longest shoot, average length of the shoots and number of leaves per shoot between the cultures kept under dark and light. However, the shoots and leaves appeared pale when kept under dark culture condition.

Table 23. Effect of cobaltous chloride on shoot proliferation *via* enhanced release of axillary buds from cotyledon explants of *Aegle marmelos*

* Treatments	No. of shoots per culture	Length of the longest shoot (cm)	Average length of the shoots (cm)	No. of leaves per shoot
CC 1	47.17	4.53	2.65	5.33
CC 2	47.67	4.73	2.38	5.33
Control	49.00	4.93	2.67	5.50
CD at 5% level	NS	NS	NS	NS

The data represents mean value of the six replications
*Treatment details are given in Table 7.

Culture medium : MS + BA (0.50 mgl^{-1}) + Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 gl^{-1})

Table 24. Effect of agar on shoot proliferation *via* enhanced release of axillary buds from cotyledon explants of *Aegle marmelos*

* Treatments	No. of shoots per culture	Length of the longest shoot (cm)	Average length of the shoots (cm)	No. of leaves per shoot
AG 1	60.33	5.28	2.73	5.67
AG 2	55.33	5.07	2.55	5.50
AG 3	50.67	4.97	2.38	5.33
AG 4	49.00	4.93	2.67	5.50
CD at 5% level	NS	NS	NS	NS

The data represents mean value of the six replications
*Treatment details are given in Table 8.

NS – Not Significant

Culture medium : MS + BA (0.50 mgl^{-1}) + Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1})

Table 25. Effect of mode of culture on shoot proliferation *via* enhanced release of axillary buds from cotyledon explants of *Aegle marmelos*

Treatments	No. of shoots per culture	Length of the longest shoot (cm)	Average length of the shoots (cm)	No. of leaves per shoot
Solid	49.00	4.93	2.67	5.50
Liquid	13.50	2.83	1.52	4.00
CD at 5% level	14.19	1.28	0.70	1.25

The data represents mean value of the six replications

Culture medium (Solid) : MS + BA (0.50 mgl^{-1}) + Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 gl^{-1})
Culture medium (liquid) : MS + BA (0.50 mgl^{-1}) + Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1})

Table 26. Effect of culture conditons on shoot proliferation *via* enhanced release of axillary buds from cotyledon explants of *Aegle marmelos*

Treatments	No. of shoots per culture	Length of the longest shoot (cm)	Average length of the shoots (cm)	No. of leaves per shoot
Dark	20.33	4.28	2.21	4.33
Light	49.00	4.93	2.67	5.50
CD at 5% level	14.30	NS	NS	NS

The data represents mean value of the six replications

NS – Not Significant

Culture medium : MS + BA (0.50 mgl^{-1}) + Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 gl^{-1})

4.2 Somatic Organogenesis

4.2.1 Direct Somatic Organogenesis

4.2.1.1 Cotyledons

4.2.1.1.1 Initiation

4.2.1.1.1.1 Plant growth substances

Eighteen treatments with combinations of plant growth substances (BA and IAA) at different levels were tried to assess the effect on initiation of direct organogenesis from cotyledonary explant.

Wide variation was noticed with regard to the per cent of cultures initiating direct organogenesis, the values ranging from 16.66 to 83.33. The highest percentage (83.33 per cent) of cultures initiating direct organogenesis was observed in DSO1 (BA 0.10 mgl^{-1}) and DSO 4 (BA 0.20 mgl^{-1}) (Plate 12). The least percentage of initiation was shown by DSO 11, DSO 12, DSO 14, DSO 15, DSO 17 and DSO 18 (Table 27). Control, which had no plant growth substances failed to initiate direct organogenesis.

With regard to the number of shoots produced *via* direct organogenesis, maximum number (22.25) was recorded by the treatment DSO 7 (BA 0.40 mgl^{-1}) which was closely followed by DSO 4 (BA 0.20 mgl^{-1}). The least number of shoots (4.00) was produced by DSO 18 (BA 2.50 mgl^{-1} and IAA 4.00 mgl^{-1}).

Table 27.Effect of plant growth substances on initiation of direct organogenesis from cotyledon explants of *Aegle marmelos*

*Treatments	cultures initiating direct organogenesis(%)	Number of shoots per culture
DSO 1	83.33	19.20
DSO 2	50.00	19.00
DSO 3	50.00	15.30
DSO 4	83.33	21.00
DSO 5	50.00	20.30
DSO 6	50.00	12.00
DSO 7	66.66	22.25
DSO 8	33.33	19.00
DSO 9	33.33	14.00
DSO 10	50.00	16.30
DSO 11	16.66	13.00
DSO 12	16.66	11.00
DSO 13	50.00	11.30
DSO 14	16.66	9.00
DSO 15	16.66	6.00
DSO 16	50.00	5.30
DSO 17	16.66	5.00
DSO 18	16.66	4.00
Control	0.00	0.00

The data represents mean value of the six replications
*Treatment details are given in Table 9.

Culture medium : MS + Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 gl^{-1})

4.2.1.1.2 Shoot proliferation

4.2.1.1.2.1 Plant growth substances

The shoots obtained *via* direct organogenesis were subcultured into media with eighteen treatment combinations of BA and IAA at various levels to study the effect on shoot proliferation.

With respect to the number of shoots, significantly superior value (46.83) was recorded by DSO 5 (BA 0.20 mgl^{-1} and IAA 2.00 mgl^{-1}) (Plate 13). This was on par with DSO 1, DSO 2 and DSO 4. DSO 18 (BA 2.50 mgl^{-1} and IAA 4.00 mgl^{-1}) recorded the minimum number (8.33) of shoots (Table 28, Fig. 5).

Length of the longest shoot was also highest (5.80 cm) in DSO 5. DSO 1, DSO2, DSO 3 and DSO 4 were on par with it. The least value (1.73 cm) for the length of the longest shoot was recorded by DSO 17 (BA 2.50 mgl^{-1} and IAA 2.00 mgl^{-1}).

Regarding the average length of the shoots, DSO 2 (BA 0.10 mgl^{-1} and IAA 2.00 mgl^{-1}) recorded the highest value (3.35 cm) and was on par with DSO 1, DSO3, DSO 4 and DSO 5. DSO 16 (BA 2.50 mgl^{-1}) registered the least value (0.45 cm) for the average length of the shoots which was significantly inferior.

The treatment DSO 2 registered the maximum number of leaves per shoot (7.83). Treatments DSO 1, DSO 3, DSO 4, DSO 5 and DSO 6 were on par with it. The least number of leaves per shoot (3.00) was observed in the

Table 28. Effect of plant growth substances on shoot proliferation *via* direct organogenesis from cotyledon explants of *Aegle marmelos*

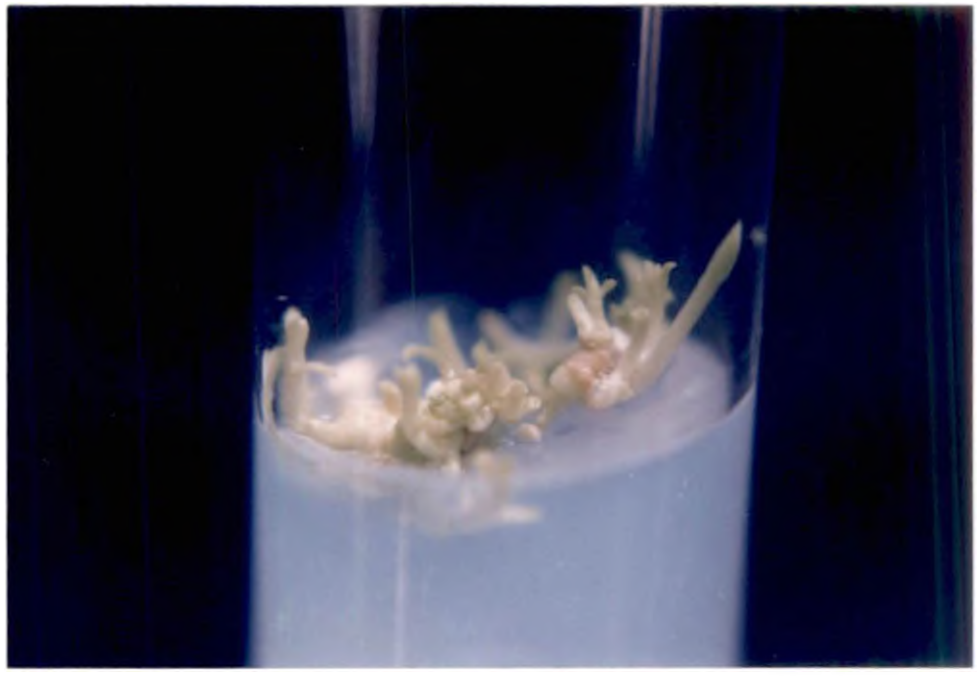
*Treatments	No. of shoots per culture	Length of the longest shoot (cm)	Average length of the shoots (cm)	No. of leaves per shoot
DSO 1	41.33	5.23	3.25	6.50
DSO 2	43.50	5.73	3.35	7.83
DSO 3	24.16	5.30	2.82	6.00
DSO 4	42.50	5.76	3.15	6.83
DSO 5	46.83	5.80	3.28	7.33
DSO 6	22.17	4.70	2.28	6.83
DSO 7	25.00	4.27	2.25	6.00
DSO 8	28.33	4.50	2.18	5.17
DSO 9	19.17	4.38	1.98	5.17
DSO 10	21.83	3.41	2.08	5.00
DSO 11	20.17	4.38	1.97	5.50
DSO 12	16.67	3.75	1.98	5.17
DSO 13	11.50	2.27	1.45	4.17
DSO 14	13.67	2.42	1.15	3.83
DSO 15	10.17	2.10	1.25	4.17
DSO 16	9.50	1.83	0.45	3.00
DSO 17	9.83	1.73	1.22	3.33
DSO 18	8.83	1.87	1.42	3.00
Control	8.33	3.23	2.03	5.67
CD at 5% level	7.48	0.95	0.62	0.61

The data represents mean value of the six replications
*Treatment details are given in Table 9.

