ROOTING OF MICROSHOOTS AND *EX VITRO* ESTABLISHMENT OF PLANTLETS OF BAEL [*Aegle marmelos* (L.) Corr.]

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DECLARATION

I hereby declare that this thesis entitled **"Rooting of microshoots and *ex vitro* establishment of plantlets 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 **"Rooting of microshoots and *ex vitro* establishment of plantlets of bael [*Aegle marmelos* (L.) Corr.]"** is a record of research work done independently by **Mrs. Surya D. Aniyan (2003-12-13)** 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

%	–	Per cent
°C	–	Degree Celsius
µg	–	Microgram
µM	–	Micro molar
2,4-D	–	2,4-dichloro phenoxy acetic acid
AC		Activated charcoal
AMF	–	Arbuscular mycorrhizal fungi
B5	–	Gamborg *et al.*
BA	–	Benzyl adenine
CD	–	Critical difference
CGR	–	Crop growth rate
cm	–	Centimetre
cm S^{-1}	–	Centimetre per Siemens
cm^2	–	Square centimetre
$CoCl_2$	–	Cobalt chloride
CRD	–	Completely Randomised Design
DAP	–	Days after planting
Fig.	–	Figure
g	–	Gram
h	–	Hours
HCl	–	Hydrochloric acid
IAA	–	Indole-3-Acetic Acid
IBA	–	Indole-3-Butyric Acid
KOH	–	Potassium hydroxide
LAI	–	Leaf area index
mg	–	Milligram
mg l^{-1}	–	Milligram per litre
min.	–	Minutes
MS	–	Murashige and Skoog
NAA	–	α - Naphthalene acetic acid
NS	–	Not significant
s	–	Seconds
SC	–	Stomatal conductance
SH	–	Schenk and Hildebrandt
v/v	–	Volume by volume
WPM	–	Woody plant medium

Introduction

1. INTRODUCTION

Aegle marmelos (L.) Corr., commonly known as bael (koovalam) belonging to the family Rutaceae, is an important medicinal fruit tree distributed throughout the plains and hilly tracks of India. It is one among the thirty two medicinal plants identified by the National Medicinal Plants Board, Government of India for commercial cultivation, conservation and development (Rawat and Uniyal, 2003).

Bael has great demand in the indigenous systems of medicine, especially ayurveda and is used for treating wide range of ailments (CSIR, 1985). The root is a constituent of 'Dasamula', which is commonly used in ayurveda. The different plant parts and their extracts posses a number of pharmacological properties. The officinal plant parts are roots, leaves and fruits (Plate 1). They have anti-helminthic, anti-microbial, anti-diarrhoeal and cardiotonic properties. Leaves have anti-ashtmatic properties while fruit pulp can be used in the treatment of leucoderma. The medicinal property of the plant is due to the presence of the active principle 'marmelosin'. The pulp of ripe fruit is used in preparing 'sherbet' which is a popular aromatic summer drink in North India. Bael has considerable traditional and socio-cultural values also. The leaves of the tree are traditionally used as sacred offering to Lord Shiva. Bael can also be used in the afforestation of dry areas due to its high drought tolerance.

Generally bael is propagated by seed. But seeds have short viability and give rise to highly heterozygous population. Vegetative propagation through root suckers is slow, difficult and cumbersome (Ajithkumar and Seeni, 1998). Propagation by methods such as grafting and cutting are also not commercially feasible (Nambiar *et al.*, 2000). Over exploitation of the plant as well as habitat destruction, has significantly reduced the population of bael in natural habitat. Since the supply of planting material is

inadequate, more emphasis need to be given for the large scale multiplication of quality planting material.

Evolving protocols for the *in vitro* propagation and *ex vitro* establishment of bael becomes relevant in this context as it can overcome the disadvantages of conventional vegetative multiplication methods and also ensures high rate of multiplication. Micropropagation techniques in bael has already been standardised (Arya *et al.*, 1981; Hossain *et al.*, 1994a; Islam *et al.*, 1996a; Hazeena, 2001). But *in vitro* rooting and *ex vitro* establishment needs further refinement.

Success of micropropagation is mainly influenced by the field establishment of *in vitro* derived plantlets. Within the *in vitro* system, the plantlets are heterotrophic and get very favourable conditions for their growth. During *ex vitro* establishment, the plantlets have to switch over to autotrophic nutrition, involving normal photosynthetic activity and water relations. Serious field mortality is encountered while planting out of tissue culture derived plantlets which is very severe in case of woody species.

Hardening and *ex vitro* establishment of plantlets are the most difficult stages in micropropagation (Razdan, 2003). Micropropagation on a large scale would be successful only when plantlets after transfer from culture to soil show high survival rate. Standardising the techniques for *ex vitro* establishment of bael plantlets will streamline the supply of elite planting material in sufficient number for large scale cultivation.

Hence the present study "Rooting of microshoots and *ex vitro* establishment of plantlets of bael (*Aegle marmelos* (L.) Corr.)" was undertaken with the objectives of evolving protocols for *in vitro* rooting, *ex vitro* rooting and *ex vitro* establishment.

Plate 1. Twig of *Aegle marmelos* with fruit

Review of Literature

2. REVIEW OF LITERATURE

Micropropagation has become a valuable tool for large scale multiplication of horticultural crops. Considerable efforts have been directed to optimise the *in vitro* stages of micropropagation. However, the process of acclimatization and *ex vitro* establishment of plantlets, especially those of woody species, remain critical.

Only very few reports are available on the rooting and *ex vitro* establishment of bael which is a woody medicinal plant. This review encompasses the research work on various aspects of rooting with reference to growth hormone, basal media, sucrose, agar, activated charcoal and *ex vitro* establishment with reference to potting media and mycorrhization of tissue cultured plantlets of bael and other related crops.

There are different steps in *in vitro* plantlet production, namely culture establishment, multiplication of propagules, rooting and hardening of plantlets, planting out and *ex vitro* establishment.

2.1 *IN VITRO* PLANT REGENERATION

According to Murashige (1974) there are three possible routes available for *in vitro* propagule multiplication *viz.*, enhanced release of axillary buds, somatic organogenesis and somatic embryogenesis. The basal media and supplements are critical for *in vitro* plant regeneration.

2.1.1 Plant Growth Substances

Callus was initiated from stem explants of *Aegle marmelos* on medium supplemented with 1.00 mg l^{-1} kinetin and 5.00 mg l^{-1} NAA (Varghese *et al.*, 1993).

Hossain *et al.* (1994a) induced regeneration of plantlets from *in vitro* cultured cotyledons of *Aegle marmelos*. He also observed the shoot regeneration capacity of BA to be superior to other cytokinins.

Direct organogenesis from hypocotyl explants of *Aegle marmelos* was obtained on MS medium supplemented with BAP 0.10 mg l^{-1} (Hossain *et al.*, 1995).

Cotyledonary nodal explants of *Aegle marmelos* differentiated to multiple shoots on MS medium supplemented with different concentrations of BAP, kinetin and NAA. The highest number of shoots per explant was observed on MS medium supplemented with BAP 3.00 mg l^{-1} in *Aegle marmelos* (Arumugam and Rao, 1996).

Islam *et al.* (1996b) reported that a medium containing 1.00 mg l^{-1} and 0.20 mg l^{-1} NAA produced high frequency adventitious plant regeneration from radicle explants of *Aegle marmelos*. They also reported somatic embryogenesis and plant regeneration using zygotic embryos.

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*.

Successful callus free release of axillary buds were reported from single node segments of *Aegle marmelos* by Ajithkumar and Seeni (1998).

Arumugam and Rao (2000) reported the induction of somatic embryogenesis in *Aegle marmelos* in solid medium containing 2,4-D, BA and ABA.

Hazeena (2001) reported that maximum multiple shoot proliferation from nodal segments was obtained with BA 2.50 mg l^{-1} and IAA 1.00 mg l^{-1}. For enhanced release of axillary buds from cotyledon, BA 0.50 mg l^{-1} registered maximum number of shoots per culture. For shoot proliferation via direct organogenesis, maximum number of shoots were obtained within MS basal media supplemented with BA 0.20 mg l^{-1} and IAA 2.00 mg l^{-1}. Maximum shoot proliferation from callus was obtained by combining BA 2.00 mg l^{-1} and IAA 0.50 mg l^{-1}. Among the different plant growth substances tried to initiate direct organogenesis from cotyledon, BA 0.40 mg l^{-1} was ideal for producing maximum

number of shoots. The highest number of shoots produced via direct organogenesis from *in vitro* root was observed in MS basal media supplemented with BA 0.50 mg l^{-1}. Maximum elongation of shoot was noticed at lower concentration of BA 0.20 mg l^{-1}.

2.1.2 Basal Media

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 basal medium.

Komalavalli and Rao (2000) reported that MS media was best for shoot sprouting in *Gymnema sylvestre*.

Full strength MS basal medium was best for shoot proliferation in *Aegle marmelos* when compared to half strength MS, SH, B5 and WPM (Hazeena, 2001). Addition of gibberellic acid (3.00 mg l^{-1}) in the medium produced lengthy shoots. Supplementation of adenine sulphate at 20.00 mg l^{-1} improved shoot proliferation.

2.1.3 Solidifying Agent

Hossain *et al.* (1994a) reported that media was gelled with 0.70 per cent agar for regeneration of plantlets from *in vitro* cultured cotyledons of *Aegle marmelos*. Arumugam and Rao (1996) reported the use of medium containing 0.80 per cent agar for production of plantlets from cotyledonary node cultures of *Aegle marmelos*. Hazeena (2001) observed that there was an increase in shoot number with decrease in agar concentration. Agar at 5.00 g l^{-1} recorded maximum number of shoots.

2.1.4 Culture Conditions

Murashige (1977) observed that light intensity, both quality and duration affect the growth of *in vitro* grown cultures. The optimum day length period was considered to be 16 h for a wide range of plants. Maintenance of cultures in 16 h light and 8 h dark cycle was reported by Mumtaz *et al.* (1990) in *Catharanthus roseus*. Cultures were maintained at 25 ± 2°C with 60.00 per cent

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

2.1.5 Sucrose

Hossain *et al.* (1994a) observed that media supplemented with 40.00 g l^{-1} sucrose was best for shoot induction and elongation in *Aegle marmelos.* Sucrose was found to be superior to glucose with respect to shoot number. Sumana *et al.* (1999) reported that sucrose at three per cent was the sugar of choice for shoot regeneration in *Holarrhena pubescens.* According to Hazeena (2001) maximum number of shoots per culture was produced by sucrose at 50.00 g l^{-1}.

2.2 ROOTING

2.2.1 *In vitro* Rooting

2.2.1.1 Effect of Plant Growth Substances

Hutchinson (1981) found IBA as superior auxin to IAA or NAA for *in vitro* rooting of apple shoots, while Amin and Jaiswal (1987) observed its superiority for rooting in guava and jack fruit microcuttings.

Ranjit and Kester (1988) reported that GA at lower concentration improved the rooting of tissue cultured cherry root stocks. However, rooting did not occur in the absence or at higher concentrations of GA.

William and Taji (1989) observed that when NAA was supplemented in MS basal medium, roots produced were thin in the case of *Cheiranthera volubilis.* IBA produced thicker roots which reduced the establishment of plantlets during transplanting.

Islam *et al.* (1992) found that plantlets developed roots in *Aegle marmelos* when they were subcultured in half strength MS basal medium with IBA 1.00 mg l^{-1}.

According to Islam *et al.* (1994), *in vitro* regenerated shoots of *Aegle marmelos* rooted when cultured on half strength MS medium. Hossain *et al.* (1994b) found that callus derived shoots produced roots and developed into plantlets when transferred to half strength MS medium supplemented with IBA 0.50 mg l^{-1} and NAA 0.50 mg l^{-1}. Approximately five months were required for the full regenerative process of *Aegle marmelos*.

NAA favoured rooting compared to other auxins in *Clitoria ternatea* (Kumar *et al.*, 1993) and *Mucuna pruriens* (Chattopadhyay *et al.*, 1995).

Islam *et al.* (1996a) reported that shoots grown in medium containing 25.00 mg l^{-1} IBA for one week, when transferred to basal medium produced adventitious roots. Maximum rooting (80.00 per cent) with 3-6 roots per shoot was achieved.

Shoot cutting (3.00-5.00 cm) was best rooted in half strength MS basal medium supplemented with 0.50 mg l^{-1} IAA or 10.00 mg l^{-1} IBA in *Aegle marmelos* (Ajithkumar and Seeni, 1998).

Rhizogenesis in *Acorus calamus* was initiated in the presence of high concentrations of NAA and produced short stumpy roots (Anu *et al.*, 2001).

Efficacy of IBA at lower concentrations in *in vitro* rooting has been reported in medicinal plants like *Wedelia chinensis* (Bhuyan *et al.*, 2000), *Swainsonia salsula* (Yang *et al.*, 2001) and *Plumbago* spp. (Das and Rout, 2002).

Indrani and Bhanja, 2002, reported that in *Flacourtia jangomas,* rhizogenesis was achieved in 1.00 mg l^{-1} NAA from both internode and leaf explants.

According to Rai, 2002, *in vitro* rooting was best induced (87.00 per cent) in shoots excised from proliferated shoot cultures in one fourth MS medium augmented with 1.20 mg l^{-1} IAA and 2.40 mg l^{-1} IBA in *Nothapodytes foetida.*

Vengadesan *et al.* (2002) reported that *in vitro* regenerated shoots in *Acacia sinuata* produced roots when transferred to half strength MS medium supplemented with 7.31 µM IBA and success rate in the field was 55.00 per cent.

Shoots rooted best (87.00 per cent) on MS medium containing 2.00 mg l^{-1} IBA in *Withania somnifera* (Rani *et al.*, 2002).

According to Azad *et al.* (2003) elongated shoots of *Adhatoda vasica* rooted successfully on half strength MS medium with 0.10 to 0.20 mg l^{-1} IBA. In *Cichorium intybus* L., shoots were rooted in MS basal medium augmented with 0.20 µM IBA (Rehman *et al.*, 2003). Saha *et al.* (2003) found that half strength MS basal medium supplemented with IBA 1.50 mg l^{-1} was best for inducing maximum number of roots in *Hemidesmus indicus*. On the other hand, NAA was found inferior and favoured callus formation at the base.

Radha and Chandra Roy (2003) reported rooting on the shoots of *Holarrhena antidysenterica* Wall., using MS medium with 5.00 mg l^{-1} IBA. IBA treatment was more effective for root induction and root growth compared to NAA in Panan Ginseng C.A. Meyer (Kim *et al.*, 2003).

Among the different types of auxins used, IBA was found to be the best for root induction in Native Olive (Rahman *et al.*, 2004). The maximum percentage of cultures that produced root was 80.00 when the medium contained 0.20 mg l^{-1} IBA. Percentage of root induction and number of roots per shoot were also highly influenced by concentration and type of auxin. No rooting was found in auxin free media. The *in vitro* elongated shoots were rooted with 80.00 per cent success by treating them in MS basal medium supplemented with 0.20 mg l^{-1} IBA and incubated under 30° C in dark for initial one week in Native Olive.

According to Deepa (2004) in *Clitoria ternatea* maximum percentage of root initiation was obtained in NAA 1.00 mg l^{-1}. IBA 2.50 mg l^{-1} recorded maximum root initiation in *Mucuna pruriens* and IAA 1.50 mg l^{-1} in *Indigofera tinctoria*.