Culture medium : MS + Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 gl^{-1})

Plate 12. Initiation of direct organogenesis from cotyledon

Plate 13. Shoot proliferation *via* direct organogenesis from cotyledon on MS basal medium supplemented with BA 0.20 mgl^{-1} and IAA 2.00 mgl^{-1}



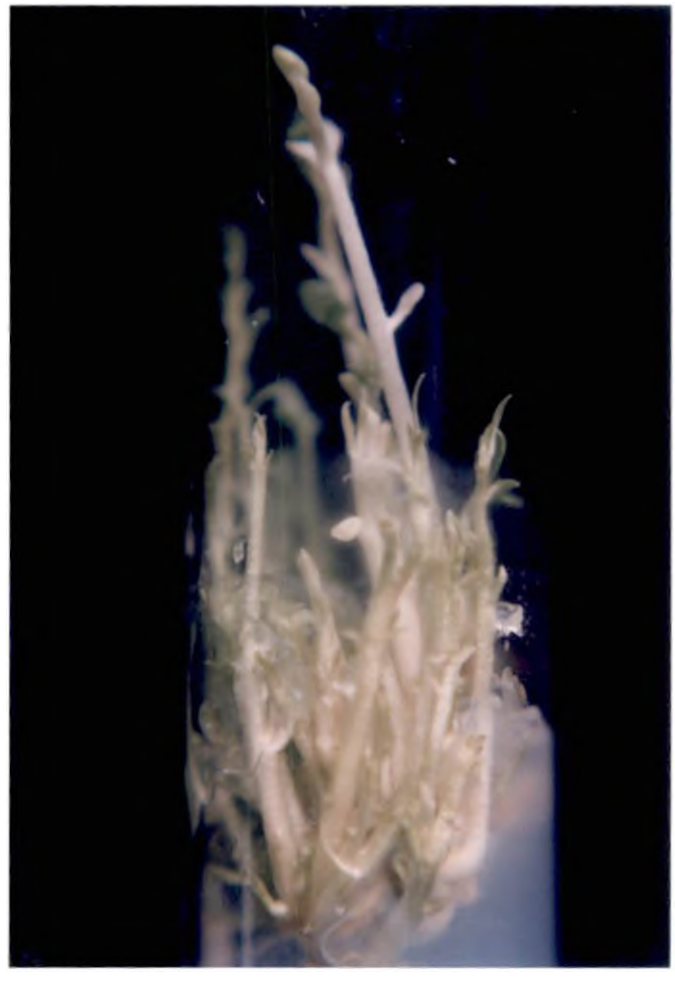
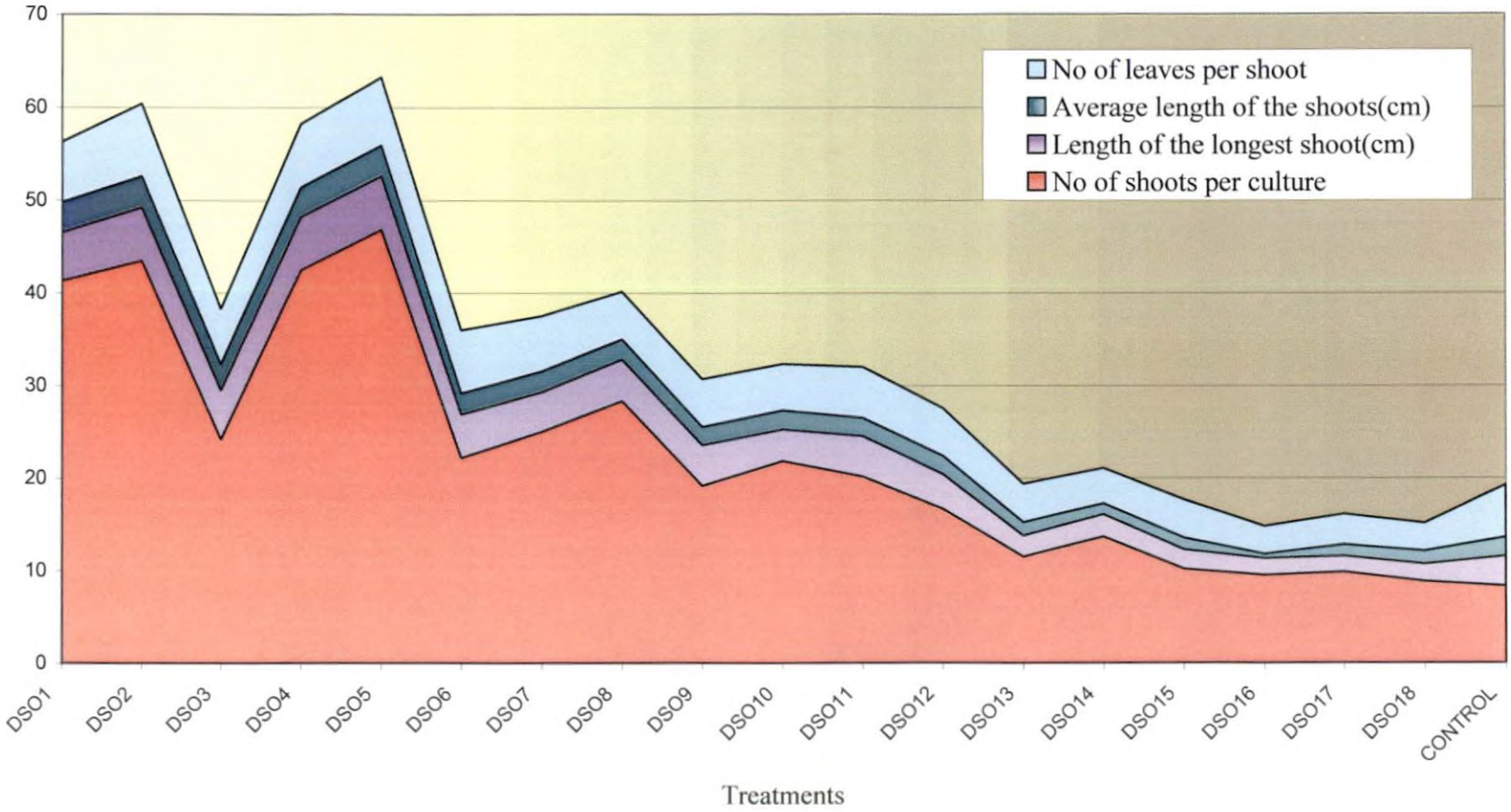


Fig. 5 Effect of plant growth substances on shoot proliferation *via* direct organogenesis from cotyledon explants of *Aegle marmelos*



treatments DSO 16 (BA 2.50 mgl^{-1}) and DSO 18 (BA 2.50 mgl^{-1} and IAA 4.00 mgl^{-1}).

4.2.1.2 Root

In some of the treatments tried for direct organogenesis and enhanced release of axillary buds, intact seedling with root was formed along with the multiple shoots, from the cotyledonary node. In many of these cases, adventitious buds were found to grow directly from the stunted roots which lacked any secondary roots (Plate 14). By four to five weeks, these adventitious buds proliferated and elongated to produce substantiate number of shoots. Shoots were formed from all parts of the root but maximum proliferation was observed from the root tip. The data pertaining to the effect of different plant growth substances on the direct organogenesis from root is presented in Table 29 (Fig. 6).

Highest percentage of cultures showing direct organogenesis (33.33) from root was observed in cultures supplemented with BA 0.20 mgl^{-1} and 0.50 mgl^{-1}.

Maximum number of shoots (35.00) was recorded by BA at 0.50 mgl^{-1} followed by BA at 0.80 mgl^{-1} (Plate 15). IAA at 2.00 mgl^{-1} produced the least number of shoots (5.00).

The treatment with BA at a lower concentration of 0.20 mgl^{-1} exhibited the maximum elongation of shoot (6.10 cm) while BA at 1.60 mgl^{-1} level, produced the shortest shoot (0.80 cm).

Table 29. Effect of plant growth substances on shoot proliferation *via* direct organogenesis from root of *Aegle marmelos*

Treatments (mgl^{-1})	Cultures showing direct organogenesis from root (%)	Number of shoots per culture	Length of the longest shoot (cm)
BA 0.1	16.66	6.00	3.50
BA 0.2	33.33	18.00	6.10
BA 0.5	33.33	35.00	3.20
BA 0.8	16.66	21.00	2.60
BA 1.6	16.66	13.00	0.80
IAA 2	16.66	5.00	4.20
BA 2.5 + IAA 2	16.66	19.00	3.60

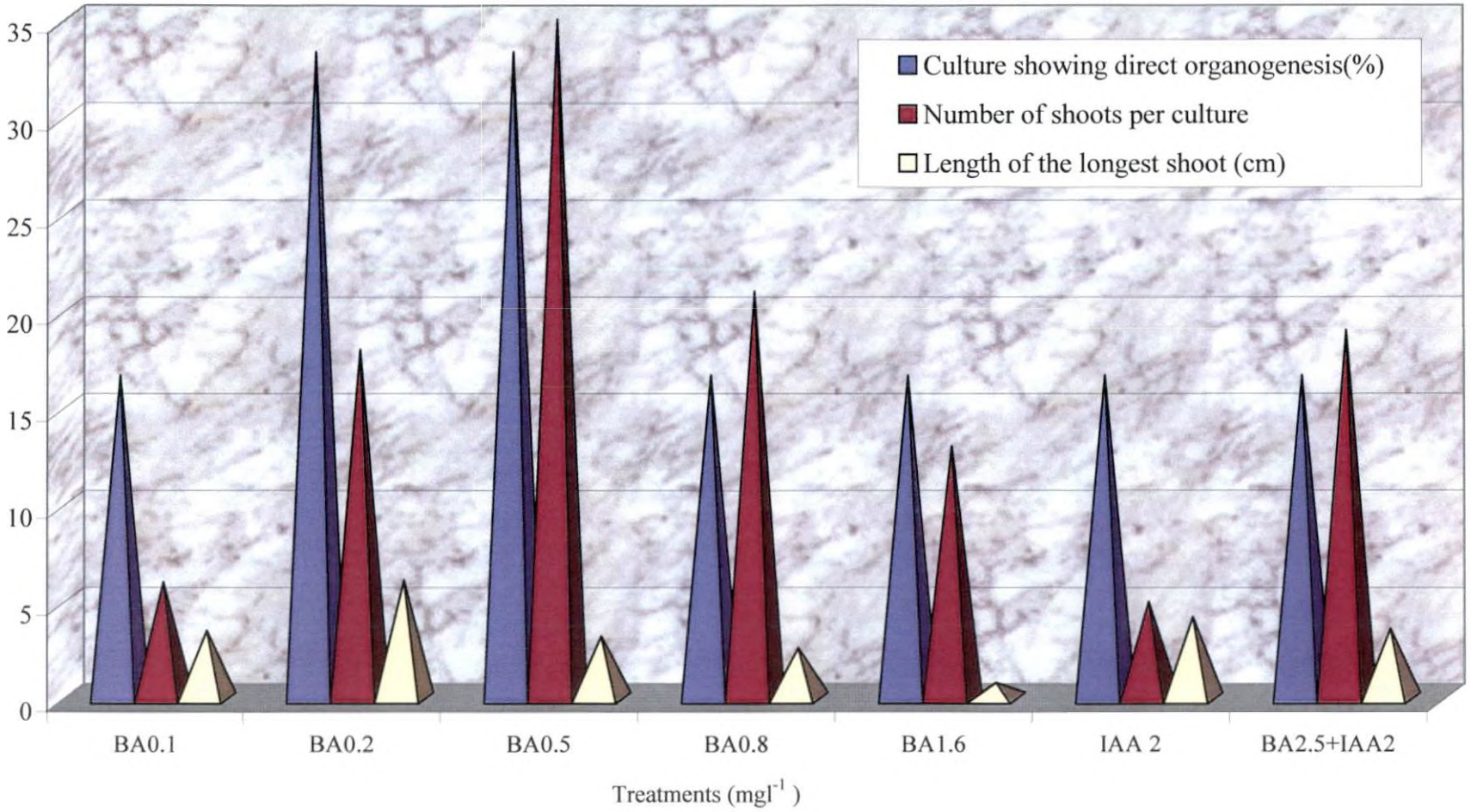
The data represents mean value of the six replications

Culture medium : MS + Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 gl^{-1})

Plate 14. Initiation of direct organogenesis from *in vitro* root

Plate 15. Shoot proliferation *via* direct organogenesis from *in vitro* root on MS basal medium supplemented with BA 0.50 mgl^{-1}

Fig. 6 Effect of Plant Growth Substances on Shoot Proliferation *via* Direct Organogenesis from root of *Aegle marmelos*



4.2.2 Indirect Somatic organogenesis

4.2.2.1 Callus initiation

Fourteen treatments with different combinations of plant growth substances (BA, 2,4-D and NAA) were tried to assess its effect on callus initiation, using cotyledon as explant.

Cent percent cultures in the treatments C 3, C 6, C 12, C 13 and C 14 initiated callus. But C 12, C 13 and C 14 registered very low growth score. Highest growth score (3.50) was recorded by the treatment C 6 (BA 0.50 mgl^{-1} and 2, 4 –D 0.50 mgl^{-1}) followed by C 3 (BA 0.10 mgl^{-1} and 2, 4-D 0.50 mgl^{-1}). The lowest growth score (1.00) was registered by C 5 (BA 0.50 mgl^{-1} and 2, 4-D 0.10 mgl^{-1}) and C 10 (NAA 1.00 mgl^{-1} and BA 0.10 mgl^{-1}).

Treatment C 6 recorded the highest callus index (350.00) followed by the treatment C 3 (283.00). C 1 (BA 0.10 mgl^{-1}), C 4 (BA 0.50 mgl^{-1}) and control failed to initiate callus (Table 30).

4.2.2.2 Shoot proliferation

4.2.2.2.1 Plant growth substances

The calli obtained in the initiation media were sub cultured into twenty treatments with various combinations of cytokinins (BA and kinetin) and auxins (2,4–D, NAA and IAA) in the culture medium.

The per cent of calli cultures developing into shoots varied widely (0.00 to 100.00). Cent per cent cultures differentiated in to shoots in the treatments ISO 4, ISO 5, ISO 6, ISO 8, ISO 9, ISO 10, ISO 11 and ISO 12 (Plate 16). ISO 19 (NAA 1.00 mgl^{-1}), ISO 20 (NAA 2.00 mgl^{-1}) and control failed to develop shoots (Table 31).

Plate 16. Shoot regeneration from callus

Plate 17. Shoot regeneration from callus on MS basal medium supplemented with BA 2.00 mgl^{-1} and IAA 0.50 mgl^{-1}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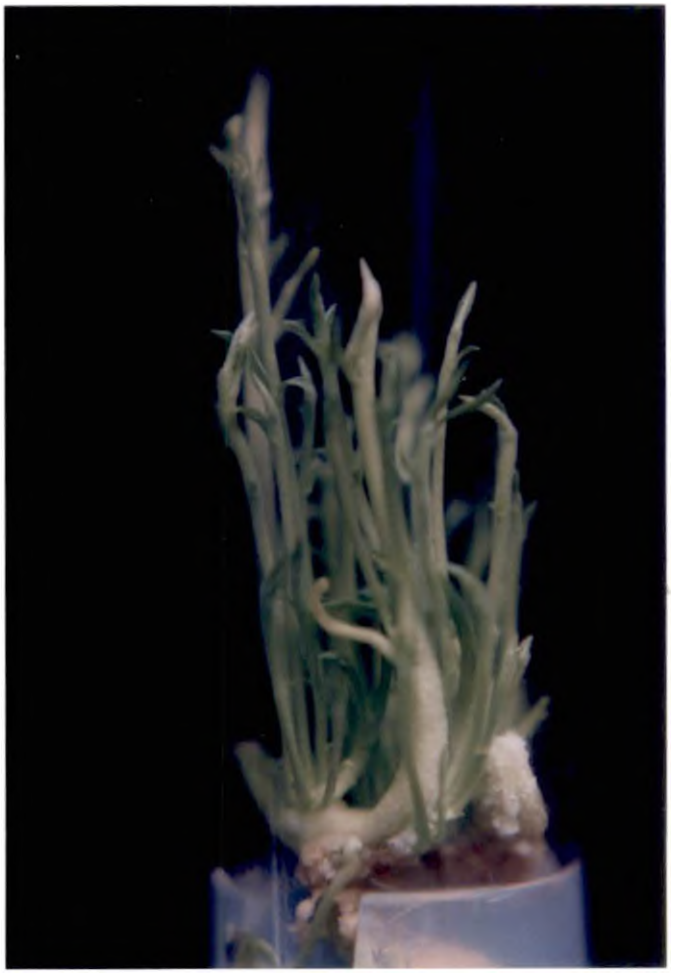

Fig. 7 Effect of Plant Growth Substances on Shoot Proliferation *via* Indirect Somatic Organogenesis from Cotyledon Explants of *Aegle marmelos*