2.2.1.2 Effect of Sucrose

Root induction in apple was decreased proportionately with decreasing sucrose levels (Lane, 1978).

Desjardins and Tiessen (1985) observed that very low sucrose concentration in the medium reduced the rooting percentage. At higher sucrose concentration, the rooting percentage and subsequent shoot growth were better. At higher sucrose concentration, time required for rooting was found to be reduced in asparagus.

Independent growth could not be achieved on medium without sucrose during proliferation of rose shoots (Longford and Wainwright, 1987). Also, reduction of sucrose below 10.00 g l^{-1} affected chlorophyll content of leaves and growth of shoots. Lowering sucrose level in rose shoot cultures increased their photosynthetic ability.

Short *et al.* (1987) reported that complete omission of sucrose in the medium increased the photosynthetic ability in cauliflower and chrysanthemum plantlets.

Hainwright and Scrace (1989) studied the effect of sucrose concentration in *in vitro* plant growth. Two to four per cent gave the maximum shoot height, fresh weight and dry weight of plantlets and registered 97.50 per cent *ex vitro* establishment.

According to Ramesh (1990) the number of days taken for root initiation increased with increase in sucrose concentration from 20.00 to 40.00 g l^{-1} in jack plantlets. Highest intensity root branching and survival was registered at 30.00 g l^{-1} sucrose concentration. Sucrose at 20.00 g l^{-1} registered lower survival rate. Sucrose at 10.00 g l^{-1} was inferior with respect to *in vitro* rooting and *ex vitro* establishment.

Ticha *et al.* (1998), found that the total biomass and leaf area of tobacco plants were increased by the addition of sucrose to the medium.

Height reduction was observed when pre-conditioned with 60.00 g l^{-1} sucrose compared to optimum sucrose dose of 30.00 g l^{-1} in jack plants (Rahman and Blake, 1998) and citrus plants (Hazarika *et al.*, 2004).

Hazarika *et al.* (2000) reported maximum *ex vitro* survival per cent (89.20 to 97.30) in citrus microshoots in medium supplemented with 30.00 g l^{-1} sucrose. Explants grown in the medium with low concentration of sucrose or medium without sucrose showed significant reduction in total chlorophyll. Hazarika observed that sucrose levels should be maintained at 30.00 g l^{-1} or even increased, prior to acclimatization to maximize plant quality. Though, leaves of plants derived from sucrose free medium have a greater ability to photosynthesise, the acclimatization environment may not permit appreciable photosynthesis to take place resulting in very high mortality when transferred to *in vivo*. Very high *ex vitro* survival rate (97.30 per cent) in *Citrus volkameriana* was observed when grown in 60.00g l^{-1} sucrose compared to those (43.80 per cent) grown in sucrose free medium before transferring to *ex vitro* environment.

An increase in sucrose from 20.00 to 35.00 g l^{-1} increased the multiplication rate of rooting and percentage survival in *Malus* and *Prunus* microcuttings (Kunneman and Albers, 2003).

Hazarika *et al.* (2004) reported that maximum values for shoot height, root length and root number was obtained after transferring citrus plantlets to *in vivo* conditions when previously conditioned with 3.00 per cent sucrose. The addition of sucrose to the medium had a positive effect on the formation of biomass and leaf area of citrus plants *ex vitro*.

2.2.1.3 Effect of Agar

Leshem (1983), Marin and Gella (1987) and Short *et al.* (1987) reported that higher concentration of agar in the rooting medium increased the *ex vitro* establishment of plantlets; but reduced the number of roots in carnation, cherry and chrysanthemum.

Survival percentage was found to be more at agar concentrations of 4.00, 5.00, and 6.00 g l^{-1} and low at 7.00 and 8.00 g l^{-1}. Maximum rooting prcentage was obtained when agar 5.00 g l^{-1} was used. The number of days taken for root

initiation was less when 4.00 g l^{-1} agar was used but the percentage of rooting was less in jack (Ramesh, 1990).

Phillip *et al.* (2001) observed that best rooting was obtained in paper shell almond in a medium containing 0.60 per cent agar.

Increasing agar concentration upto 20.00 g l^{-1} eliminated hyper hydration but decreased the number of shoots regenerated to half. Cent per cent of the cultured shoots initiated roots in solid MS basal medium with 8.00 g l^{-1} agar in *Globularia alypum* (Bertsoukhi *et al.*, 2003).

According to Rahman *et al.* (2004) maximum rooting has been obtained in medium gelled with 0.70 per cent agar in Native Olive.

2.2.1.4 Effect of Activated Charcoal

The number of roots per shoot and rooting intensity showed a decreasing pattern with increasing concentration of activated charcoal. The maximum *ex vitro* survival percentage of plantlets (66.60) was observed in charcoal 1.00 g l^{-1} while the least (16.70 per cent) was in the case of 10.00 g l^{-1}.Minimum days for root initiation was observed in the case of 1.00 g l^{-1} activated charcoal, followed by 5.00 and 10.00 g l^{-1} in jack plantlets (Ramesh, 1990).

Naomita and Rai (2001) reported that in *Ochreinauclea missionis* individual shoots rooted best in MS medium with 0.30 per cent activated charcoal.

According to Gomes *et al.* (2003), root formation in *Maclura tinctoria* was found to be the best when 4.70 g l^{-1} activated charcoal was used in WPM medium.

2.2.1.5 Mode of Culture

Filter paper bridge technique was developed by Goodwin (1966) for the rapid growth of potato buds.

Hoque *et al.* (2001) reported that shoots produced *in vitro* rooted in a liquid half strength MS medium in chestnut.

Pateli *et al.* (2003) found that liquid media compared to solid media gave much longer seedlings, as well as higher percentage of protocorm like bodies and

branched seedlings in *Epidendrum radicans*. They reported that the percentage of *ex vitro* establishment was higher when grown on liquid MS medium.

2.2.2 *Ex vitro* Rooting

Direct rooting of tea shoots was achieved by dipping the cut ends in IBA 50.00 mg l^{-1} for 20 min and subsequently planting these in a soil: peat moss (1:1) mixture. Shoots which are directly rooted in soil showed higher per cent survival in the field than those rooted under *in vitro* conditions (Das *et al.*, 1990).

John (1996) reported *ex vitro* rooting to be better than *in vitro* rooting in *Holostemma annulare.*

According to Gonclaves *et al.* (2001), survival rate reached 100.00 per cent for plantlets with *ex vitro* rooting. *Ex vitro* rooting was associated with a vigorous growth of the plant expressed by an increase in relative growth, total dry weight, leaf area, number of new leaves formed, relative growth rate and net assimilation rate in chestnut plants.

Naomita and Rai (2001) reported that individual shoots with a minimum of one node was excised and successfully rooted *ex vitro* by treating with 49.00 µM IBA for 30 min in *Ochreinauclea missionis.*

Rai (2002) reported that *in vitro* developed shoots of *Nothapodytes foetida* rooted *ex vitro* by dipping in 49.00 µM IBA for 10 min .

Arya *et al.* (2003) observed that 90.00 per cent of *in vitro* produced shoots rooted *ex vitro* when they were pulse treated with 123.00 µM each of IBA and NAA in *Leptadenia reticulata.*

In Bogainvillea, the transplanting losses during acclimatization were higher for plantlets derived from *in vitro* rooted microcuttings than *ex vitro* rooted microcuttings (Hatzilazarou *et al.*, 2003a). In Nerium, for *ex vitro* rooting, the microcuttings were first dipped in various concentrations of IBA (0.00, 0.13, 0.25 and 0.50 g l^{-1}) and then planted in perlite and rooting was upto 70.00 per cent

with 0.50 g l^{-1} IBA. No losses were noticed in those plantlets derived from *ex vitro* rooting (Hatzilazarou *et al.*, 2003b).

Cent per cent survival of regenerated plantlets under *ex vitro* conditions were reported in *Wedelia chinensis* (Martin *et al.*, 2003).

There was no effect of IBA pre-treatments (25.00, 50.00, 100.00 and 200.00 mg l^{-1}) on *ex vitro* rooting of *Malus* and *Prunus* microcuttings (Kunneman and Albers, 2003)

For *in vivo* rooting, IBA 1.00 to 3.00 mg l^{-1} was found to be the best combination in *Leucospermum cordifolium* (Tal *et al.*, 2003).

According to Deepa (2004), in *Clitoria ternatea* maximum survival rate (83.33 per cent) was obtained when the shoots were dipped in IBA 1000.00 mg l^{-1} for 20 s and least (50.00 per cent) in IBA 100.00 mg l^{-1} kept overnight. In *Mucuna pruriens*, pre-treatments with IBA 500.00 mg l^{-1} for 20 s recorded maximum survival rate (83.33 per cent) and least (58.33 per cent) in IBA 100.00 mg l^{-1} kept overnight. IBA 1000.00 mg l^{-1} for 20 s registered maximum survival rate (75.00 per cent) and IBA 100.00 mg l^{-1} kept overnight recorded only 41.67 per cent survival after four weeks in *Indigofera tinctoria*.

According to Romano and Martins (2004), the rooting percentage was improved to 95.00 per cent by basal immersion of shoots in 0.50 g l^{-1} IBA for 2 min. Roots formed by dipping were more branched.

2.3 *EX VITRO* ESTABLISHMENT

2.3.1 Potting Media

According to Ramesh (1990), sand supported 53.30 per cent survival of jack plantlets *ex vitro*. Vermiculite: peat as well as sand: soil (1:1) recorded 40.00 per cent survival. Soilrite, coirdust and sawdust did not support the survival of plantlets.

Acclimatization was carried out on benches in a green house, in 6.00 cm plastic pots in a mixture of peat : garden soil : perlite (1:1:1) in lemon balm with 90.00 to 98.00 per cent survival rate (Annamaria *et al.*, 1999).

Rooted plantlets of *Acorus calamus* when transferred to pots containing sand, soil and organic manure (cow dung) in the ratio 3:1:1, registered 80.00 to 90.00 per cent survival rate (Anu *et al.*, 2001).

Hazeena (2001) reported that in *Aegle marmelos*, sand and soilrite registered cent per cent survival of plantlets two weeks after planting and sand recorded maximum survival rate (75.00) one month after planting.

Naomita and Rai (2001) observed that regenerance of *Ochreinauclea missionis* acclimated in soilrite exhibited 65.00 per cent survival in the green house.

In *Salvadora persica*, the regenerated plantlets were transferred to sterile pots containing a mixture of soil and vermiculite (3:1) and showed a transplantation success of 60.00 to 70.00 per cent (Mathur *et al.*, 2002).

Yusuf (2002), reported that in *Anogeissus sericea* var.nummularia, plantlets were hardened and acclimatized by transferring to autoclaved soilrite moistened with one fourth strength MS mineral salts in glass bottles and then to soil mixture containing sandy soil, black soil and pond soil (1:1:1 v/v) and all these plants survived in the field.

Rooted plantlets of *Adhatoda vasica* were initially planted in polybags containing garden soil and compost (1:1) or garden soil, sand and compost (2:1:2) with 60.00 and 80.00 per cent survival rate respectively (Azad *et al.*, 2003).

In a 1:1 peat : perlite medium, 37.50 per cent of micropropagated citrus plantlets formed lateral roots. On soil:manure:peat (1:1:1), 12.50 per cent of the plantlets died and those survived formed a poor root system. The plants grown on sand- peat, sand-perlite and peat-perlite (1:1) developed good root system (Plastira and Karetsos, 2003).

In *Leptadenia reticulata* plantlets were transferred to bottles containing sterile soilrite moistened with half strength MS macrosalts. 90.00 per cent of the plantlets were hardened and these plants were transferred to black polybags in the nursery (Arya *et al.*, 2003).

The rooted plantlets of kinnow mandarin when transferred to potting mixture containing garden soil and vermiculite (1:1) showed 80.00 per cent survival rate (Praveen *et al.*, 2003).

According to Nath and Buragohain (2003), rooted plants of *Centella asiatica* were transferred from culture tubes into plastic cups containing vermiculite: soil : sand in 1:1:1 ratio and the acclimatized plants were successfully established in field with only 1.00 to 2.00 per cent mortality rate.

Saha *et al.* (2003) found that the rooted plantlets of *Hemidesmus indicus* when transferred to pots containing garden soil and sand in the ratio 1:3 under greenhouse conditions registered 85.00 per cent survival rate.

According to Rahman *et al.* (2004), *in vitro* regenerated plantlets of Native Olive acclimatized better under *ex vitro* conditions when they were transferred on specially made plastic trays containing coco-peat as potting mixture.

Aloe vera plantlets planted in plastic pots containing soil and FYM (1:1) were kept in green house for ten days and they survived when shifted to shade house (Aggarwal and Barna, 2004).

Sharma *et al.* (2004) reported that in *Anethum graveolens*, an important medicinal herb, rooted plantlets when transferred to pots containing sterile vermiculite registered 64.00 per cent survival rate.

According to Misra *et al.* (2004), rooted plantlets of *Piper betle* were hardened by transferring them into pots containing a sterile mixture of soil, cowdung and neem cake in the ratio 1:1:1.

2.3.2 ACCLIMATIZATION

Podwyszynska and Hampel (1988) found that acclimatization was better when lower levels of BA was used in the rooting media and when IAA was omitted from the rooting media in rose hybrids.

Ajithkumar and Seeni (1998) reported that 88.00 per cent of the rooted plants of *Aegle marmelos* got established in polybags after hardening.

No humid chamber phase was required to harden tissue culture plants of *Acorus* (Anu *et al.*, 2001).

Ahuja *et al.* (2002) reported that the potted plants of *Atropa acuminata* were maintained inside an intermittent mist chamber under high humid conditions (80.00 - 90.00 per cent RH) for three weeks and irrigated with one fourth strength Knop's nutrient solution for acclimatization and afterwards transferred to the green house.

According to Mathur *et al.* (2002), regenerated plantlets of *Salvadora persica* were covered with inverted glass beakers during first week to provide high humidity and irrigated after 24 h with a solution containing MS salts at half strength for hardening. Transplantation success was 60.00 to 70.00 per cent. Subsequently these plantlets were transferred to the garden soil and after one month they were planted in the field for establishment.

Rani *et al.* (2002) reported that in *Withania somnifera*, rooted plantlets when transferred to the field after acclimatization showed 60.00 per cent survival.

Saha *et al.* (2003) reported that the fully developed plantlets of *Hemidesmus indicus* were initially hardened for 15 days in small pots containing vermiculite at a light intensity of 3000 lux before transferring to pots.

Rahman *et al.* (2004) observed that rooted shoots were gradually acclimatized and successfully established under natural conditions with about 50.00 per cent survival rate in *Elaeocarpus robustus*. He also observed that in the first week of transplantation, plantlets were moistened uniformly at periodic

intervals and kept covered in polythene tents for providing conditions of high humidity and sufficient light.

Sharma *et al.* (2004) reported that in *Anethum graveolens*, rooted plantlets after transferring to pots were covered with a perforated polythene bag for one week to retain moisture.

2.3.3 Mycorrhiza

According to Heslin and Douglas (1986) ectomycorrhizal fungi increased shoot height, plant dry weight and NPK content of eucalyptus.

According to Dixon *et al.* (1988), endogenous biosynthesis of different phytohormones specially cytokinin was increased several times when mycorrhiza was applied in *Citrus jambhiri* Lush.