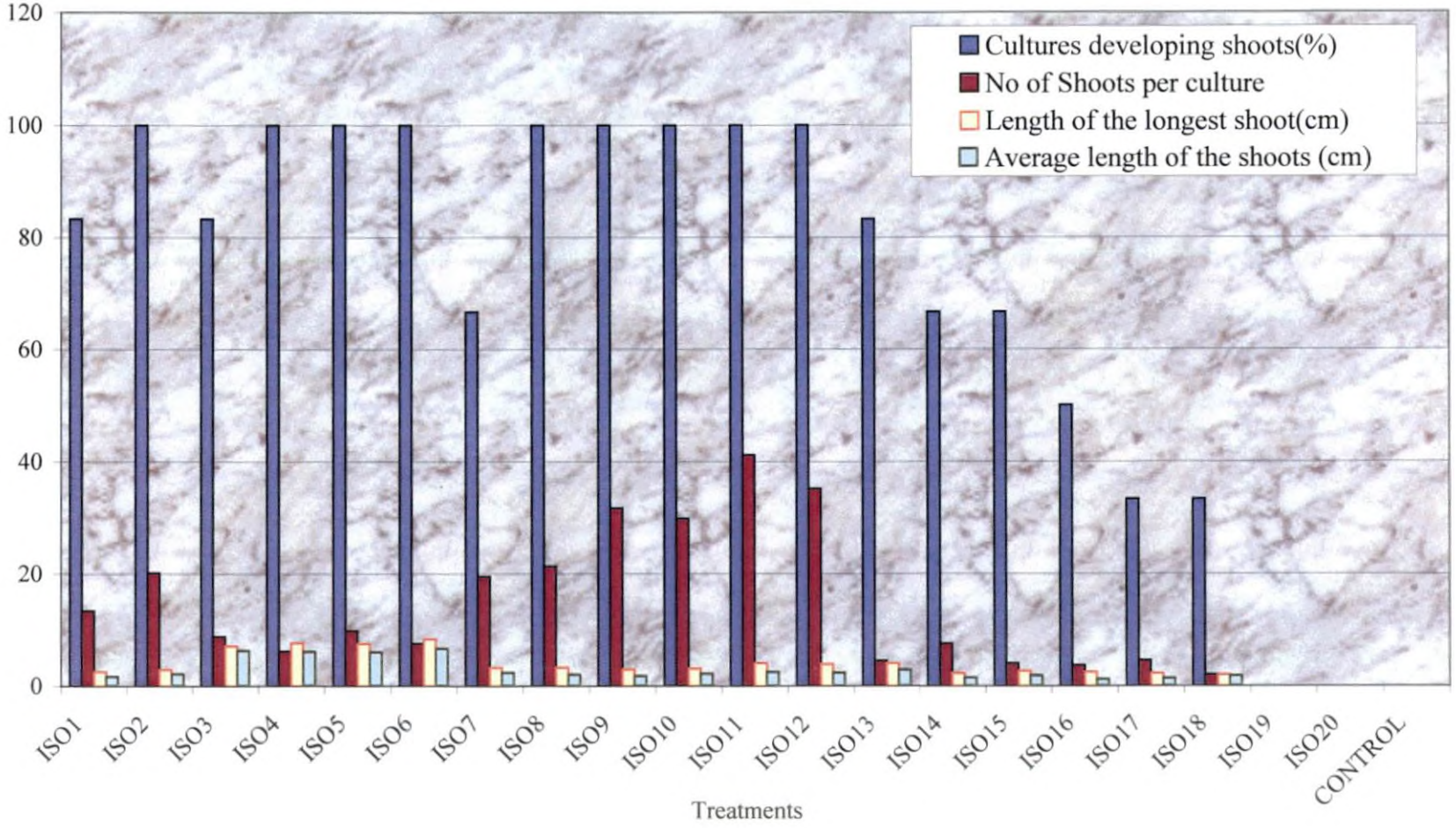

Plate 18. Non-embryogenic callus in the treatment tried for somatic embryogenesis

Fig. 8 Effect of plant growth substances on *in vitro* rooting of *Aegle marmelos*

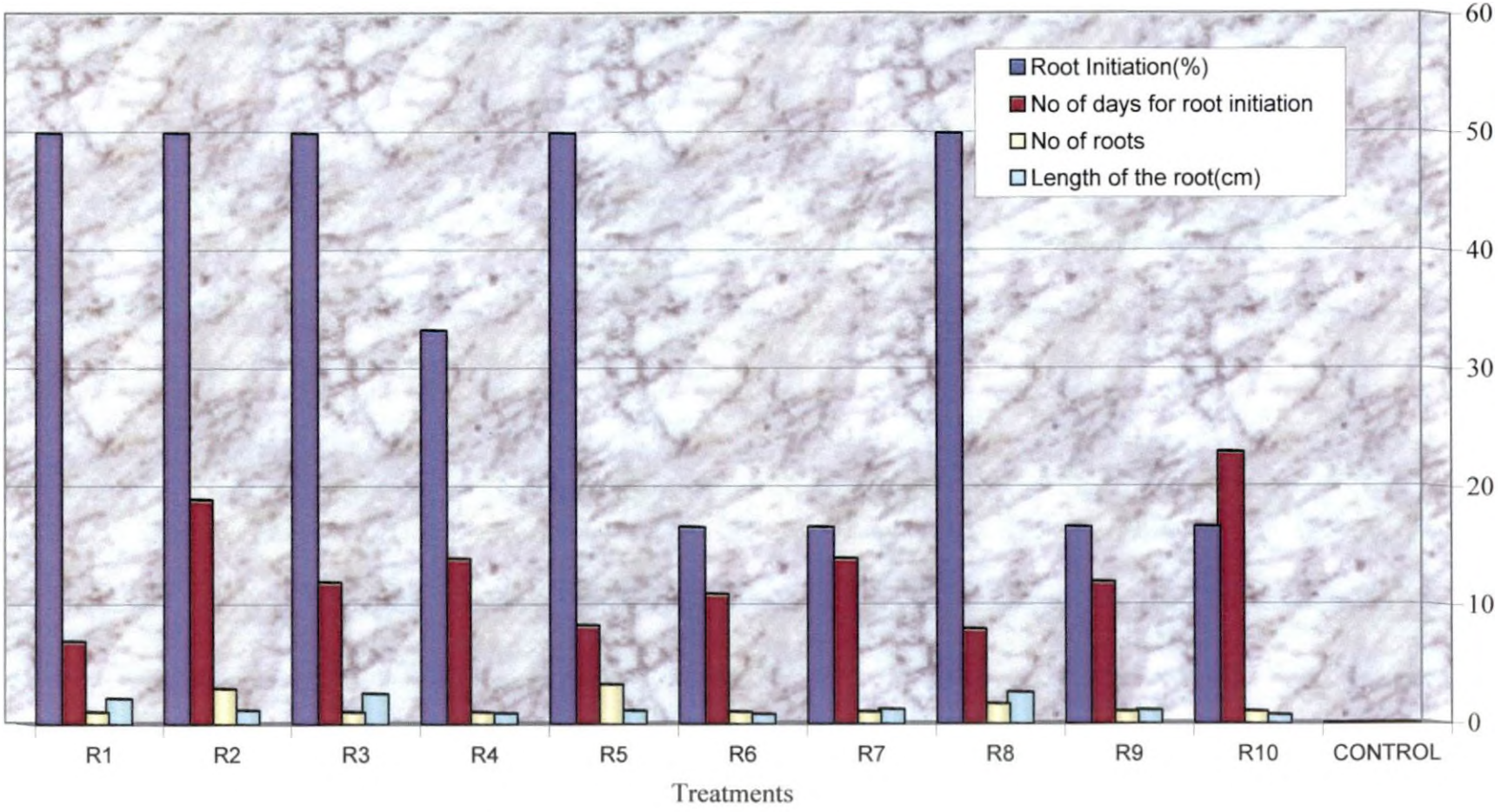

Table 30 Effect of plant growth substances on callus initiation from cotyledon explants of *Aegle marmelos*

*Treatments	Cultures initiating callus (%)	Growth score	Callus Index
C 1	0.00	0.00	0.00
C 2	66.66	1.75	116.55
C 3	100.00	2.83	283.00
C 4	0.00	0.00	0.00
C 5	66.66	1.00	66.66
C 6	100.00	3.50	350.00
C 7	50.00	1.33	66.50
C 8	83.33	2.40	119.99
C 9	83.33	1.40	116.66
C 10	50.00	1.00	50.00
C 11	33.33	1.50	49.99
C 12	100.00	1.33	133.00
C 13	100.00	1.67	167.00
C 14	100.00	1.17	117.00
Control	0.00	0.00	0.00

The data represents the mean value of six replications
*Treatment details are given in Table 10.

Culture medium : MS + Inositol (100.0 mgl^{-1}) + Sucrose (30.0 gl^{-1}) + Agar (8.00 gl^{-1})

Maximum number of shoots per culture (41.17) was recorded by the treatment ISO 11 (BA 2.00 mgl^{-1} and IAA 0.50 mgl^{-1}) (Plate 17) followed by ISO 12 (BA 2.00 mgl^{-1} and IAA 1.00 mgl^{-1}) which produced 35.17 shoots. The least number of shoots (2.00) was observed in ISO 18 (Kinetin 2.00 mgl^{-1} and NAA 2.00 mgl^{-1}) (Table 31, Fig. 7). The treatments with NAA alone, ISO 19 and ISO 20 failed to regenerate any shoots but exhibited rhizogenesis. The roots formed were thick and spongy.

With regard to the length of the longest shoot, ISO 6 (BA 0.50 mgl^{-1} and NAA 2.00 mgl^{-1}) recorded the highest value (8.33 cm). The lowest value (1.95 cm) was recorded by the treatment ISO 18 (Kinetin 2.00 mgl^{-1} and NAA 2.00 mgl^{-1}).

Average length of the shoot was also highest (6.58 cm) in the treatment ISO 6 and the least (1.10 cm) in the treatment ISO 16 (kinetin 2.00 mgl^{-1}).

4.3 Somatic embryogenesis

Fourteen treatments with various combinations of plant growth substances (BA, 2,4-D and NAA) were tried to initiate somatic embryogenesis (Table 10). But embryogenic callus could not be initiated even after two months (Plate 18).

4.4 Rooting

4.4.1 *In vitro* rooting

4.4.1.1 Plant growth substances

Ten treatments with various levels of auxins alone (IBA, IAA and NAA) were tried to study its effect on *in vitro* rooting.