Puthur *et al.* (1988) observed cent per cent survival rate in mycorrhiza treated tissue cultured plantlets of *Leucaena leucocephala.*

Inoculation of potting medium with *Glomus etunicatum* and *Glomus fasciculatum* favoured 100.00 and 80.00 per cent *ex vitro* establishment of micropropagated jack plantlets. In the treated plants, plant height, fresh weight, dry weight, number of leaves and total leaf area were significantly increased (Ramesh, 1990).

Chang (1992) observed that mycorrhizal plants enter reproductive phase early because of increased phosphorus nutrition and greater development of water conducting tissues.

Glomus mosseae was most effective in stimulating plant growth in jack plantlets. It recorded significantly higher plant height, shoot and root fresh weight (Sivaprasad *et al.*, 1995).

It was observed that cardamom plants inoculated with *Glomus monosporum* performed best in improved plant growth as well as nutrient uptake (Sreeramulu and Bhagyaraj, 1997).

Schultz *et al.* (1998) reported that the survival rate of micropropagated oil palm clones during acclimatization was increased from 70.00 to 90.00 per cent when vesicular arbuscular fungi was inoculated.

Kothari *et al.* (1999) observed that inoculation of *Glomus intraradices* to *Mentha citrata* substantially increased biomass and nutrient uptake.

Rauvolfia tetraphylla plants inoculated with *Glomus caledonium* showed maximum plant height, leaf number and stem girth (Earanna *et al.*, 2002).

Singh *et al.* (2004) reported that mycorrhizal plantlets were found to be faster in growth and possessed high shoot and root fresh and dry weights in grape plantlets. Mycorrhization resulted in high plantlet survival of over 85.00 per cent compared to 55.00 per cent in non–mycorrhizal control.

Singh and Singh (2004) reported that inoculation of VAM significantly increased the growth of banana plants compared to non-mycorrhizal control and was effective in increasing nutrient uptake by the plant. VAM influenced growth attributing characters (plant height, girth, production of functional leaves) and yield attributing components (finger length, number of fingers per bunch and bunch weight) which contributed towards higher yield.

Materials and Methods

3. MATERIALS AND METHODS

Investigations were carried out at the Department of Plantation Crops and Spices and Department of Plant Biotechnology, College of Agriculture, Vellayani with the objective to develop a protocol for the *in vitro* and *ex vitro* rooting of microshoots and to standardise techniques for the *ex vitro* establishment of plantlets of bael commonly known as koovalam (*Aegle marmelos* (L.) Corr.) during 2003 to 2005.

The materials and methods tried for rooting and *ex vitro* establishment are being described in this chapter.

3.1 MICROCUTTINGS

Microcuttings needed for the study were obtained from the cultures of previous study kept in the Plant Biotechnology Laboratory, College of Agriculture, Vellayani, Thiruvananthapuram.

3.1.1 Subculturing

The microcuttings were subcultured in MS basal media supplemented with BA 0.20 mg l^{-1} and $CoCl_2$ 10.00 mg l^{-1}.

3.2 ROOTING

3.2.1 Basal Media

The basal media used for the study were MS (Murashige and Skoog, 1962), 1/2 MS and WPM. The chemicals used for the preparation of the culture media were of analytical grade from Sisco Research Laboratories (Bombay), Merck (Bombay) and British Drug House (Bombay).

Standard procedures were followed for the preparation of the basal media (Thorpe, 1980). Stock solutions of major and minor nutrients and plant growth substances were prepared by dissolving the required quantity of chemicals in

specific volume of double glass distilled water and were stored under refrigerated conditions (4° C).

The glass ware used for the preparation of the media was washed with labolene and rinsed with double glass distilled water. Specific quantities of the stock solutions were pipetted out into 1000 ml beaker. Sucrose and inositol were added fresh and dissolved. The pH of the medium was adjusted between 5.60 and 5.80 using 0.10 N NaOH or 0.10 N HCl with the aid of an electronic pH meter. Agar (in the case of solid medium) was added to the medium and final volume made upto 1000.00 ml.

The solution was then heated by placing the beaker on a heating mantle and stirring thoroughly for uniform mixing, till agar melted. Activated Charcoal (AC) 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 (25×150 mm) and jam bottles were used as culture vessels. The test tubes and jam bottles were filled with 15 and 50 ml of the medium, respectively. The culture vessels containing the medium were plugged tightly with cotton. They were then autoclaved at 121° C and 1.06 kg cm^{-2} pressure for 20 min.

3.2.2 *In vitro* Rooting

3.2.2.1 Plant Growth Substances

The microcuttings were subjected to different combinations of plant growth substances. MS, ½ MS and WPM were used as the basal media. Different combinations of auxins *i.e.*, IBA (0.50 to 2.50 mg l^{-1}), IAA (1.00 to 2.50 mg l^{-1}) and NAA (1.00 to 2.50 mg l^{-1}) were tried (Table 1). The treatments were replicated ten times. The cultures were kept in darkness initially and after observing root initiation, they were transferred to light with an intensity of 3000 lux and illumination for six hours.

Table 1. Plant Growth Substances tried for *in vitro* rooting of *Aegle marmelos*

Medium : MS / ½ MS / WPM + Inositol (100.00mg l^{-1}) + Sucrose (30.00 g l^{-1}) + Agar (6.00 g l^{-1})

Treatments	Plant Growth Substances (mg l^{-1})
T_1	IBA 0.50
T_2	IBA 1.00
T_3	IBA 1.50
T_4	IBA 2.00
T_5	IBA 2.50
T_6	IAA 1.00
T_7	IAA 1.50
T_8	IAA 2.00
T_9	IAA 2.50
T_{10}	NAA 1.00
T_{11}	NAA 1.50
T_{12}	NAA 2.00
T_{13}	NAA 2.50

The observations taken under *in vitro* rooting were number of cultures initiating roots, number of days taken for root initiation, number of roots, length of roots developed and their nature.

3.2.2.2 Basal Media

Three different media were tried, full and half strength MS and WPM. Composition of MS and WPM is given in Appendix 1.

3.2.2.3 Sucrose

Varying levels of sucrose were tried to study their effect on *in vitro* rooting (Table 2).

3.2.2.4 Gelling Agent (Agar)

The effect of different levels of agar on *in vitro* rooting were studied (Table 3).

3.2.2.5 Activated Charcoal

The cultures were transferred to varying levels of activated charcoal to study its effect on *in vitro* rooting (Table 4).

3.2.2.6 Mode of Culture

Liquid as well as solid media were tried in order to asses their effect on *in vitro* rooting.

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

Filter paper bridge technique

The filter paper bridge prevents direct contact of the plant material with the media. A filter paper bridge in the shape of letter 'M' was made and then inserted into the test tube. The test tube was then plugged tightly with cotton and autoclaved at 121° C and 1.06 kg cm^{-2} pressure for 40 min. Liquid medium was

Table 2. Sucrose levels tried for *in vitro* rooting of *Aegle marmelos*

Medium : MS + Inositol (100.00 mg l^{-1}) + Agar (6.00 g l^{-1}) + IBA (2.50 mg l^{-1}) / NAA (1.00 mg l^{-1})

Treatments	Sucrose (g l^{-1})
S_1	10.00
S_2	20.00
S_3	30.00
S_4	40.00

Table 3 Agar levels tried for *in vitro* rooting of *Aegle marmelos*

Medium : MS + Inositol (100.00 mg l^{-1}) + Sucrose (30.00 g l^{-1}) + IBA (2.50 mg l^{-1}) / NAA (1.00 mg l^{-1})

Treatments	Agar (g l^{-1})
AG_1	4.00
AG_2	5.00
AG_3	6.00
AG_4	7.00
AG_5	8.00

Table 4. Activated charcoal levels tried for *in vitro* rooting of *Aegle marmelos*

Medium : MS + Inositol (100.00 mg l^{-1}) + Sucrose (30.00 g l^{-1})+ Agar (6.00 g l^{-1}) + IBA (2.50 mg l^{-1}) / NAA (1.00 mg l^{-1})

Treatments	Charcoal (g l^{-1})
AC_1	0.50
AC_2	1.00
AC_3	2.00
AC_4	3.00
AC_5	4.00
AC_6	5.00

prepared with specific quantity of plant growth substance. The sterilized media was then poured into the test tube containing paper bridge in such a way that two arms of the filter paper dip into the medium. The bridge with the explant was kept above the medium.

3.2.3 *Ex vitro* Rooting

3.2.3.1 Quick Dip Method

The basal portion of the plantlets was dipped in three different concentrations of IBA for 20 s (Table 5).

3.2.3.2 Slow Dip Method

The basal portion of the microcuttings was dipped in three different concentrations of IBA overnight (Table 6).

3.2.3.3 Microcuttings

Healthy microcuttings of 3.00 to 5.00 cm length and 3.00 to 4.00 leaves were taken.

3.2.3.4 Potting Media

The microcuttings were planted in plastic pots with sterile sand. The pots were filled with sand upto three-fourth height and the shoots planted at the center. The pots were provided with two holes for proper drainage.

3.2.3.5 Plant Growth Hormones

Different concentrations of IBA were tried for *ex vitro* rooting (Table 5 and 6). Different concentrations of IBA were prepared and the basal portion of the microcuttings was dipped in the respective solutions for 20 s in quick dip method. In slow dip method, the lower portions of the microcuttings were dipped in different concentrations of IBA and kept overnight. Then they were planted directly in sterile sand for rooting. Each treatment was replicated six times.

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

Table 5. IBA levels tried for *ex vitro* rooting of *Aegle marmelos* (quick dip method)

Treatments	IBA (mg l^{-1})
ERQ_1	500.00
ERQ_2	1000.00
ERQ_3	1500.00

Table 6. IBA levels tried for *ex vitro* rooting of *Aegle marmelos* (slow dip method)

Treatments	IBA (mg l^{-1})
ERS_1	50.00
ERS_2	100.00
ERS_3	150.00

Table 7. Potting media tried for *ex vitro* establishment of *Aegle marmelos* plantlets

Treatments	Media
P_1	Sand
P_2	Soilrite
P_3	Sand:soil (1:1)
P_4	Sand:soil:coirpith (1:1:1)
P_5	Sand:soil:leafmould (1:1:1)

3.3 *EX VITRO* ESTABLISHMENT

3.3.1 Potting Media

Five different potting media were prepared using different proportions of sand, soilrite, soil, coirpith and leaf mould (Table 7). The potting media were sterilized by autoclaving at 1.06 kg cm^{-2} for 45 min.

3.2.2 Mycorrhizal Fungi

Two mycorrhizal species *viz.*, *Glomus etunicatum* and *Glomus fasciculatum* were used. They were obtained from Department of Plant Pathology, College of Agriculture, Vellayani. The *in vitro* grown plantlets were planted in sterile potting media inoculated with a mixture of 5.00 g AMF infected root bits of guinea grass.

3.3.3 *Ex vitro* Establishment

In vitro rooted plantlets were taken out from the culture vessels using sterilized forceps. The agar adhering to the roots was completely removed by thorough washing. For this first the plants were kept under running tap water and then washed in distilled water. During all these processes, care was taken for not damaging the roots. The plantlets were then dipped in 0.10 per cent Indofil (Dithane M-45), a fungicicide, for 30 min. These plantlets were then planted in disposable cups filled with different sterilized potting media according to treatments. The plantlets were kept inside a humidity chamber covered with polythene sheets of 350 guage thickness for a period of thirty days at a RH of 75.00 per cent and temperature of 35° C.

3.3.3.1 Aftercare of Plantlets

After seven days of planting out, the plantlets were irrigated twice a week with 0.10 MS solution. Humidity inside the chamber was adjusted by lifting the polythene sheet and plantlets were gradually exposed to sunlight. After the observation period, some of the plants were transferred to pots and placed outside.

3.3.4 Observations

3.3.4.1 Survival of Plantlets

Survival of plantlets was observed at fortnightly intervals.

3.3.4.2 Arbuscular Mycorrhizal Colonization (AMF)

Mycorrhizal colonization pattern in the roots was studied according to the method described by Phillips and Hayman (1970). FAA solution (formaldehyde: acetic acid: alchohol) was prepared and roots were cut into pieces of 1.00 cm length and immersed in this solution and kept overnight. After draining this solution, ten per cent KOH solution was poured into the root bits, autoclaved for ten min for softening the roots so as to make them vulnerable to staining. After draining KOH solution, roots were treated with one per cent HCl for 10 min. for neutralizing the effect of KOH.

The root bits were stained in lactophenol tryptan blue (stain) powder 0.05 per cent (lactophenol composition: lactic acid 20 parts, phenol 20 parts, glycerol 40 parts, distilled water 40 parts) for staining mycorrhiza. The roots were treated with the dye for one to five min. The dye was then drained and the root bits were observed under microscope for mycelia, vesicles and arbuscules. Fifty bits were examined under each treatment for better precision.

$$\text{AMF Colonization (\%)} = \frac{\text{Number of root bits positive for AMF Colonization}}{\text{Total number of root bits observed}} \times 100$$

3.3.4.3 Biometric Observations

The following biometric observations were recorded at fortnightly intervals for a period of one month.

3.3.4.3.1 Height of the Plant

This was measured from the collar region to the tip of the microshoot and the mean length was expressed in centimetre. The observations were made at fifteen days interval.

3.3.4.3.2 Number of Leaves

The number of fully opened leaves per plantlet was counted at fifteen days interval.

3.3.4.3.3 Number of Roots

The number of roots per plantlet was counted and recorded at fifteen days interval.

3.3.4.3.4 Fresh Weight

Fresh weight of individual plants were recorded at fifteen days interval and expressed as g plant^{-1}. The plants were uprooted and weighed using an electronic balance.

3.3.4.3.5 Dry weight

Plants were dried at 70° C for 48 h till constant weight was obtained, then dry weight recorded and expressed as g plant^{-1}.

3.3.4.4 Physiological Observations

3.3.4.4.1 Stomatal Conductance (SC)

Stomatal resistance of individual plants were recorded using a Porometer (Delta T devices-Cambridge-UK) at fortnightly interval during 10:00 to 12:00 am.

$$\text{Stomatal Conductance (SC)} = \frac{1}{\text{Stomatal Resistance}}$$

Stomatal Conductance was expressed in cm S^{-1}

3.3.4.4.2 Leaf Area Index (LAI)

Leaf area was calculated by tracing the area of the leaf on a graph sheet and Leaf Area Index was worked out as per the method suggested by Williams (1946).

$$\text{Leaf Area Index (LAI)} = \frac{\text{Total leaf area of the plant (cm}^2\text{)}}{\text{Area of land covered by the plant (cm}^2\text{)}}$$

3.3.4.4.3 Crop Growth Rate (CGR)

The CGR was worked out using the formula of Watson(1971) and expressed in mg cm^{-2} day^{-1}.

$$\text{Crop Growth Rate (CGR)} = \frac{W_2 - W_1}{p(t_2 - t_1)}$$

where W_1 andW_2 – whole plant dry weight at t_1 and t_2 respectively.

t_1 and t_2 - time in days

p – ground area on which W_1 and W_2 was estimated.

3.3.5 Statistical Analysis

The data was statistically analysed as per the procedure outlined by Panse and Sukhatme (1985) for Completely Randomised Block Design.

Results

4. RESULTS

Investigations were carried out for standardising the rooting of microshoots and *ex vitro* establishment of plantlets of *Aegle marmelos* (L.) Corr., at the Department of Plantation Crops and Spices and Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2003 to 2005. The results of the studies are presented in this chapter.

4.1 ROOTING

Rooting is a crucial step in the propagation of woody species and there is great variation in the rooting ability of different species. For conducting this experiment, microshoots were taken from the cultures of previous studies on bael, maintained in the Plant Biotehnology Laboratory, College of Agriculture, Vellayani.

4.1.1 *In vitro* Rooting

4.1.1.1 Plant Growth Substances

Thirteen treatments involving various levels of auxin (IBA, NAA and IAA) were tried in three basal media (full strength and half strength MS and WPM) to study their effect on *in vitro* rooting. In addition, combination treatments were also tried.

The percentage of rooting varied from 30.00 to 50.00 per cent for the various plant growth substances tried in MS basal medium. The treatments T_5 (IBA 2.50 mg l^{-1}), T_2 (IBA 1.00 mg l^{-1}) and T_{10} (NAA 1.00 mg l^{-1}) recorded the highest per cent of rooting (50.00) and the treatments T_1, T_4, T_6, T_7, T_8, T_9 and T_{13} recorded the least (30.00). There was significant difference among the treatments with respect to root initiation percentage. T_5 and T_{10} were found to be superior to that of others (Table 8, Fig. 1). Control without any plant growth substance failed to initiate rooting.

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

Treatments	Root initiation per cent	Days for root initiation	Number of roots	Length of roots (cm)
T_1	30.00	8.00	1.00	0.89
T_2	50.00	15.80	1.67	1.16
T_3	40.00	13.00	1.67	1.26
T_4	30.00	12.50	1.00	1.20
T_5	50.00	10.00	2.67	1.30
T_6	30.00	13.00	1.00	0.87
T_7	30.00	13.00	1.00	1.13
T_8	30.00	15.33	1.00	0.70
T_9	30.00	13.50	1.00	0.89
T_{10}	50.00	11.25	1.67	1.03
T_{11}	40.00	13.50	1.67	1.53
T_{12}	40.00	13.75	1.67	1.27
T_{13}	30.00	14.00	2.00	1.20
Control	–	–	–	–
CD at 5% level	3.08	NS	3.50	NS

The data represents the mean value of ten replications

Culture medium : MS + Inositol (100.00 mg l^{-1}) + Sucrose (30.00 g l^{-1}) + Agar (6.00g l^{-1})

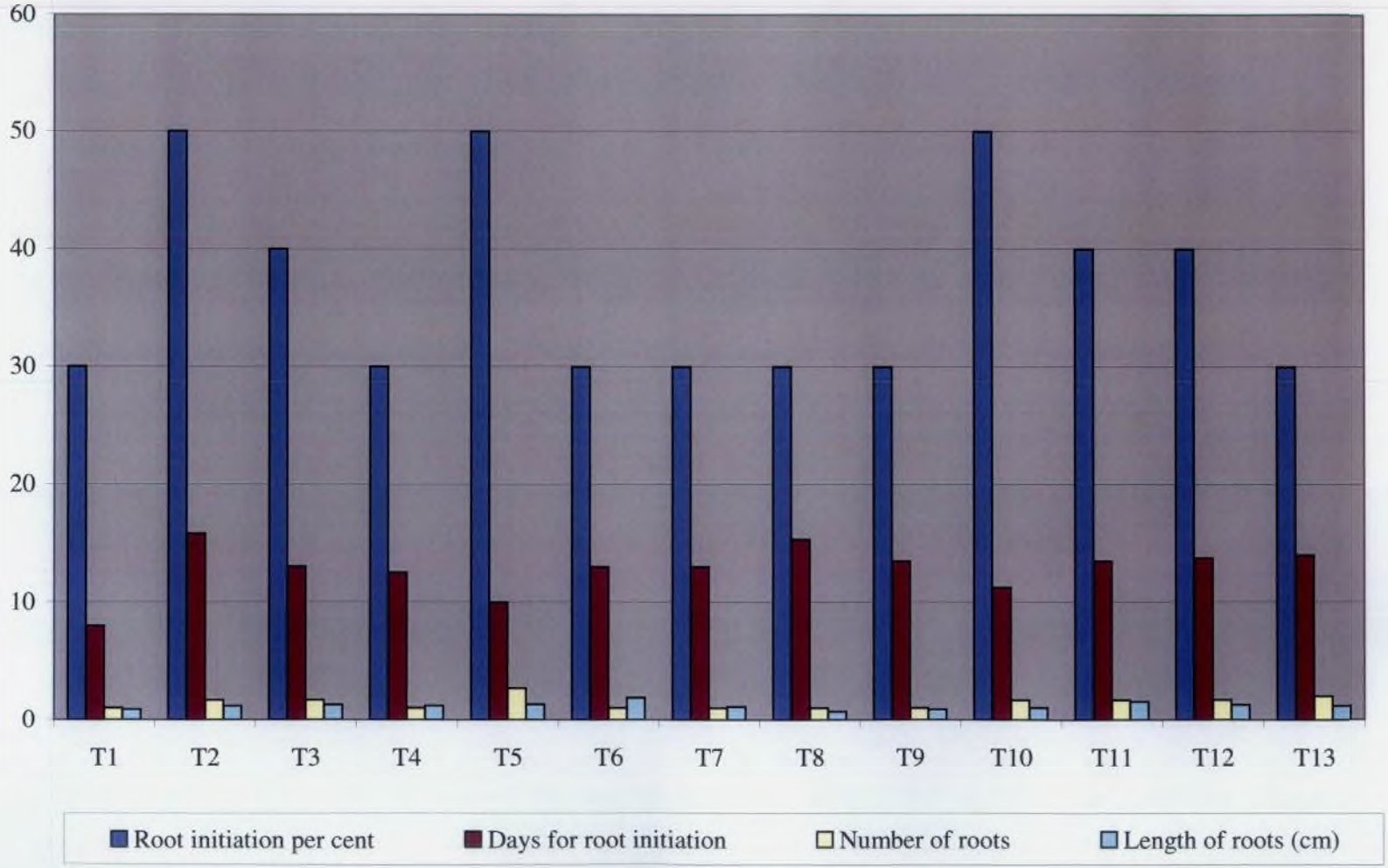


Fig. 1. Effect of plant growth substances on *in vitro* rooting of *Aegle marmelos*

Wide variation was noticed in the case of number of days taken for root initiation (8.00 to 15.80 days). The earliest rooting (8.00 days) was observed in T_1 (IBA 0.50 mg l^{-1}) followed by 10.00 days in T_5. The highest number of days (15.80) was taken by T_2 (IBA 1.00 mg l^{-1}) followed by T_8 (IAA 2.00 mg l^{-1}) recording 15.33 days (Table 8).

The number of roots produced varied significantly. The highest number of roots (2.67) was formed in the treatment T_5 (Plate 2). This was statistically on par with T_{13} (NAA 2.50 mg l^{-1}). The treatments T_5 and T_{13} were superior to all others. Treatments T_8, T_4, T_1, T_6 and T_7 gave the least number of roots (1.00) (Table 8).

With regard to the length of roots, treatment T_{11} (NAA 1.50 mg l^{-1}) showed the highest value of 1.53 cm followed by T_5 (1.30 cm). The mean values varied from 0.87 to 1.53 cm. The least value for length of roots (0.87 cm) was obtained in T_6 (IAA 1.00 mg l^{-1}) (Table 8).

The roots produced with IBA were pure white in colour, comparatively thin and long. Whereas roots produced with NAA and IAA were creamy white in colour, thick and short. Very high callusing was also observed in plantlets kept in different levels of IAA (Plate 3).

In addition to single hormone treatments, combination of plant growth substances gave the following results (Table 9). The number of microshoots initiating roots were the highest (70.00 per cent) in TC_3 (IBA 1.00 mg l^{-1} + NAA 1.00 mg l^{-1}) and the lowest (40.00per cent) in TC_2 (IBA 1.50 mg l^{-1} + NAA 1.00 mg l^{-1}). The number of days for root initiation was the earliest (11.00) in TC_3 and the slowest (13.00) in TC_2. Number of roots produced was also found to be high (3.00) in TC_2 and TC_3 and less (2.00) in TC_1 (IBA 1.00 mg l^{-1} + NAA1.50 mg l^{-1}) and TC_4 (IBA 1.50 mg l^{-1} + NAA 1.50 mg l^{-1}). Length of roots was the highest (1.70 cm) in TC_3 followed by TC_2 (1.40 cm) and the least in TC_1 and TC_4 (1.20 cm).

In half strength MS basal medium, the percentage of cultures showing *in vitro* rooting was the highest (50.00) in T_5 (IBA 2.50 mg l^{-1}) and T_{11} (NAA

Table 9. Effect of combinations of plant growth substances on *in vitro* rooting of *Aegle marmelos*

Treatments	IBA (mg l^{-1})	NAA (mg l^{-1})	Root initiation per cent	Days for root initiation	Number of roots	Length of roots (cm)
TC_1	1.00	1.50	50	12.00	2.00	1.20
TC_2	1.50	1.00	40	13.00	3.00	1.40
TC_3	1.00	1.00	70	11.00	3.00	1.70
TC_4	1.50	1.50	50	12.00	2.00	1.20

The data represents the mean value of ten replications
Culture medium : MS + Inositol (100.00 mg l^{-1}) + Sucrose (30.00 g l^{-1}) + Agar (6.00 g l^{-1})

Table 10. Effect of plant growth substances on *in vitro* rooting of *Aegle marmelos*

Treatments	Root initiation per cent	Days for root initiation	Number of roots	Length of roots (cm)
T_1	30.00	9.00	1.00	0.80
T_2	20.00	8.00	1.00	0.83
T_3	40.00	9.50	1.30	0.97
T_4	30.00	9.00	1.00	1.06
T_5	50.00	11.00	1.00	1.16
T_6	30.00	10.00	1.00	0.90
T_7	10.00	8.00	1.00	0.89
T_8	10.00	10.00	1.00	0.83
T_9	20.00	9.00	1.00	0.80
T_{10}	30.00	12.00	1.30	0.97
T_{11}	50.00	10.60	1.67	1.13
T_{12}	30.00	9.00	1.33	1.10
T_{13}	30.00	9.50	1.67	1.10
Control	_	_	_	_
CD at 5% level	2.16	NS	NS	3.1

The data represents the mean value of ten replications
Culture medium : ½ MS + Inositol (100.00 mg l^{-1}) + Sucrose (30.00 g l^{-1}) + Agar (6.00 g l^{-1})

Plate 2. Different stages of *in vitro* rooting in MS basal medium suppplemented with IBA 2.50 mg l^{-1}

1.50 mg l^{-1}) and the lowest (10.00) with treatments T_7 (IAA 1.50 mg l^{-1}) and T_8 (IAA 2.00 mg l^{-1}) (Table10). Significant difference was observed among the treatments with respect to root initiation percentage (Fig. 2). T_5 was superior to that of others. The number of days taken for root initiation was found to be more (12.00) in T_{10} (NAA 1.00 mg l^{-1}) and less (8.00) in T_2 (IBA 1.00 mg l^{-1}) and T_7 (IAA 1.50 mg l^{-1}) (Table 10).

Regarding the number of roots produced per microshoot, the mean values varied from 1.00 to 1.67. The highest value (1.67) was obtained for T_{11} (NAA 1.50 mg l^{-1}) and T_{13} (NAA 2.50 mg l^{-1}) and the least value (1.00) was obtained for T_1, T_2, T_4 ,T_5, T_6 , T_7, T_8 and T_9 (Table 10).

Observations on length of root were also taken. The mean values for length of roots varied from 0.80 to 1.16 cm. The highest was obtained for T_5 and the lowest for T_1 (IBA 0.50 mg l^{-1}) and T_9 (IAA 2.50 mg l^{-1}). Significant difference was obtained with respect to the length of roots for the treatments. The treatments T_4, T_5, T_{11}, T_{12} and T_{13} were on par with T_5 which recorded the highest value for length of roots (Table 10).

When WPM was used as the basal medium, the percentage of root initiation varied from 10.00 to 60.00 per cent (Table 11). The highest rooting occurred with T_5 and the lowest with T_6. Significant difference was observed among the treatments with respect to root initiation percentage. The average number of days for root initiation was found to vary from 8.00 to 14.00. The longest time was taken by T_6 (14.00 days) and the least (8.00 days) by T_1 (Table 11, Fig. 2).

Regarding the number of roots produced, the mean values ranged from 1.00 to 2.00. The highest was produced by T_8 (IAA 2.00 mg l^{-1}) and T_{12} (NAA2.00 mg l^{-1}) and the lowest by T_6 (IAA 1.00 mg l^{-1}) and T_7 (IAA 1.50 mg l^{-1}) (Table 11).

The mean values for the length of roots produced varied from 0.80 to 2.27 cm. The longest root was obtained with T_{13} (NAA 2.50 mg l^{-1}) and the

Table 11. Effect of plant growth substances on *in vitro* rooting of *Aegle marmelos*

Treatments	Root initiation per cent	Days for root initiation	Number of roots	Length of roots (cm)
T_1	20.00	8.00	1.30	0.80
T_2	20.00	9.00	1.67	1.23
T_3	20.00	12.00	1.67	1.33
T_4	40.00	12.00	1.30	1.03
T_5	60.00	13.00	1.30	1.27
T_6	10.00	14.00	1.00	0.80
T_7	20.00	10.50	1.00	1.43
T_8	30.00	11.50	2.00	1.70
T_9	40.00	12.00	1.67	1.70
T_{10}	40.00	13.00	1.67	1.23
T_{11}	50.00	13.50	1.67	1.60
T_{12}	40.00	12.00	2.00	1.70
T_{13}	40.00	12.00	1.33	2.27
Control	–	–	–	–
CD at 5% level	2.20	NS	NS	8.41

The data represents the mean value of ten replications
Culture medium : WPM + Inositol (100.00mg l^{-1}) + Sucrose (30.00g l^{-1}) + Agar (6.00g l^{-1})