Table 31. Effect of plant growth substances on shoot proliferation *via* indirect somatic organogenesis from cotyledon explants of *Aegle marmelos*

* Treatment	Cultures developing shoots (%)	No. of shoots per culture	Length of the longest shoot (cm)	Average length of the shoots (cm)
ISO 1	83.33	13.40	2.56	1.64
ISO 2	100.00	20.16	2.88	2.08
ISO 3	83.33	8.80	7.10	6.26
ISO 4	100.00	6.17	7.62	6.03
ISO 5	100.00	9.83	7.53	5.97
ISO 6	100.00	7.50	8.33	6.58
ISO 7	66.66	19.50	3.23	2.33
ISO 8	100.00	21.33	3.32	2.00
ISO 9	100.00	31.67	2.95	1.75
ISO 10	100.00	29.83	3.12	2.12
ISO 11	100.00	41.17	4.05	2.45
ISO 12	100.00	35.17	3.87	2.30
ISO 13	83.33	4.40	4.02	2.80
ISO 14	66.66	7.50	2.25	1.43
ISO 15	66.66	4.00	2.63	1.78
ISO16	50.00	3.67	2.43	1.10
ISO 17	33.33	4.50	2.20	1.25
ISO 18	33.33	2.00	1.95	1.75
ISO 19	0.00	0.00	0.00	0.00
ISO 20	0.00	0.00	0.00	0.00
Control	0.00	0.00	0.00	0.00

The data represents the mean value of six replications
*Treatment details are given in Table 11.

Culture medium : MS + Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 gl^{-1})

The percentage of rooting varied from 16.66 to 50.00. Treatments R 1, R 2, R 3, R 5 and R 8 recorded the highest per cent (50.00) of rooting. Control without any plant growth substance failed to initiate rooting (Table 32, Fig. 8).

Wide variation was noticed in the case of number of days taken for root initiation (7.00 to 23.00 days). Earliest rooting (7.00 days) was observed in R 1 (IBA 0.50 mgl^{-1}) followed by R 8 (IAA 2.00 mgl^{-1}) and R 5 (IBA 2.50 mgl^{-1}) with 8.00 and 8.33 days, respectively. The maximum number of days (23.00) for rooting was taken by R 10 (NAA 1.00 mgl^{-1}) followed by R 2 (IBA 1.00 mgl^{-1}).

The number of roots formed was maximum (3.33) in the treatment R 5 (IBA 2.50 mgl^{-1}) followed by R 2 (1.00 mgl^{-1}) with 3.00 roots (Plate 19). Only one root per shoot was formed in majority of the treatments.

With respect to the length of the roots, the maximum length (2.63 cm) was recorded by R 8 (IAA 2.00 mgl^{-1}). R 10 (NAA 1.00 mgl^{-1}) produced the shortest root (0.70 cm).

The roots formed in all the treatments were white in colour and without secondaries and root hairs.

4.4.1.2 Basal media

Basal media such as MS (half and full strength), SH, B5 and WPM supplemented with the best plant growth substance standardised were tried to assess their effect on *in vitro* rooting (Table 33).

Table 32. Effect of plant growth substances on *in vitro* rooting of *Aegle marmelos*

* Treatment	Root initiation (%)	No. of days for root initiation	No. of roots	Length of theroot (cm)
R 1	50.00	7.00	1.00	2.16
R 2	50.00	19.00	3.00	1.16
R 3	50.00	12.00	1.00	2.56
R 4	33.33	14.00	1.00	0.90
R 5	50.00	8.33	3.33	1.10
R 6	16.66	11.00	1.00	0.80
R 7	16.66	14.00	1.00	1.20
R 8	50.00	8.00	1.66	2.63
R 9	16.66	12.00	1.00	1.10
R 10	16.66	23.00	1.00	0.70
Control	0.00	0.00	0.00	0.00

The data represents the average value of 6 replications
* Treatment details are given in Table 12.

Culture medium : MS + Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 gl^{-1})

The per cent cultures showing *in vitro* rooting was maximum (66.66 per cent) in SH, B5 and WPM (Plate 20). Cultures in full strength and half strength MS basal media recorded 50.00 per cent rooting.

There was not much variation in the number of days taken for root initiation. The earliest root initiation (8 days) was in WPM and half strength MS, whereas delayed root initiation (9.25 days) was observed in SH.

Maximum number of roots per shoot (3.33) was produced in MS and minimum (1.00) was recorded in half strength MS and SH.

Length of the root was maximum (2.52 cm) in B5 while half strength MS registered the least elongation of root (0.80 cm).

4.4.1.3 Sucrose

Data pertaining to the effect of sucrose at various levels (10.00, 20.00, 30.00 and 40.00 gl^{-1}) is presented in Table 34.

Highest per cent of rooting (50 per cent) was recorded in SR 2 (20.00 gl^{-1}) and SR 3 (30.00 gl^{-1}). The treatment SR 4 (40.00 gl^{-1}) showed the least per cent (16.66) of rooting.

The number of days taken for root initiation was minimum (7.00 days) in SR 2 while it was maximum in SR 4(11.00 days).

Treatment SR 3 (30.00 gl^{-1}) recorded the maximum number of roots (3.33) followed by SR 2 (3.00) SR1 (10.00 gl^{-1}) and SR 4 (40.00 gl^{-1}) recorded only one root per shoot in all treatments.

Shoots in the treatment SR 2 could produce lengthy roots (2.06 cm) but in SR 4 the shoots produced only short roots (1.50 cm).

Table 33. Effect of basal media on *in vitro* rooting of *Aegle marmelos*

Treatments	Root initiation (%)	No. of days for root initiation	No. of roots	Length of the root (cm)
1/2 MS	50.00	8.00	1.00	0.80
MS	50.00	8.33	3.33	1.10
SH	66.66	9.25	1.00	0.93
B5	66.66	8.50	1.25	2.52
WPM	66.66	8.00	1.25	1.68

The data represents the mean value of six replications

Supplements : IBA (2.50 mgl^{-1}) + Inositol (100.00 mgl^{-1}) + Sucrose (30.00 gl^{-1}) + Agar (8.00 gl^{-1})

Table 34. Effect of sucrose on *in vitro* rooting of *Aegle marmelos*

* Treatments	Root in initiation (%)	No. of days for root initiation	No. of roots	Length of the root(cm)
SR 1	33.33	10.50	1.00	1.80
SR 2	50.00	7.00	3.00	2.06
SR 3	50.00	8.33	3.33	2.03
SR 4	16.66	11.00	1.00	1.50

The data represents the mean value of six replications
* Treatment details are given in Table 13.

Culture medium : MS + IBA (2.50 mgl^{-1}) + Inositol (100.00 mgl^{-1}) + Agar (8.00 gl^{-1})

Plate 19. ***In vitro*** **rooting on MS basal medium supplemented with IBA 2.50 mgl^{-1}**

Plate 20. Effect of SH, B5 and WPM on *in vitro* rooting

4.4.2 *Ex vitro* rooting

Among the pre-treatments given with IBA, to the unrooted micro shoots, IBA 1000.00 mgl^{-1} and IBA 500.00 mgl^{-1} for 20 seconds, cent per cent survival was recorded after two weeks. Shoots subjected to overnight treatment with IBA 100.00 mgl^{-1} showed only 50.00 per cent survival.

After four weeks, highest survival rate (75.00 per cent) was noticed in plants treated with IBA 1000.00 mgl^{-1} for 20 seconds while the least (25 per cent) was registered by IBA 100.00 mgl^{-1} given overnight (Table 35).

4.5 Planting out and acclimatization

The *in vitro* rooted plantlets were taken out carefully from the culture vessels and planted out into various potting media to study the effect on *ex vitro* establishment (Plate 21).

PO 1 and PO 2 registered cent per cent survival of plantlets after two weeks, while PO 3 recorded 75.00 per cent survival. After four weeks of planting out, maximum survival rate (75.00 per cent) was observed in PO 1 (Plate 22) while PO 2 and PO 3 recorded 50.00 per cent survival (Table 36).

Table 35 Effect of IBA pre-treatments on *ex vitro* rooting of *Aegle marmelos*

IBA (mg l^{-1})	Survival rate after two weeks (%)	Survival rate after four weeks (%)
1000.00 (20 seconds)	100.00	75.00
500.00 (20 seconds)	100.00	50.00
100.00 (overnight)	50.00	25.00

The data represents the mean value of four replications

Table 36 Effect of potting media on *ex vitro* establishment of *Aegle marmelos*

Media	Survival rate after two weeks (%)	Survival rate after four weeks (%)
PO 1	100.00	75.00
PO 2	75.00	50.00
PO 3	100.00	50.00

The data represents the mean value of four replications

*Treatment details are given in Table 14

Plate 21. *Aegle marmelos* plantlets established in different potting media

Plate 22. *Ex vitro* establishment of plantlets in sand

Plate 23. Effect of plant growth substances on *in vitro* rooting

Discussion

5. DISCUSSION

Aegle marmelos (L.) Corr. commonly known as bael, is a multipurpose fruit tree with immense medicinal value. Fruit, leaves and roots of the tree possess medicinal properties and have been extensively used in ayurvedic and folk medicine. Due to it's high drought tolerance, bael is also used in afforestation of dry areas.

Bael is routinely propagated by seeds. But being an outbreeding crop, seed progeny is highly heterozygous. Moreover, the seeds have short viability and are prone to insect attack. Propagation by vegetative methods such as grafting and cutting are also not commercially feasible. Propagation through root suckers is slow, difficult and cumbersome.

In vitro culture techniques provide a tool for rapid and large scale propagation of crop species. Micropropagation can be achieved by enhanced release of axillary buds, direct or indirect somatic organogenesis and somatic embryogenesis. Genetic uniformity is favoured and ensured in enhanced release of axillary buds, direct organogenesis and somatic embryogenesis. Hence studies were undertaken to standardise rapid and more prolific rate of vegetative multiplication, using modern techniques of cell, tissue and organ culture, in order to meet the increasing demand for planting material. The three possible routes of *in vitro* propagule multiplication were tried in the present study. The results of the investigations are discussed in the following pages.

Micropropagation by axillary bud proliferation has proved to be the most reliable method for large scale clonal production of many crop plants (Sathyakala *et al.*, 1995). In the present study, enhanced release of axillary buds was attempted using nodal segments and cotyledons as explants.

Growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators supplied in the medium and growth substances produced endogenously by the cultured cells. A balance between auxin and cytokinin is required for shoot proliferation. In order to standardise an optimum hormone concentration for inducing multiple shoot formation from these explants, studies were carried out using BA and IAA at various concentrations. In the case of nodal explants, the best hormone combination for multiple shoot induction was found to be BA 2.50 mgl^{-1} and IAA 1.00 mgl^{-1} with regard to both shoot number and elongation. Similar results, where combination of BA and IAA was found to be ideal for axillary bud proliferation from nodal segments was reported by Ajithkumar and Seeni (1998) in *Aegle marmelos*. Varghese *et al.* (1993) tried kinetin and NAA for multiple shoot induction from nodal explants, but callus formation and poor shoot regeneration was observed. In the present study, further maintenance and elongation of the shoots were not possible due to precocious leaf fall and tip necrosis. Arya *et al.* (1981) reported that *Aegle marmelos* was difficult to propagate *in vitro* from mature plant tissues (nodal segments and shoot apices) due to phenolic exudation and precocious leaf fall. Similar reports of leaf drop which inhibited the conversion of multiple buds into shoots was reported in *Gymnema sylvestre* by Komalavalli and Rao (2000).

The cotyledons as explants have been shown to possess high morphogenetic potential (Fazekas *et al.*, 1986). They are more responsive than the explants derived from mature trees, and many tree species have been successfully propagated in vitro through axillary proliferation from cotyledonary node (Purohit and Dave, 1996 ; Bhuyan *et al.*, 1997). Morphogenic response of cotyledon explants to various combinations of BA and IAA was investigated in the present study. The best treatment for axillary shoot proliferation was found to be BA 0.50 mgl^{-1} with regard to the maximum number of shoots per culture (49.00). This response of BA is in agreement with the studies on axillary shoot proliferation from cotyledon explants of *Aegle marmelos* reported by Hossain *et al.* (1994a) and Arumugam and Rao (1996). But there is contradiction regarding the optimum concentration of BA to be incorporated. Hossain *et al.* (1994a) reported that the optimum concentration for regeneration of plantlets from cotyledonary node of *Aegle marmelos* was BA 2.00 mgl^{-1}. According to Arumugam and Rao (1996) highest number of shoots was observed on MS medium supplemented with 3.00 mgl^{-1} of BA. In contrast, during the present investigation significant reduction in the number of shoots per culture was observed when BA concentration was increased from 0.50 mgl^{-1} to 2.50 mgl^{-1}. The number of shoots per culture at 0.50 mgl^{-1} was 49.00 while that at 2.50 mgl^{-1} was 9.50. These results complement the poor shoot regeneration ability (11.50 shoots per culture) reported by Hossain *et al.* (1994a). With regard to the length of the longest shoot also, significant difference was noticed between BA 0.50 mgl^{-1} (4.93 cm) and BA 2.50 mgl^{-1} (1.83 cm) (Plate 7). Similar reports on inhibitory effect of high concentrations of BA on shoot growth was observed

in *Trichopus zeylanicus* (Krishnan *et al.*, 1995) and *Citrus halimii* (Normah *et al.*, 1997).

Hossain *et al.* (1994a) observed that shoot regeneration from cotyledon explants was always preceded by an early stage of callus growth. During the present investigation, no callus formation was observed in the initial stages eventhough after several subcultures slight callusing occurred in some cultures.

Varied concentrations of BAP was required for maximum shoot formation from nodal explants (2.50 mgl^{-1}) and cotyledons (0.50 mgl^{-1}). This differential response could be due to the difference in the physiological age and state of the explants. According to Bonga (1982) and Hackett (1985) *in vitro* response of perennials was strongly affected by the age of the explant.

Gibberellic acid is a growth regulator which is commonly used for shoot elongation. Progressive increase in average length of the shoots was noticed at higher GA_3 concentrations (2.00 mgl^{-1} and 3.00 mgl^{-1}). Average height of the shoots was the highest (4.33 cm) at GA_3 3.0 mgl^{-1} which was significantly superior to the treatment lacking GA_3 (2.67 cm). Addition of GA_3 along with BA was found to be beneficial for the elongation of shoots in *Murraya koenigii* (Bhuyan *et al.*, 1997). Sahoo and Chand (1998) also observed that shoot elongation was dependent on the synergistic influence of gibberellic acid along with BA in *Vitex negundo.*

The basal medium requirement depends upon the plant species and the purpose of cell, tissue or organ culture. In the present study, full strength MS medium was found to be significantly superior to half strength MS, B5, SH

and WPM with respect to the number of shoots and length of the shoots. This is in agreement with the findings of Komalavalli and Rao (2000) who reported that among the different basal media tested, MS was the best for shoot sprouting, more number of shoots and length followed by B5, SH, and WPM in *Gymnema sylvestre*. In *Lagestroemia reginae* also, MS was found to be better for shoot regeneration when compared to B5 and WPM (Sumana and Kaveriappa, 2000).

Sucrose is the most utilisable carbon form and energy source as well as an osmoregulatory factor (George and Sherrington, 1984). In the present investigation, effect of various concentrations of sucrose on shoot proliferation was studied. Sucrose at 50.00 gl^{-1} produced maximum number of shoots (54.67) and was on par with sucrose at 40.00 gl^{-1} (50.00). These results are supported by earlier reports. Hossain *et al.* (1994a) observed that the media supplemented with 40.00 gl^{-1} sucrose was best for shoot growth in *Aegle marmelos*. Sucrose at 50.00 gl^{-1} evoked best response in studies on direct shoot regeneration from cotyledon explants of *Citrus sinensis* (Daming *et al.*, 2000). In the present study, glucose was also tried at various levels in the medium but was found to be significantly inferior to sucrose in proliferating shoots. Sumana *et al.* (1999) also opined that sucrose was the sugar of choice for shoot regeneration when compared to glucose in *Holarrhena pubescens*.

Amino acid supplements are often incorporated in to the tissue culture media for obtaining better proliferation of shoots. Casein hydrolysate at different concentrations was supplemented to the media to study the proliferation rate. But there was no significant difference in the number of

shoots produced. Similar result has been reported in *Gymnema Sylvestre* by Komalavalli and Rao (2000) where casein hydrolysate addition did not significantly improve shoot sprouting frequency.

Adenine sulphate is an amino acid supplement which when added to the medium often enhanced growth and shoot proliferation (Skoog and Tsui, 1948). Adenine sulphate at five different levels were tried to study it's effect on shoot proliferation. Number of shoots was significantly improved when adenine sulphate was supplemented at 20.00 mgl^{-1}. This is in agreement with the findings of Arumugam and Rao (1996) that shoot proliferation was considerably increased on adenine sulphate (10.00 mgl^{-1}) supplemented medium in *Aegle marmelos*. In the present study, it was observed that at higher concentration of adenine sulphate (50.00 mgl^{-1}) there was significant reduction in the number of shoots. This may be due to the antagonistic effect of adenine sulphate when the concentration exceeds optimum level as is seen in the case of cytokinin activity also.

The optimum agar concentration create an osmotic potential favourable for the uptake of nutrients. The different concentrations of agar tried in the present study failed to show any statistically significant difference in shoot proliferation. But gradual increase in the number of shoots was observed at the lower levels of agar. Maximum number of shoots (60.33) was produced by agar at 5.00 gl^{-1}. The probable reason may be the increased absorption of nutrients from the media due to the lowered osmotic potential. Osmotic potential can play a critical role in shoot proliferation.

Light had significant influence on shoot proliferation. Cultures under lighted condition produced an average of 49.00 shoots, while under dark

produced only 20.33 shoots. Also, the shoots and leaves appeared pale and chlorotic under dark conditions. This can be attributed to the lack of chlorophyll development required for photosynthesis and in turn shoot regeneration which might have contributed to the reduced rate of shoot proliferation of cultures under dark.

In direct somatic organogenesis, adventitious shoots arise directly from the tissues of the explant and not from previously formed callus. The induction of direct shoot regeneration depends on the plant organ from which the explant is derived and above all, on the plant species. Explants taken from newly originated organs are most likely to be capable of direct organogenesis.

In the present investigation, direct organogenesis was initiated from cotyledon explants. In order to standardise an optimum hormone concentration, studies were carried out using BA and IAA at various levels. With regard to initiation, BA at 0.10 mgl^{-1} and 0.20 mgl^{-1} were found to record the maximum per cent of cultures initiating direct organogenesis. BA at 0.40 mgl^{-1} was found to be the best with respect to the number of shoots produced. However, for the further shoot proliferation a lower concentration of BA (0.20 $mgl^{-1)}$ along with IAA (2.00 mgl^{-1}) was found to be ideal. The result is in agreement with the only report on direct organogenesis in *Aegle marmelos* by Hossain *et al.* (1995). They obtained shoot proliferation from hypocotyl surface when BA 0.10 mgl^{-1} was supplemented in the medium. Normah *et al.* (1997) also obtained direct organogenesis from hypocotyl explant of *Citrus halimii* with BA at 0.50 mgl^{-1}.

Direct organogenesis from root of intact seedlings was also observed during the present study. Adventitious shoots were found to differentiate from

the entire root eventhough maximum shoot proliferation was from the root tip. Similar phenomenon was observed in *Aegle marmelos* by Islam *et al.* (1996a) during the direct culture of mature seed. *In vitro* regeneration of plantlets from root segments of *Aegle marmelos* was reported by Bhati *et al.* (1992). In the present study, among the different treatments that exhibited direct organogenesis from root, maximum shoot number per culture (35.00) was observed at BA 0.50 mgl^{-1}. In contrast, Bhati *et al.* (1992) reported less number of shoots (3.00 to 10.00) when BA alone was used alone in the medium. He observed that the maximum number of shoots (22.00) was produced on medium containing 0.50 mgl^{-1} each of BA and kinetin. This can be attributed to the differential endogenous levels of cytokinin. Also in such cases, the possibility of genotypic differences between the individuals, especially of an outbreeding tree species contributing to varied shoot multiplication rates cannot be ruled out.