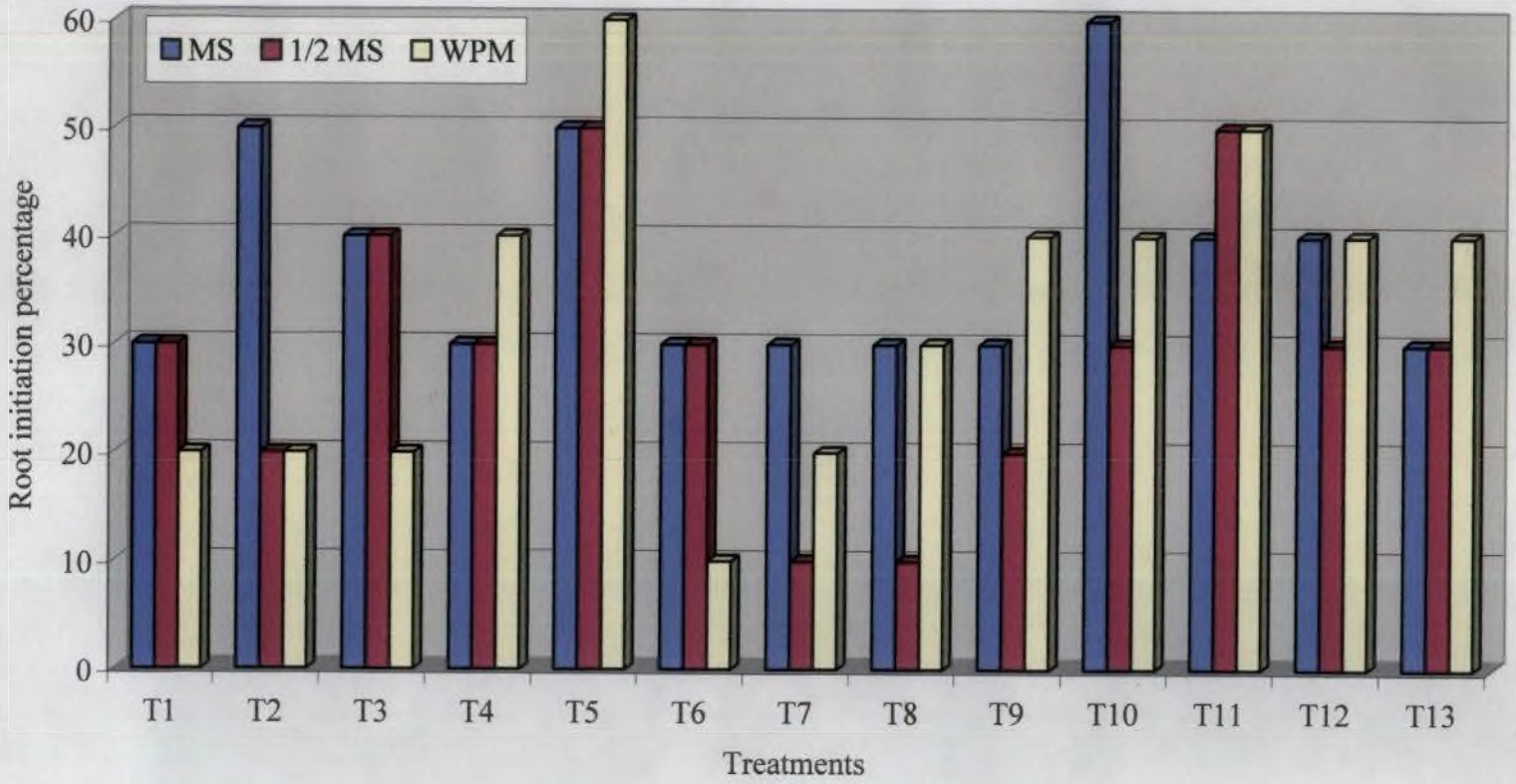


Fig. 2. Effect of plant growth substances and basal media on *in vitro* root initiatiion in *Aegle marmelos*

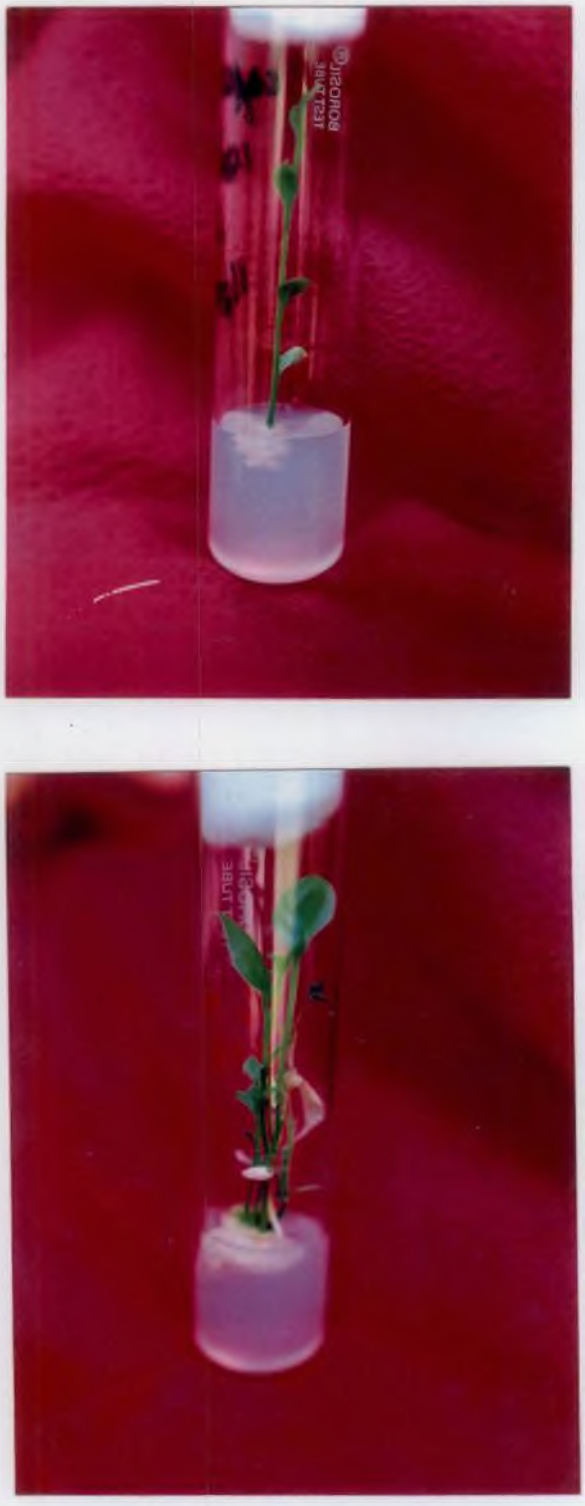


Plate 3. Callusing in MS medium supplemented with IAA 2.00 mg l^{-1}

shortest with T_6 (IAA 1.00 mg l^{-1}). Significant difference was obtained among the treatments with respect to length of roots. Treatment T_{13} was significantly different from the treatments T_8, T_9 and T_{12} which were on par (Table 11).

4.1.1.2 Basal Media

Among the different basal media tried, the percentage cultures showing *in vitro* rooting was the highest (60.00) in WPM, whereas only 50.00 per cent of the microshoots initiated roots in MS, both full and half strength. Statistical significance was observed between the treatments with respect to root initiation percentage (Table 12). The number of days taken for root initiation varied significantly. The earliest root initiation of 10.00 days was obtained in B_1 (MS) and delayed root initiation (13.00 days) in B_3 (WPM) (Table 12). The highest number of roots per shoot (2.67) was produced in B_1 (MS) and was statistically superior to the least (1.00) produced in B_2 (half MS) (Table 12). The average length of root was the highest in B_1 (1.30 cm). Half strength MS and WPM produced roots with length of 1.16 and 1.27 cm respectively (Table 12).

From the results obtained, IBA 2.50 mg l^{-1} was standardised as the best plant growth substance and MS as the best basal medium for inducing *in vitro* rooting. Hence further studies were conducted in this medium.

4.1.1.3 Effect of Sucrose

The microshoots were cultured in MS basal medium supplemented with IBA 2.50 mg l^{-1} and sucrose was tried at four levels (10.00, 20.00, 30.00 and 40.00 g l^{-1}) to asses its effect on *in vitro* rooting.

The percentage of root initiation was the highest (50.00) in S_3 (30.00 g l^{-1}) which was superior to the least (20.00) obtained in S_1 (10.00 g l^{-1}). The average number of days taken for root initiation was the least (10.00) in S_3 and the highest (13.00) in S_2 (20.00 g l^{-1}) (Table 13).

S_3 recorded the highest number of roots produced per microshoot (2.67) and S_1 recorded the least number of roots produced per microshoot (1.00), when the mean values were taken into consideration (Table 13).

Table 12. Effect of basal media on *in vitro* rooting of *Aegle marmelos*

Treatments	Media	Root initiation per cent	Days for root initiation	Number of roots	Length of roots (cm)
B_1	MS	50.00	10.00	2.67	1.30
B_2	1/2 MS	50.00	11.00	1.00	1.16
B_3	WPM	60.00	13.00	1.30	1.27
CD at 5% level		4.86	NS	4.40	NS

The data represents the mean value of ten replications

Culture medium : Inositol (100.00mg l^{-1}) + Sucrose (30.00 g l^{-1}) + Agar (6.00 g l^{-1}) + IBA (2.50 mg l^{-1})

Table 13. Effect of Sucrose on *in vitro* rooting of *Aegle marmelos*

Treatment	Concentrations ($g\ l^{-1}$)	IBA 2.50 mg l^{-1}				NAA 1.00 mg l^{-1}			
		Root initiation per cent	Days for root initiation	Number of roots	Length of roots (cm)	Root initiation per cent	Days for root initiation	Number of roots	Length of roots (cm)
S_1	10.00	20.00	12.00	1.00	0.93	25.00	17.00	1.00	0.70
S_2	20.00	40.00	13.00	1.67	1.57	25.00	14.00	1.30	0.97
S_3	30.00	50.00	10.00	2.67	1.30	50.00	11.00	1.30	1.40
S_4	40.00	30.00	10.00	1.67	1.43	50.00	12.00	1.00	1.23
CD at 5% level		3.67	NS	NS	NS	NS	NS	NS	6.40

The data represents the mean value of ten replications

Culture medium : MS + Inositol (100.00mg l^{-1}) + Agar (6.00 g l^{-1})

The length of roots was the highest (1.57 cm) for S_2 (20.00 g l^{-1}) and the lowest (0.93 cm) for S_1 (10.00g l^{-1}) (Table 13).

The same levels of sucrose were also tried in MS media supplemented with NAA 1.00 mg l^{-1} to study its effect on the *in vitro* rooting of microshoots. In this case, the highest root initiation percentage (50.00) was obtained in S_3 (30.00 g l^{-1}) and S_4 (40.00 g l^{-1}) and the lowest (25.00) in S_1 (10.00 g l^{-1}) and S_2 (20.00 g l^{-1}) (Table 13).

Treatment S_3 showed the least number of days (11.00) and S_1 showed the highest number of days (17.00) for root initiation (Table 13).

The highest number of roots (1.30) was produced by S_2 and S_3 and the lowest (1.00) by S_1 and S_4 (Table 13).

There was significant difference among the treatments with respect to the length of roots produced. The longest root (1.40 cm) was obtained in S_3 and the shortest (0.70 cm) in S_1. The values for treatments S_3 and S_4 (1.40 and 1.23 cm respectively) were found to be on par but were superior to that of S_1 and S_2 (0.70 and 0.97 cm respectively) (Table 13).

4.1.1.4 Gelling Agent : Agar

Agar was tried at five different levels (4.00, 5.00, 6.00, 7.00 and 8.00 g l^{-1}) to assess its effect on *in vitro* rooting (Table 14). The basal media used was MS supplemented with IBA 2.50 mg l^{-1}.

The highest percentage of root initiation (50.00) was obtained in AG_2 (5.00 g l^{-1}) and AG_3 (6.00 g l^{-1}), whereas the lowest (25.00) was recorded by AG_1, AG_4 and AG_5.

The treatment AG_1 (4.00 g l^{-1}), recorded the least number of days (9.00) taken for root initiation and AG_5 (8.00 g l^{-1}) the highest (17.00).

With regard to the number of roots produced per microshoot it was observed that AG_3 produced an average of 1.67 roots. But AG_1, AG_4 and AG_5 produced only one root. Significant difference was observed among the treatments with respect to the number of roots.

Table 14. Effect of agar on *in vitro* rooting of *Aegle marmelos*

Treatments	Concentrations (g l^{-1})	IBA 2.50 mg l^{-1}				NAA 1.00 mg l^{-1}			
		Root initiation per cent	Days for root initiation	Number of roots	Length of roots (cm)	Root initiation per cent	Days for root initiation	Number of roots	Length of roots (cm)
AG_1	4.00	25.00	9.00	1.00	1.07	25.00	10.00	1.00	1.40
AG_2	5.00	50.00	9.50	1.33	1.13	50.00	10.50	1.33	1.03
AG_3	6.00	50.00	11.00	1.67	1.20	50.00	11.00	1.33	1.33
AG_4	7.00	25.00	14.00	1.00	0.73	25.00	15.00	1.67	1.33
AG_5	8.00	25.00	17.00	1.00	0.93	25.00	16.00	1.00	1.20
CD at 5% level		NS	NS	1.97	7.00	NS	NS	NS	NS

The data represents the mean value of four replications
Culture medium : MS + Inositol (100.00mg l^{-1}) + Sucrose (30.00 g l^{-1})

The length of roots was found to be the highest (1.20 cm) for the treatment AG_3 and the lowest (0.73 cm) for AG_4. There was statistical significance among the treatments with respect to the length of roots. AG_3 was found to be significantly different from rest of the treatments. But AG_3 and AG_2 were found to be on par (Table 14).

The effect of same levels of agar was also studied in MS basal medium supplemented with NAA 1.00 mg l^{-1} which gave the following results. The root initiation percentage was the highest (50.00) in AG_2 and AG_3. But AG_1, AG_4 and AG_5 showed only 25.00 per cent root initiation. Days for root initiation was the least (10.00) in AG_1 and the highest (16.00) in AG_5 (Table 14).

AG_4 showed the highest value (1.67) for the number of roots produced and AG_1 and AG_5 showed the least (1.00). The average length of root was the highest (1.40 cm) for AG_1 followed by AG_3 and AG_4 (1.33 cm).

4.1.1.5 Activated Charcoal

Charcoal was tried at six different levels (0.50, 1.00, 2.00, 3.00, 4.00, 5.00g l^{-1}) to assess its effect on *in vitro* rooting (Table 15). The basal media used was MS supplemented with IBA 2.50 mg l^{-1}.

Treatment AC_2 (1.00g l^{-1}) recorded the highest percentage of root initiation (60.00) and AC_5 and AC_6 recorded the least value (20.00). Number of days taken for root initiation was found to be less (11.50) in AC_2 and the highest (14.00) in AC_6 (Fig. 3).

The microshoots in AC_2 produced the highest number of roots (2.00) followed by 1.33 in AC_3 (2.00 g l^{-1}), AC_5 (4.00 g l^{-1}) and AC_6 (5.00 g l^{-1}) and AC_1 (0.50 g l^{-1}) and AC_4 (3.00 g l^{-1}) showed the least number of roots (1.00).