Indirect somatic organogenesis is characterised by the redetermination of differentiated cells leading to callus formation and subsequent formation of separate shoot and root initials from it. The main disadvantage in callus mediated organogenesis compared to other routes is genetic variation developing in many of it's component cells. However, the regenerated variants can be used to complement the existing natural variability.

Cotyledons excluding the embryo axes were used as the explant in the present investigation. Different combinations of plant growth substances (BA, 2,4-D and NAA) were tried to assess the effect on callus initiation. Highest callus index (350.00) was recorded for the medium supplemented with BA 0.50 mgl^{-1} and 2,4-D 0.50 mgl^{-1} followed by BA 0.10 mgl^{-1} and 2,4-D 0.50

mgl^{-1}. The present study confirm with the findings of Islam *et al.* (1995) who also reported that combination of BA and 2,4-D was best for inducing callus formation from embryonic tissues in *Aegle marmelos*. Similar reports were obtained by Arumugam *et al.* (1997).

Shoot proliferation from the callus is again dependent on the optimum plant growth substance combination. Various combinations of cytokinins (BA and kinetin) and auxins (2,4-D, NAA and IAA) were tried to standardise the optimum concentration needed. The best combination for the maximum shoot proliferation was found to be BA 2.00 mgl^{-1} and IAA 0.50 mgl^{-1}. Similar result in *Azadirachta indica* by Zypman *et al.* (1997) and in *Plumbago zeylanica* by Rout *et al.* (1999) support the present findings. Hossain *et al.* (1994b) recorded highest shoot number per culture from nucellar callus on medium with BA 2.00 mgl^{-1} and NAA 0.10 mgl^{-1} in *Aegle marmelos*.

The plantlets produced *in vitro* should have a strong and functional root system. Auxins frequently used for inducing rooting are IAA, IBA and NAA (George and Sherrington, 1984). In the present study, IBA was found to be superior to other plant growth substances with respect to earliest root induction and formation of roots. Similar results on IBA being good for inducing rooting in *Aegle marmelos* were reported by several scientists (Hossain *et al.*, 1993 ; Islam *et al.*,1993 ; Arumugam and Rao, 1996 ; Islam *et al.*, 1996a). MS media without any growth regulator failed to induce root initials during the trials. Rout *et al.* (1999) reported a similar observation in *Plumbago zeylanica*.

All cytokinins, especially BA inhibit root induction. BA sometimes delays rooting, even after transferring to cytokinin free medium (Yeoman,

1986). In the present investigation, it was observed that the root initiation occurred even after three weeks on root induction medium. This delay can be attributed to the persisting effect of BA. Islam *et al.* (1996a) reported that no rooting occurred in the microshoots of *Aegle marmelos* cultured on auxin-free media containing BA alone. Higher per cent of rooting (66.66) was recorded during the present study. Islam *et al.* (1996a) and Arumugam and Rao (1996) obtained 25.00 per cent and 30.00 per cent of *in vitro* rooted shoots, respectively, in *Aegle marmelos.* This can also be attributed to the higher level (2.00 mgl^{-1} and 3.00 mgl^{-1}) of BA that was optimized for shoot regeneration in their studies.

The number of roots produced per shoot varied from 1.00 to 3.33 (Plate 23). This result is in agreement with the studies conducted on *in vitro* rooting of *Aegle marmelos* by other scientists (Hossain *et al.*, 1993 ; Hossain *et al.*, 1994a ; Arumugam and Rao, 1996 ; Arumugam *et al.*, 1997). During the present study, single-root formation was observed in majority of the treatments. Similar observation was made in bael by Ajithkumar and Seeni (1998). Usually, single-root formation in *in vitro* plants has been reported to occur in tree species (Mascarenhas *et al.*, 1981).

The root elongation phase is very sensitive to auxin concentration. Higher concentrations of auxin inhibited root elongation (Thimann, 1977). In the present study it was observed that IBA at 2.00 mgl^{-1} and 2.50 mgl^{-1} produced shorter roots when compared to lower levels of IBA. Hossain *et al.* (1994a) also observed that at higher concentrations of IBA, there was reduction in root length.

The major cost of producing *in vitro* plants lies in the rooting and hardening stages. *Ex vitro* rooting is preferred in many crops with a view to save time and resources. In the present study, pretreatment with IBA 1000.00 mgl^{-1} for 20 seconds recorded highest survival rate (75 per cent) after four weeks. John (1996) reported similar result in *Holostemma annulare* where IBA 1000.00 mgl^{-1} was found to be best for *ex vitro* rooting. *Ex vitro* root induction was achieved with IBA (260.00 μM) in *Vitex negunda* (Kannan and Jasrai, 1998). *In vitro* developed shoots of *Murraya koenigii* were rooted *ex vitro* by pulse treatment with IBA at 2.46 μM (Babu *et al.*, 2000).

Acclimitisation is important in the case of mircopropagated plants because *in vitro* raised plant is not adapted for *ex vitro* conditions. A proper hardening method is a major factor, which determines plantlet survival and establishment in the field. Various potting media were tried in the present investigation to study the effect on the survival of *in vitro* plantlets. Sand was the ideal potting media registering maximum survival rate (75 per cent) after four weeks (Plate 22). Successful *ex vitro* establishment of *in vitro* plantlets in sand was peviously reported in *Holostemma annulare* (John, 1996) and in *Murraya koenigii* (Babu *et al.*, 2000).

In the present investigation *in vitro* techniques could be standardised for the rapid propagation of *Aegle marmelos via* enhanced release of axillary buds, direct organogenesis and indirect organogenesis. But somatic embryoids could not be initiated in any of the treatments tried. Further studies on standardisation and refinement of media is thus necessary for *in vitro* propagation of *Aegle marmelos via* somatic embryogenesis.

Summary

6. SUMMARY

Attempts were made in the Plant Molecular biology and Biotechnology Centre, College of Agriculture, Vellayani during 1999-2001 for evolving techniques for the *in vitro* propagation of *Aegle marmelos* (L.) Corr.

Standardisation of basal media, media components and culture conditions was attempted for shoot proliferation *via* enhanced release of axillary buds. Standardisation of plant growth substances for initiation and shoot proliferation stages of direct and indirect somatic organogenesis was tried. Studies were also conducted on *in vitro* rooting, *ex vitro* rooting and *ex vitro* establishment.

The salient findings of the studies are summarised below.

1. Two types of explants *viz.*, shoot nodal segments and cotyledons were tried for enhanced release of axillary buds. Cotyledons responded better with respect to survival percentage (100.00) and shoot proliferation (49.00 shoots per culture). Nodal segments recorded a maximum of 50.00 per cent survival and 7.33 shoots per culture.

2. Among the different plant growth substances tried for multiple shoot proliferation from nodal segments, maximum proliferation was obtained with BA 2.50 mgl^{-1} and IAA 1.00 mgl^{-1}.

3. Out of the different plant growth substances tried for enhanced release of axillary buds from cotyledon, BA 0.50 mgl^{-1} registered maximum number of shoots per culture. Hence this was used for standardisation of other media components. However, the length of the shoots was

maximum in the treatments with auxin (IAA) alone. IAA 2.00 mgl^{-1} registered the highest value for the length of the longest shoot (8.25 cm).

4. Full strength MS basal medium was best for shoot proliferation when compared to half strength, SH, B5 and WPM.

5. Addition of gibberellic acid 3.00 mgl^{-1} in the medium produced lengthy shoots.

6. Among the different levels of sucrose tried, maximum number of shoots per culture was produced by sucrose at 50.00 gl^{-1}. Sucrose was found to be superior to glucose with respect to the shoot number.

7. Addition of casein hydrolysate did not evoke any significant effect on shoot proliferation.

8. Supplementation of adenine sulphate at 20.00 mgl^{-1} improved shoot proliferation.

9. Addition of cobaltous chloride had no beneficial effect on shoot proliferation.

10. Agar at various levels, showed no statistically significant difference. But there was an increase in shoot number with decreasing agar concentration. Agar at 5.00 mgl^{-1} recorded maximum number of shoots.

11. Solid medium was found superior to liquid medium with respect to number as well as length of the shoots.

12. Light was essential for maximum proliferation of healthy green shoots.

13. Among the different plant growth substances tried to initiate direct organogenesis from cotyledon, BA 0.40 mgl^{-1} was ideal for producing maximum number of shoots.

14. Out of the different plant growth substances attempted for shoot proliferation *via* direct organogenesis, the best response with respect to number of shoots was evoked by the combination BA 0.20 mgl^{-1} and IAA 2.00 mgl^{-1}.

15. Highest number of shoots produced *via* direct organogenesis from *in vitro* root was observed in media supplemented with BA 0.50 mgl^{-1}. Maximum elongation of shoot was noticed at lower concentration of BA (0.20 mgl^{-1}).

16. Among the plant growth substance combinations tried to initiate callus, highest callus index was recorded by BA 0.50 mgl^{-1} and 2,4-D 0.50 mgl^{-1}.

17. Plant growth substance combination of BA 2.00 mgl^{-1} and IAA 0.50 mgl^{-1} ideal for maximum shoot proliferation from callus.