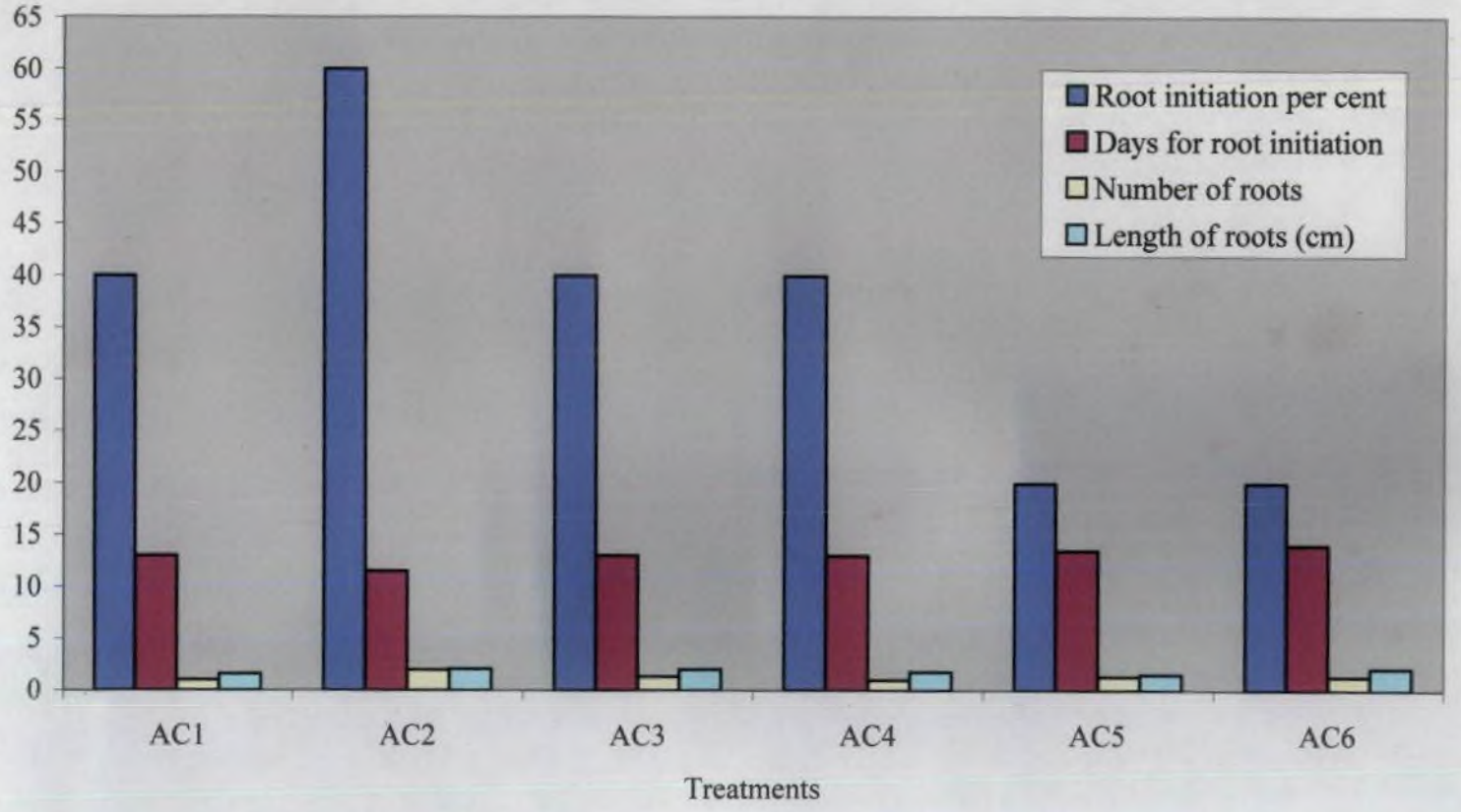
With regard to the length of roots, AC_6 (5.00 g l^{-1}) showed the highest value (2.13 cm) and AC_5 (4.00 g l^{-1}) showed the least (1.56 cm). There was significant difference between the treatments. AC_6 was superior to all other treatments expect AC_2, AC_3, and AC_4 which were on par.

Table 15. Effect of activated charcoal on *in vitro* rooting of *Aegle marmelos*

Treatments	Concentrations ($g\ l^{-1}$)	IBA 2.50 mg l^{-1}				NAA 1.00 mg l^{-1}			
		Root initiation per cent	Days for root initiation	Number of roots	Length of roots (cm)	Root initiation per cent	Days for root initiation	Number of roots	Length of roots (cm)
AC_1	0.50	40.00	13.00	1.00	1.60	20.00	12.00	1.00	1.33
AC_2	1.00	60.00	11.50	2.00	2.10	60.00	12.50	2.00	1.90
AC_3	2.00	40.00	13.00	1.33	2.00	20.00	12.00	1.33	1.70
AC_4	3.00	40.00	13.00	1.00	1.80	60.00	11.50	1.00	1.96
AC_5	4.00	20.00	13.50	1.33	1.56	20.00	11.50	1.33	1.76
AC_6	5.00	20.00	14.00	1.33	2.13	20.00	12.00	1.30	1.56
CD at 5%level		NS	NS	NS	4.68	2.64	NS	NS	4.33

The data represents the mean value of five replications

Culture medium : MS + Inositol ($100.00mg\ l^{-1}$) + Sucrose ($30.00g\ l^{-1}$)+ Agar ($6.00\ g\ l^{-1}$)



AC_1	0.50 g l^{-1}	AC_4	3.00 g l^{-1}
AC_2	1.00 g l^{-1}	AC_5	4.00 g l^{-1}
AC_3	2.00 g l^{-1}	AC_6	5.00 g l^{-1}

Fig. 3. Effect of activated charcoal on *in vitro* rooting in *Aegle marmelos*

The effect of charcoal on MS basal media supplemented with NAA 1.00 mg l^{-1} was also assessed. Root initiation percentage was the highest (60.00) in AC_2 and AC_4 which were statistically superior to the least value (20.00) in AC_1, AC_3, AC_5 and AC_6. The average number of days for root initiation was the least (11.50) in AC_4 and AC_5 and the highest (12.50) in AC_2 (Table 15).

The highest number of roots (2.00) was produced by AC_2 and the least (1.00) by AC_1 and AC_4. AC_4 showed the highest root length (1.96 cm) which is on par with AC_2 and AC_5 but superior to AC_1, AC_3 and AC_6.

4.1.1.6 Mode of Culture

The percentage initiation of roots in solid medium was 50.00 and that in liquid medium was 40.00. The number of days taken for root initiation was found to be one day less (11.00 days) in liquid medium when compared to that in solid medium (12.00). The number of roots was found to be more (2.67) in solid medium compared to that in liquid medium (1.00). The root length was found to be more (1.44 cm) for plantlets cultured in liquid medium compared to that in solid medium (1.30cm). But no significant difference could be observed with respect to number of roots and length of roots between these two modes of culture (Table 16).

4.1.1.7 Culture Conditions

The plantlets maintained in darkness for one week and then transferred to light when root initials were observed gave better rooting percentage (50.00). But the culture vessels covered with aluminium foil at the basal portion and kept in light did not show any rooting.

4.1.2 *Ex vitro* Rooting

4.1.2.1 Quick Dip Method

Healthy unrooted microcuttings of 3.00 to 5.00 cm length were pre-treated with IBA (500.00, 1000.00, 1500.00 mg l^{-1}) before planting out and observations on *ex vitro* rooting were recorded after fifteen and thirty days.

Table 16. Effect of mode of culture on *in vitro* rooting of *Aegle marmelos*

Treatments	Concentrations (g l^{-1})	Root initiation per cent	Days for root initiation	Number of roots	Length of roots (cm)
Solid (Agar)	6.00	50.00	10.00	2.67	1.30
Liquid (Without agar)	0.00	40.00	11.00	1.00	1.44
CD at 5% level	-	NS	NS	NS	NS

The data represents the mean value of ten replications
Culture medium : MS + Inositol (100.00 mg l^{-1}) + Sucrose (30.00 g l^{-1}) + IBA (2.50 mg l^{-1})

Among the pre-treatments given with IBA for *ex vitro* rooting, cent per cent survival was obtained in ERQ_1 (IBA 500.00 mg l^{-1}) and ERQ_2 (IBA 1000.00 mg l^{-1}) and the least (66.67) in ERQ_3 (IBA 1500.00 mg l^{-1}), fifteen days after planting. The survival percentage thirty days after planting was the highest (66.67) in ERQ_2 (Plate 4) and the least (16.67) in ERQ_3 (Table 17, Fig. 4).

Data on the number of roots and length of roots thirty days after planting was also observed but no significant difference could be observed between the treatments. However the mean values showed that the highest number of roots (1.67) and length of roots (0.63cm) was recorded by ERQ_2 and the least number of roots (1.33) was recorded by ERQ_1 and ERQ_3 and the shortest root (0.43 cm) by ERQ_1, thirty days after planting (Table 17, Fig. 5).

4.1.2.2 Slow Dip Method

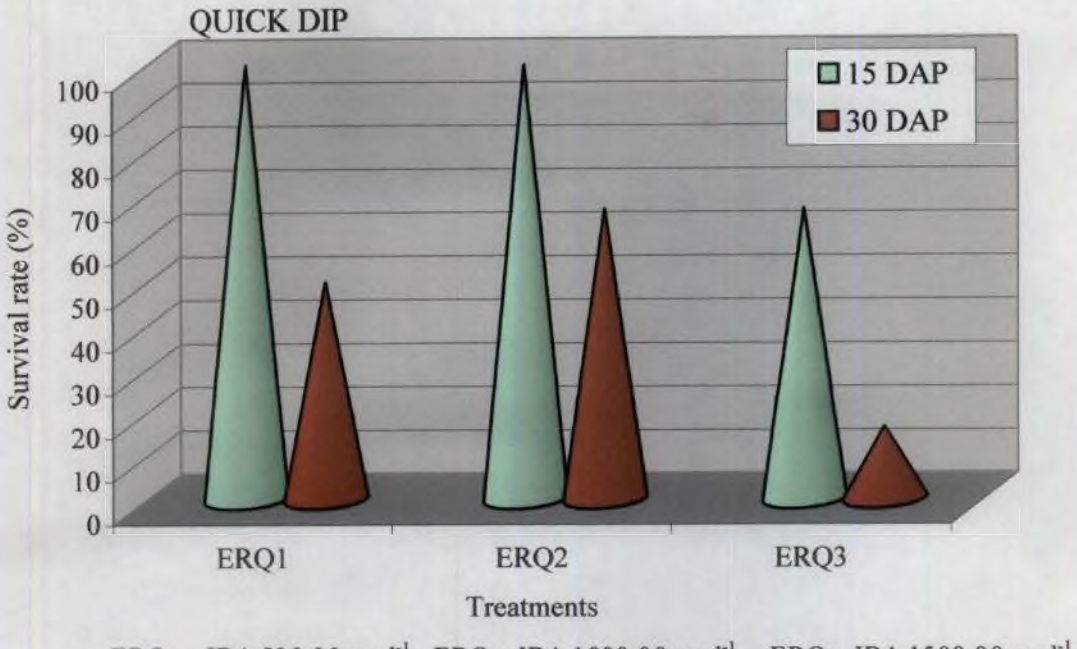
Among the pre-treatments given with IBA (50.00, 100.00, 150.00 mg l^{-1}) to the unrooted microshoots , the highest survival rate (66.67 per cent) was obtained in ERS_1 (50.00 mg l^{-1}) and ERS_2 (100.00 mg l^{-1}) and 50.00 per cent in ERS_3 (150.00 mg l^{-1}) fifteen days after planting. Thirty days after planting, the highest survival rate (33.30 per cent) was obtained in ERS_1 whereas the other two treatments showed a survival rate of 16.67 per cent (Table 18, Fig. 4).

The average number of roots produced was 1.00 for all the treatments and the length of roots varied from 0.17 to 0.23 cm, the highest being 0.23 cm in ERS_2 and the lowest 0.17 cm in ERS_1 (Table 18, Fig. 5).

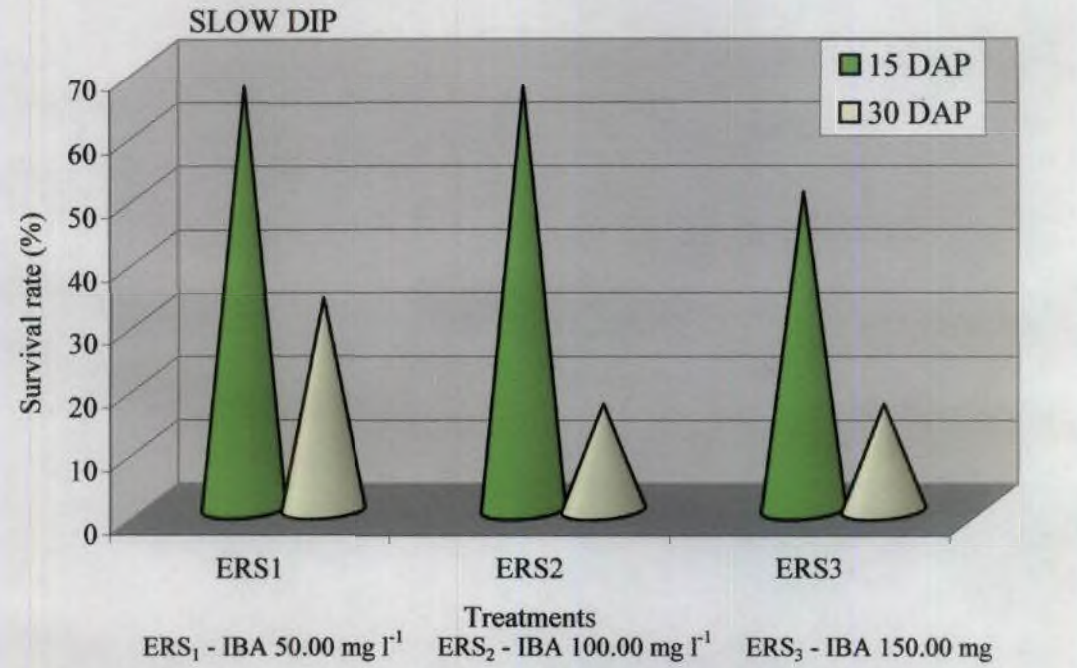
4.2 *EX VITRO* ESTABLISHMENT

4.2.1 Potting Media

The *in vitro* rooted plantlets were carefully removed from the culture vessels and planted out in different potting media (sand, soilrite, sand and soil (1:1), sand, soil and coirpith (1:1:1) and sand, soil and leaf mould (1:1:1) to study their effect on *ex vitro* establishment (Plates 5 and 6). The plantlets so obtained were kept for acclimatization in a humidity chamber with polythene sheets of



ERQ_1- IBA 500.00 mg l^{-1} ERQ_2- IBA 1000.00 mg l^{-1} ERQ_3- IBA 1500.00 mg l^{-1}

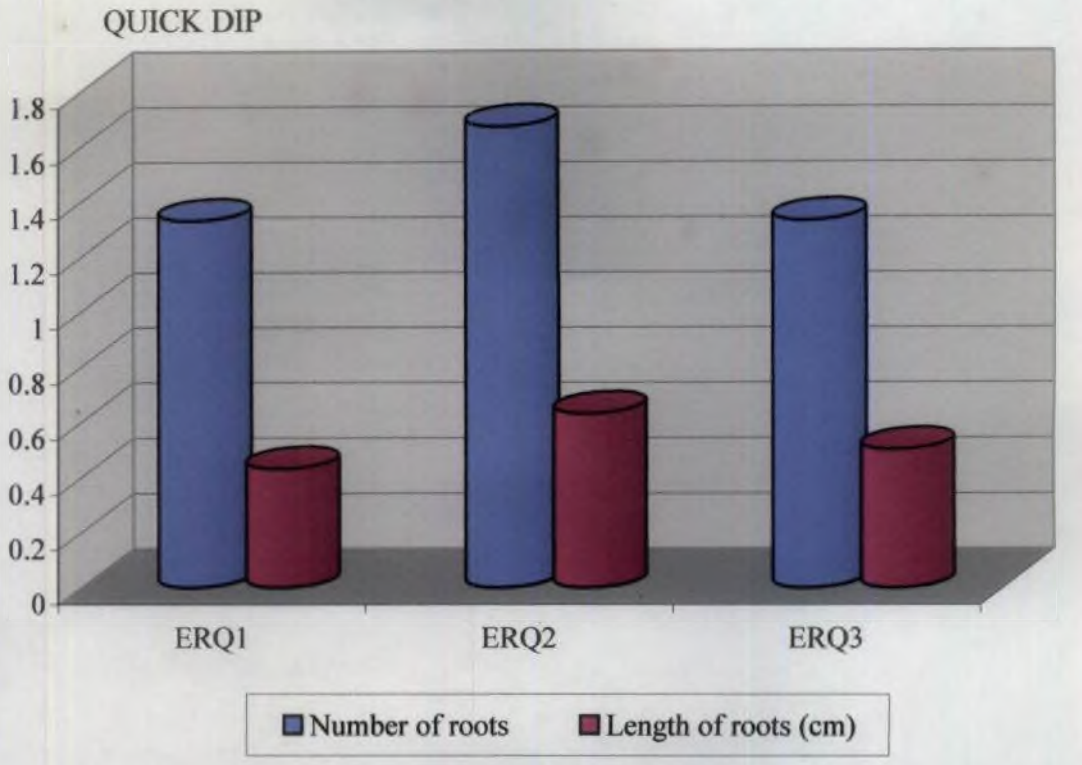


ERS_1 - IBA 50.00 mg l^{-1} ERS_2 - IBA 100.00 mg l^{-1} ERS_3 - IBA 150.00 mg

DAP - Days after planting

Fig. 4. Effect of IBA on *ex vitro* rooting of *Aegle marmelos*

Plate 4. Survival of microshoot after pre-treatment with IBA 1000.00 mg l^{-1}



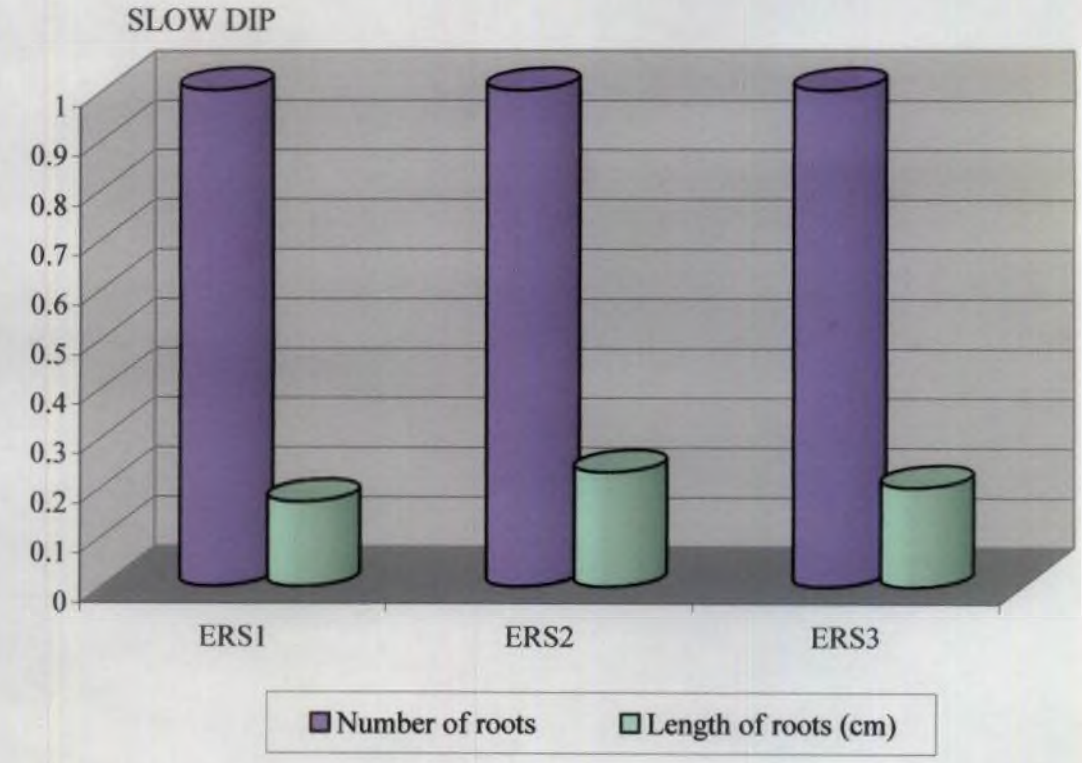


Fig. 5. Effect of IBA on *ex vitro* rooting of *Aegle marmelos*

Plate 5. Plantlets of *Aegle marmelos* in different potting media

Table 17. Effect of *ex vitro* rooting on survival rate, number of roots and length of roots of microshoots of *Aegle marmelos*

Treatments	IBA (mg l^{-1})	Duration (s)	15DAP	30DAP	Number of roots	Length of roots (cm)
ERQ_1	500.00	20	100.00	50.00	1.33	0.43
ERQ_2	1000.00	20	100.00	66.67	1.67	0.63
ERQ_3	1500.00	20	66.67	16.67	1.33	0.50
CD at 5% level					NS	NS

The data represents the mean value of six replications
Potting medium : Sterile Sand

Table 18. Effect of *ex vitro* rooting on survival rate, number of roots and length of roots of microshoots of *Aegle marmelos*

Treatments	IBA (mg l^{-1})	Duration (h)	15DAP	30DAP	Number of roots	Length of roots (cm)
ERS_1	50.00	12	66.67	33.33	1.00	0.17
ERS_2	100.00	12	66.67	16.67	1.00	0.23
ERS_3	150.00	12	50.00	16.67	1.00	0.20
CD at 5% level					NS	NS

The data represents the mean value of six replications
Potting medium : Sterile Sand

Plate 6. *In vitro* rooted microcutting of *Aegle marmelos* before planting out

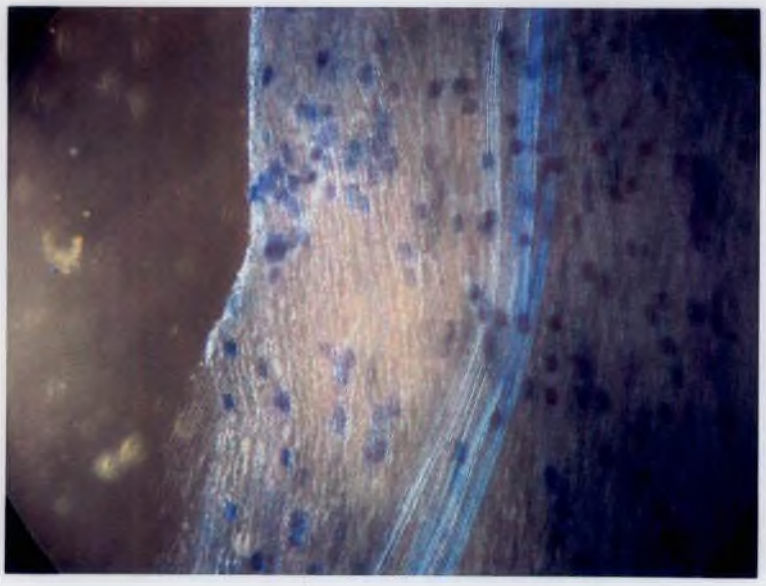


Plate 7. Roots of *Aegle marmelos* showing vesicles of AMF

350 guage thickness for a period of thirty days at a RH of 75.00 per cent and temperature of 35° C (Plate 9).

4.2.1.1 Survival Rate

Survival rate of plants was estimated at fortnightly intervals for one month (Table 19).

Among the different potting media tried for *ex vitro* establishment, sand was found to be the best which recorded the highest survival rate of 83.33 per cent, fifteen days after planting followed by 66.67 per cent in soilrite and sand : soil (1:1) and 50.00 per cent in sand, soil and coirpith (1:1:1) and sand, soil and leaf mould (1:1:1). But one month after planting, 50.00 per cent of the plantlets survived in potting media containing sand and soilrite whereas in all others only 16.67 per cent survived (Fig. 6). The survived plantlets were planted in pots filled with potting mixture containing sand, soil and cowdung in the ratio 1:1:1 (Plate 10).

4.2.1.2 Biometric Observations

4.2.1.2.1 Leaf Number

Plantlets grown in P_1 (sand) and P_5 (sand, soil and leaf mould, 1:1:1) produced the highest number of leaves (3.67), fifteen days after planting and plantlets in P_1 recorded the highest number of leaves (4.67), thirty days after planting. The least number of leaves was recorded by plantlets in P_2 (soilrite), both fifteen (2.67) and thirty days after planting (3.00) (Table 19. Fig. 7).

4.2.1.2.2 Height of Plant

Plant height was the highest (3.50 cm) for plantlets in P_5 followed by that in P_1 (3.47 cm) and the least in P_4 (2.10 cm), fifteen days after planting. Thirty days after planting, the plant height was observed the highest (3.67 cm) for plantlets in P_1 followed by P_5 (3.63cm) and the least (2.83 cm) for plantlets in P_3 (Table 19, Fig. 7).

Table 19. Effect of potting media on the survival rate, leaf number, plant height and number of roots of plantlets of *Aegle marmelos*

Treatments	Media	Survival rate		Leaf Number		Plant Height (cm)		Number of roots	
		15DAP	30DAP	15DAP	30DAP	15DAP	30DAP	15DAP	30DAP
P_1	Sand	83.33	50.00	3.67	4.67	3.47	3.67	1.33	2.00
P_2	Soilrite	66.67	50.00	2.67	3.00	3.03	3.27	1.33	1.67
P_3	Sand:soil(1:1)	66.67	16.67	3.33	4.00	2.70	2.83	1.00	1.33
P_4	Sand:soil:coirpith (1:1:1)	50.00	16.67	3.33	4.34	2.10	3.23	1.33	2.00
P_5	Sand:soil:leaf mould(1:1:1)	50.00	16.67	3.67	3.33	3.50	3.63	1.33	2.67
CD at 5% level				NS	NS	NS	NS	NS	NS

The data represents the mean value of six replications

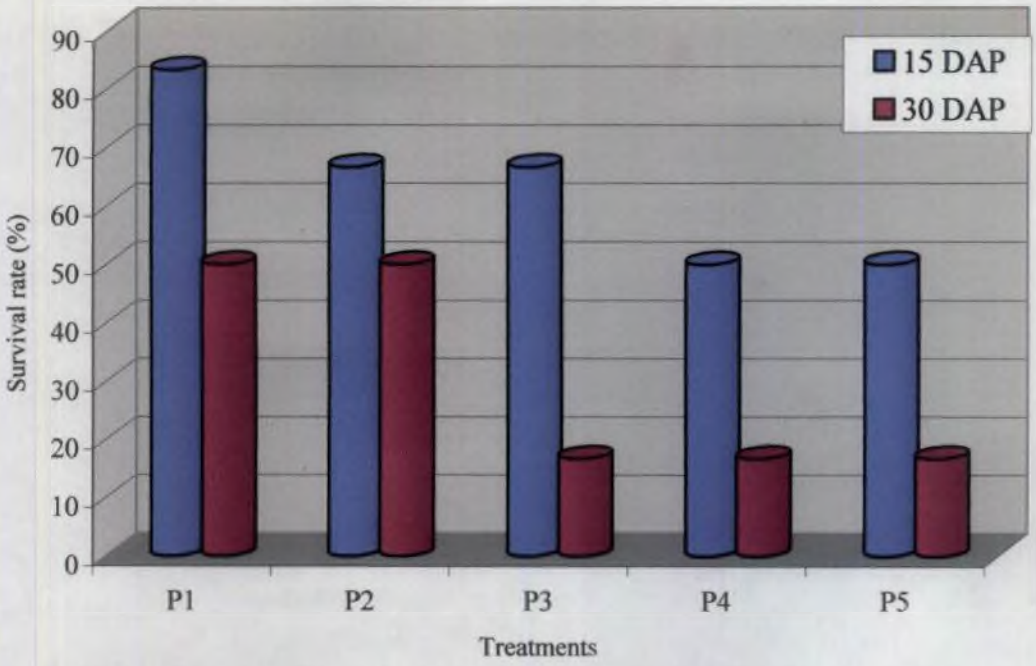


Fig. 6. Effect of potting media on *ex vitro* survival of plantlets of *Aegle marmelos*

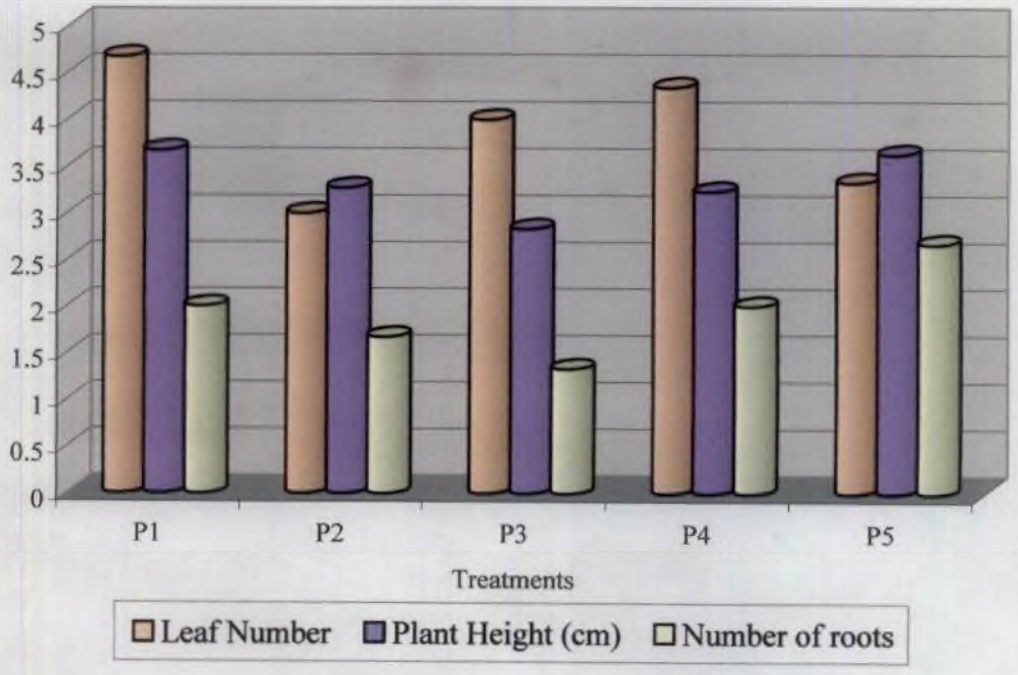


Fig. 7. Effect of potting media on the *ex vitro* establishment of *Aegle marmelos*

P_1 Sand
P_2 Soilrite
P_3 Sand:Soil(1:1)
P_4 Sand:Soil:Coirpith (1:1:1)
P_5 Sand:Soil:Leaf mould(1:1:1)

Plate 9. Plantlets of *Aegle marmelos* kept for acclimatization

Plate 10. Bael plantlets established in pots, 90 DAP

4.2.1.2.3 Number of Roots

Fifteen days after planting, the highest number of roots observed was 1.33 for all the treatments except P_3 In P_3 it was only one (Table 19). The number of roots was found to be the highest (2.67) for plantlets in P_5 followed by P_1 and P_4 (2.00) and P_2 (1.67), thirty days after planting (Table 19, Fig. 7).

4.2.1.2.4 Fresh Weight

The highest fresh weight (0.2391 g) was recorded by plantlets grown in P_1 and the lowest (0.1701 g) for plantlets in P_3, fifteen days after planting. But thirty days after planting, there was significant difference in fresh weight. The highest fresh weight (0.6501 g) was observed for plantlets in P_1 and the least (0.4751 g) for plantlets in P_3. P_1 and P_5 were superior to the treatments P_2, P_3 and P_4 which were on par (Table 20).

4.2.1.2.5 Dry Weight

P_1 (sand) was statistically superior to all other potting media with respect to dry weight. The highest dry weight of 0.0773 g was recorded by the plantlets grown in sand. Whereas the plantlets in soilrite recorded the lowest value of 0.0590 g, fifteen days after planting. Thirty days after planting, plantlets in sand recorded the highest dry weight (0.2500 g) followed by those grown in sand : soil : leaf mould (0.2081 g) and least in sand : soil (0.1583 g) (Table 20).