18. Out of the various plant growth substances tried for rooting, IBA was found to be better than others with respect to early rooting and number of roots. IBA 2.50 mgl^{-1} produced maximum number of roots (3.33).

19. Among the various basal media tried, earliest root initiation took place in half strength MS and WPM. Maximum number of roots was produced in full strength MS basal medium.

20. Sucrose at 20.00 mgl^{-1} in the rooting medium registered earliest root initiation and maximum length for the root while sucrose at 30.00 mgl^{-1} produced maximum number of roots.

21. Among the auxins tried for pre-treatment of shoots planted out for *ex vitro* rooting, IBA at 1000.00 mgl^{-1} for 20 seconds (quick dip) was the best with respect to highest survival rate (75.00 per cent) after four weeks.

22. Sand was the ideal potting media for the *ex vitro* establishment of plantlets, registering 75.00 per cent survival rate after four weeks of planting out.

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*Original not seen

Appendices

APPENDIX – I

Composition of various basal media tried for *in vitro* culture of *Aegle marmelos*

Ingredients	Quantity (mg l^{-1})			
	MS	SH	B5	WPM
Macronutrients				
$NH_4 NO_3$	1650.00	-	-	400.00
$(NH_4)_2 SO_4$	-	-	134.00	-
$NH_4 H_2PO_4$	-	300.00	-	-
KNO_3	1900.00	2500.00	2500.00	-
KH_2PO_4	170.00	-	-	340.00
$MgSO_4, 7H_2O$	370.00	400.00	250.00	1850.00
$Ca (NO_3) 4H_2O$	-	-	-	556.00
$NaH_2PO_4. H_2O$	-	-	150.00	-
$CaCl_2. 2 H_2O$	440.00	200.00	150.00	22.00
Micronutrients				
H_3BO_3	6.20	5.00	3.00	6.20
$MnSO_4. 4H_2O$	22.30	13.20	10.00	22.30
$ZnSO_4. 7H_2O$	8.60	1.00	2.00	8.60
KI	0.83	1.00	0.75	-
$Na_2 MoO_4. 2H_2O$	0.25	0.10	0.25	0.25
$CuSO_4. 5H_2O$	0.025	0.20	0.025	0.25
$CoCl_2. 6H_2O$	0.025	0.10	0.025	-
$FeSO_4. 7H_2O$	27.85	15.00	-	27.85
$Na_2EDTA. 2H_2O$	37.25	20.00	37.25	37.25
Vitamins				
Thiamine. HCl	0.10	5.00	10.00	1.00
Pyridoxine. HCl	0.50	0.50	1.00	0.50
Nicotinic acid	0.50	5.00	1.00	0.50
Amino acid				
Glycine	2.00	-	-	2.00
Others				
Inositol	100.00	1000.00	100.00	100.00
*Sucrose	30.00	30.00	20.00	30.00
*Agar	8.00	8.00	8.00	8.00

* in g l^{-1}

APPENDIX – II

Abstract of analysis of variance for the effect of different treatments

Sl. No.	Character	Treatment mean squares	Error mean squares
I.	**Shoot proliferation *via* enhanced release of axillary buds from cotyledon**		
	1.Effect of plant growth substances		
	i) Number of shoots	1220.94	43.23
	ii) Length of the longest shoot	32.09	1.36
	iii) Average length of the shoots	31.06	1.13
	iv) Number of leaves per shoot	13.42	1.66
	2.Effect of gibberellic acid		
	i) Number of shoots	6.89	112.83
	ii) Length of the longest shoot	0.20	0.64
	iii) Average length of the shoots	2.49	0.35
	iv) Number of leaves per shoot	0.72	1.25
	3.Effect of basal media		
	i) Number of shoots	1372.33	62.61
	ii) Length of the longest shoot	2.27	0.79
	iii) Average length of the shoots	0.68	0.21
	iv) Number of leaves per shoot	0.58	1.75
	4.Effect of sucrose		
	i) Number of shoots	1315.39	89.98
	ii) Length of the longest shoot	0.80	1.13
	iii) Average length of the shoots	0.38	0.33
	iv) Number of leaves per shoot	1.06	1.03
	5.Effect of glucose		
	i) Number of shoots	351.22	67.59
	ii) Length of the longest shoot	3.27	0.52
	iii) Average length of the shoots	0.99	0.21
	iv) Number of leaves per shoot	1.17	1.14
	6.Effect of casein hydrolysate		
	i) Number of shoots	2.17	113.88
	ii) Length of the longest shoot	0.20	0.64
	iii) Average length of the shoots	1.67	0.22
	iv) Number of leaves per shoot	0.22	1.23

APPENDIX – II Contd...

Sl. No.	Character	Treatment mean squares	Error mean squares
	7.Effect of adenine sulphate		
	i) Number of shoots	1771.42	67.19
	ii) Length of the longest shoot	4.70	0.65
	iii) Average length of the shoots	1.34	0.19
	iv) Number of leaves per shoot	8.22	0.73
	8.Effect of cobaltous chloride		
	i) Number of shoots	0.75	120.68
	ii) Length of the longest shoot	0.12	0.87
	iii) Average length of the shoots	0.21	0.19
	iv) Number of leaves per shoot	0.00	1.21
	9.Effect of agar		
	i) Number of shoots	155.78	113.70
	ii) Length of the longest shoot	0.14	0.93
	iii) Average length of the shoots	0.14	0.36
	iv) Number of leaves per shoot	0.11	1.28
	10.Effect of mode of culture		
	i) Number of shoots	3780.75	121.75
	ii) Length of the longest shoot	19.51	0.99
	iii) Average length of the shoots	3.97	0.30
	iv) Number of leaves per shoot	6.75	0.95
	11.Effect of light		
	i) Number of shoots	2465.33	123.53
	ii) Length of the longest shoot	1.26	1.23
	iii) Average length of the shoots	0.61	0..22
	iv) Number of leaves per shoot	4.08	0.88
II.	**Shoot proliferation *via* direct organogenesis from cotyledon**		
	1.Effect of plant growth substances		
	i) Number of shoots	2465.33	42.57
	ii) Length of the longest shoot	1.26	0.68
	iii) Average length of the shoots	0.61	0.29
	iv) Number of leaves per shoot	4.08	1.96

STANDARDISATION OF *IN VITRO* TECHNIQUES FOR THE RAPID CLONAL PROPAGATION OF BAEL [*Aegle marmelos* (L.) Corr.]

BY

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ABSTRACT

Studies were conducted for evolving *in vitro* techniques for the rapid clonal propagation of bael [*Aegle marmelos* (L.) Corr.] during 1999 – 2001 at Plant Molecular Biology and Biotechnology Centre, College of Agriculture, Vellayani.

Attempts were made to standardise *in vitro* propagation techniques of bael *via* enhanced release of axillary buds using shoot nodal segments and cotyledons as explants. Entire cotyledons were used as explants for direct organogenesis while cotyledons excluding embryoaxes were used for indirect organogenesis.

Among the explants used for enhanced release of axillary buds, cotyledons responded better than nodal segments. Cent per cent survival could be obtained in cultures with cotyledons while a maximum of 50.00 per cent was obtained in cultures with nodal segments.

Maximum shoot proliferation from nodal segments was obtained on full strength MS basal medium supplemented with BA 2.50 mgl^{-1}, IAA 1.00 mgl^{-1}, sucrose 30.00 gl^{-1} and agar 8.00 gl^{-1}.

The best treatment identified for shoot proliferation from cotyledons was full strength MS basal medium supplemented with BA 0.50 gl^{-1}, GA_3 3.00 mgl^{-1}, adenine sulphate 20.00 mgl^{-1}, sucrose 50.00 gl^{-1} and agar 5.00 gl^{-1}.

Maximum initiation of direct organogenesis from cotyledons (83.33 per cent) occurred in two treatments namely, on full strength MS basal

medium supplemented with BA 0.10 mgl^{-1}, sucrose 30.00 gl^{-1}, and agar 8.00 gl^{-1} and on the same basal medium with BA 0.20 mgl^{-1}, sucrose 30.00 gl^{-1} and agar 8.00 gl^{-1}. The best treatment identified for highest number of shoots per culture was full strength MS medium supplemented with BA 0.40 mgl^{-1}, sucrose 30.00 gl^{-1} and agar 8.00 gl^{-1}. Maximum proliferation of shoots on further subculturing was obtained on full strength MS medium supplemented with BA 0.20 mgl^{-1}, IAA 2.00 mgl^{-1}, sucrose 30.00 gl^{-1} and agar 8.00 gl^{-1}. Direct organogenesis from *in vitro* root could be best obtained on full strength MS medium supplemented with BA 0.50 mgl^{-1}, sucrose 30.00 gl^{-1} and agar 8.00 gl^{-1}.

The best treatment identified for callus initiation was full strength MS medium supplemented with BA 0.50 mgl^{-1}, 2,4-D 0.50 mgl^{-1}, sucrose 30.00 gl^{-1} and agar 8.00 gl^{-1} which recorded the highest callus index (350.00). Ideal treatment for the maximum proliferation from callus *via* indirect somatic organogenesis was found to be full strength MS medium with BA 2.00 mgl^{-1}, IAA 0.50 mgl^{-1}, sucrose 30.00 gl^{-1} and agar 8.00 gl^{-1}.

In vitro rooting occurred at its best on full strength MS medium supplemented with IBA 2.50 mgl^{-1}, sucrose 20.00 mgl^{-1} and agar 8.00 gl^{-1}. Pre-treatment with IBA 1000.00 ppm for 20 seconds proved to be the best for *ex vitro* rooting.

Sand was the ideal potting media for *ex vitro* establishment.