4.2.1.3 Physiological Observations

4.2.1.3.1 Stomatal Conductance (SC)

Stomatal conductance was recorded at fifteen and thirty days after planting. SC was found to be the highest (0.7965 cm s^{-1}) for plantlets in P_1 (sand) and the least (0.4511 cm s^{-1}) for plantlets in P_4 (sand:soil:coirpith), fifteen days after planting. There was significant difference with respect to the different potting media tried fifteen days after planting. SC was the highest (0.1165 cm s^{-1}) for plantlets in P_1 and the lowest for plantlets in P_3 (0.0827 cm s^{-1}), thirty days after planting. Treatments P_1 and P_2 were found to be superior to others and treatments P_3, P_4 and P_5 were on par (Table 20).

Table 20. Effect of potting media on fresh weight, dry weight, stomatal conductance leaf area index and crop growth rate of plantlets of *Aegle marmelos*

Treatments	Media	Fresh Weight (g plant^{-1})		Dry Weight (g plant^{-1})		Stomatal conductance (cm S^{-1})		LAI	CGR (mg cm^{-2} day^{-1})
		15DAP	30DAP	15DAP	30DAP	15DAP	30DAP	30DAP	30DAP
P_1	Sand	0.2391	0.6501	0.0773	0.2500	0.7965	0.1165	0.1370	0.0291
P_2	Soilrite	0.1785	0.5454	0.059	0.1809	0.5624	0.0867	0.084	0.0216
P_3	Sand:soil(1:1)	0.1701	0.4751	0.0596	0.1583	0.4555	0.0827	0.1202	0.0207
P_4	Sand:soil:coirpith(1:1:1)	0.2012	0.4807	0.0625	0.1602	0.4511	0.0990	0.1276	0.0248
P_5	Sand:soil:leafmould(1:1:1)	0.2174	0.6238	0.0675	0.2081	0.6492	0.1070	0.0923	0.0267
CD at 5% level		NS	4.17	NS	3.52	3.83	NS	NS	NS

The data represents the mean value of six replications

4.2.1.3.2 Leaf Area Index (LAI)

The maximum LAI (0.1370) was recorded by plantlets in P_1 followed by that in P_4 (0.1276) and the least LAI (0.084) was recorded by plantlets in P_2, thirty days after planting (Table 20).

4.2.1.3.3 Crop Growth Rate (CGR)

The highest CGR (0.0291 mg cm^{-2} day^{-1}) was observed for the rooted microshoots planted out in P_1 followed by P_5 (0.0267 mg cm^{-2} day^{-1}) and P_4 (0.0248 mg cm^{-2} day^{-1}). The least CGR (0.0207 mg cm^{-2} day^{-1}) was observed for plantlets in P_3 (Table 20).

4.2.2. Mycorrhiza

4.2.2.1 Mycorrhizal Colonization

Mycorrhizal colonization was found to be the highest (94.40 %) in P_1 (Plate 7) followed by P_2 (93.60 %), P_3 (90.00 %), P_4 (80.00 %) and P_5 (75.00 %) (Table 21, Fig. 8).

4.2.2.2 Survival Rate

Mycorrhiza inoculated plantlets in P_1 and P_4 recorded the highest survival rate (83.33) fifteen days after planting. One month after planting, P_1 recorded the highest survival rate (66.67) (Table 21, Fig. 8).

4.2.2.3 Biometric Observations

4.2.2.3.1 Leaf Number

Significant difference in the leaf number of mycorrhiza treated plantlets was observed with respect to the different potting media tried. Among the different media tried, plantlets in P_1 (sand) recorded the highest (4.00 and 6.00) (Plate 8) and plantlets in P_2 (soilrite) recorded the least number of leaves (3.00 and 3.33) at fifteen and thirty days after planting respectively. Plantlets in P_1 was found to be superior to that in other treatments with respect to leaf number, thirty days after planting and all other treatments were on par (Table 22).

Table 21. Mycorrhizal colonization and survival per cent of *Aegle marmelos* plants in different potting medium

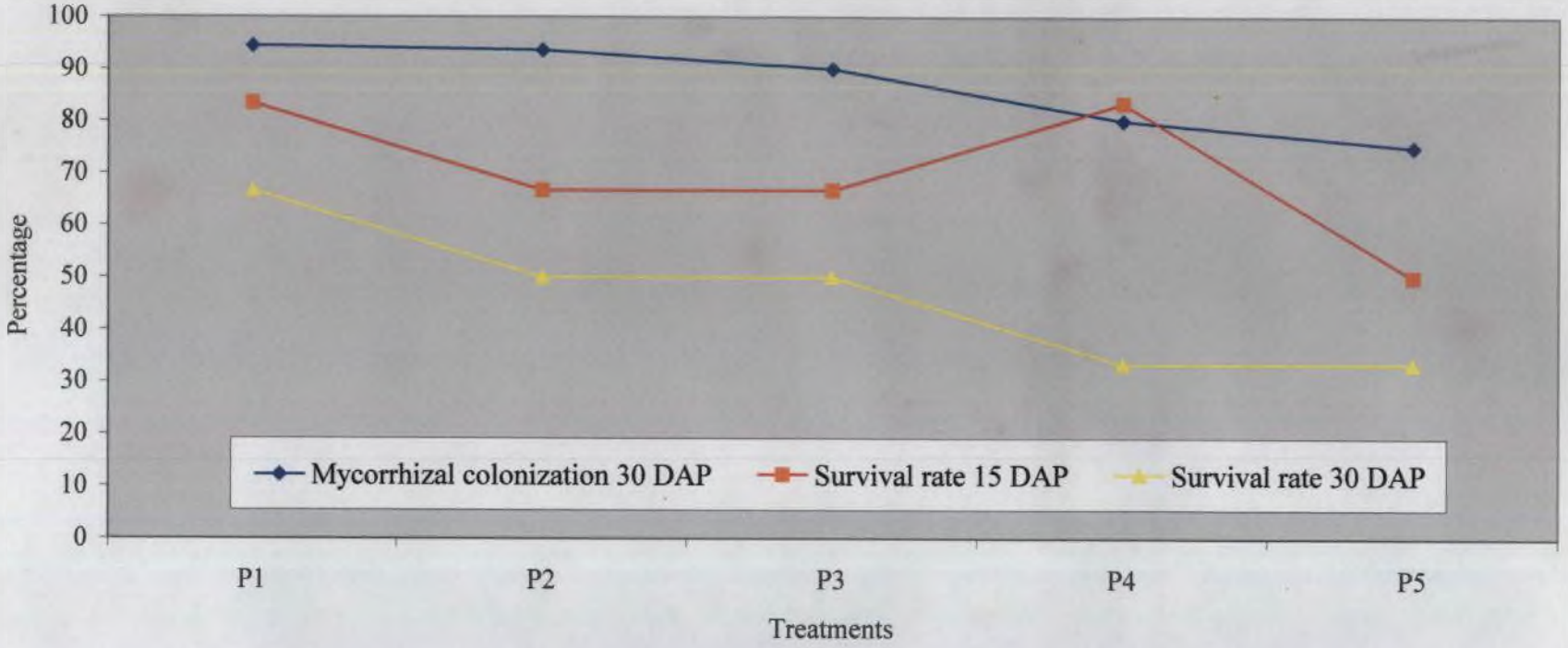
Treatments	Media	Mycorrhizal colonization 30 DAP (%)	15DAP (%)	30DAP (%)
P_1	Sand	94.40	83.33	66.67
P_2	Soilrite	93.60	66.67	50.00
P_3	Sand:soil(1:1)	90.00	66.67	50.00
P_4	Sand:soil:coirpith(1:1:1)	80.00	83.33	33.33
P_5	Sand:soil:leafmould(1:1:1)	75.00	50.00	33.33

The data represents the mean value of six replications

Table 22. Effect of mycorrhiza on the number of leaves, plant height and number of roots of *Aegle marmelos* in different potting media

Treatments	Media	Leaf Number		Plant Height (cm)		Number of roots	
		15DAP	30DAP	15DAP	30DAP	15DAP	30DAP
P_1	Sand	4.00	6.00	3.90	4.30	1.67	2.33
P_2	Soilrite	3.00	3.33	3.467	3.70	1.67	2.33
P_3	Sand:soil(1:1)	3.33	4.33	3.133	3.43	1.33	2.00
P_4	Sand:soil:coirpith(1:1:1)	3.33	4.33	3.533	3.70	1.33	2.00
P_5	Sand:soil:leafmould(1:1:1)	3.33	4.67	3.20	3.50	1.33	2.00
CD at 5% level		NS	NS	NS	NS	NS	NS

The data represents the mean value of six replications



P_1 Sand
P_2 Soilrite
P_3 Sand:Soil(1:1)
P_4 Sand:Soil:Coirpith (1:1:1)
P_5 Sand:Soil:Leaf mould(1:1:1)

Fig. 8. Effect of mycorrhiza on *ex vitro* establishment of *Aegle marmelos*

Plate 8. Growth of plantlets in sand treated and untreated with mycorrhiza

4.2.2.3.2 Plant Height

The mean values for plant height showed that plantlets in P_1 recorded the highest (3.90 and 4.30 cm) and that in P_3 recorded the lowest (3.13 and 3.43 cm) height fifteen and thirty days after planting, respectively (Table 22).

4.2.2.3.3 Number of Roots

The highest value (1.67 and 2.33) was recorded by plantlets in P_1 and P_2 and the lowest value (1.33 and 2.00) by plantlets in P_3, P_4 and P_5 both fifteen and thirty days after planting, respectively (Table 22).

4.2.2.3.4 Fresh Weight

Significant difference was observed regarding fresh weight for the different treatments tried thirty days after planting. P_1 was found to be superior to all other treatments except P_5 which was on par with P_1. Plantlets in P_1 recorded the highest (0.2583 g) and that in P_3 recorded the least (0.1812 g), fifteen days after planting (Table 23). After one month, plantlets in P_1 recorded the highest value (0.6908 g) followed by that in P_5 (0.6368 g) and P_4 recorded the lowest value (0.5018 g) (Table 23).

4.2.2.3.5 Dry Weight

Significant difference was observed with respect to dry weight for the various potting media tried, thirty days after planting. Plantlets in P_1 recorded the highest value (0.2847 g) and P4 recorded the lowest value (0.1771 g), thirty days after planting. P_1 recorded the highest value (0.0930 g) and P_4 recorded the lowest value (0.0668 g), fifteen days after planting. P_1 and P_5 were superior to other treatments (Table 23).

4.2.2.4 Physiological Observations

4.2.2.4.1 Stomatal Conductance

The highest value (0.1235 cm S^{-1}) for SC was observed for plantlets in P_1 and the lowest (0.0908 cm S^{-1}) in P_3, fifteen days after planting. The highest value for SC was for plantlets in P_1 (0.0383 cm S^{-1}) followed by that in P_4 (0.0347 cm S^{-1}) and

Table 23. Effect of mycorrhiza on fresh weight and dry weight of *Aegle marmelos* in different potting media

Treatments	Media	Fresh Weight (g plant^{-1})		Dry Weight (g plant^{-1})	
		15DAP	30DAP	15DAP	30DAP
P_1	Sand	0.2583	0.6908	0.0930	0.2847
P_2	Soilrite	0.1891	0.5604	0.0704	0.2014
P_3	Sand:soil(1:1)	0.1812	0.5055	0.0690	0.1777
P_4	Sand:soil:coirpith(1:1:1)	0.2098	0.5018	0.0668	0.1771
P_5	Sand:soil:leafmould(1:1:1)	0.2192	0.6368	0.0808	0.2341
CD at 5% level		NS	4.33	NS	9.02

The data represents the mean value of six replications

P_5 (0.0343 cm S^{-1}) and the lowest (0.0278 cm S^{-1}) for plantlets in P_3, thirty days after planting (Table 24).

4.2.2.4.2 Leaf Area Index

The highest value (0.1488) for LA1 was given by plantlets in P_4 followed by that in P_1 (0.1478), P_3 (0.1302) and P_5 (0.1239), thirty days after planting. The least value (0.0962) was observed for plantlets in P_2 (Table 24).

4.2.2.4.3 Crop Growth Rate

The mean values obtained showed that the highest CGR was recorded by those rooted microshoots planted out in P_1 (0.9921 mg cm^{-2} day^{-1}) and the lowest by those in P_4 (0.4682 mg cm^{-2} day^{-1}), one month after planting out (Table 24).

Table 24. Effect of mycorrhiza on stomatal conductance, leaf area index and crop growth rate of plantlets of *Aegle marmelos* in different potting media

Treatments	Media	SC (cm S^{-1})		LAI	CGR (mg cm^{-2} day^{-1})
		15DAP	30DAP	30DAP	30DAP
P_1	Sand	0.1235	0.0383	0.1478	0.9921
P_2	Soilrite	0.0964	0.0296	0.0962	0.5671
P_3	Sand:soil(1:1)	0.0908	0.0278	0.1302	0.5078
P_4	Sand:soil:coirpith(1:1:1)	0.1137	0.0347	0.1488	0.4682
P_5	Sand:soil:leafmould(1:1:1)	0.1153	0.0343	0.1239	0.6757
CD at 5% level		NS	NS	NS	NS

The data represents the mean value of six replications

Discussion

5. DISCUSSION

Plant Tissue culture has been successfully used to micropropagate different medicinal plants. Rare and endangered cultivars can be multiplied and prevented from becoming extinct by this method. Efficient commercial micropropagation depends on the rapid and extensive proliferation along with the use of large scale cultures for the multiplication phase. Transfer of *in vitro* plantlets to *ex vitro* conditions is the most critical stage in the wide spread use of micropropagation. To promote *ex vitro* survival and physiological competence, especially to protect the plants from various stresses and to encourage autotrophy, a transitional environment is needed during the acclimatization phase.

Bael is an important medicinal fruit tree distributed throughout the plains and hilly tracks of India. Over exploitation of the plant as well as habitat destruction, has significantly reduced the population of bael in natural habitat. Eventhough vegetative propagation methods like patch budding is practiced in North India, the rate of multiplication is not sufficient to meet the increasing demand in herbal drug industry. Tissue culture techniques are relevant in this context.

The present study was undertaken with the objective to standardise the rooting both *in vitro* and *ex vitro* and *ex vitro* establishment techniques in bael. The outcome of the investigations are discussed in this chapter.

Efficient rooting of *in vitro* regenerated plants and subsequent field establishment is the last and crucial stage of rapid clonal propagation. The plantlets produced *in vitro* should have a strong and functional root system (Razdan, 2003). Rooting appears to be an important factor for growth during acclimatization stage (Goncalves *et al.*, 2001). Auxins frequently used for inducing rooting are IAA, IBA and NAA. Being stable in nature, IBA has been the preferred auxin for root initiation (Amin and Akhteruzzaman, 2001)). In the

present study, IBA 2.50 mg l^{-1} and NAA 1.00 mg l^{-1} when tried separately in the media could induce 50.00 per cent rooting. Similar results were obtained by Hossain *et al.* (1993), Islam *et al.* (1993), Arumugam and Rao (1996), Islam *et al.* (1996a), Hazeena (2001) in *Aegle marmelos* and Deepa (2004) in *Clitoria ternatea*. In the present study, media without any growth regulator failed to induce root initials. Rout *et al.* (1999) reported similar results in *Plumbago zeylanica*.

Different combinations of auxins were also tried and it was found that a combination treatment of 1.00 mg l^{-1} of IBA and NAA to be more effective in inducing rooting. The results are in agreement with the findings of Obul Reddy *et al.* (2001) in *Decalepis hamiltonii* and Kool *et al.* (1999) in *Azadirachta excelsa*.

Wide variation was noticed in the case of number of days taken for root initiation (8.00 to 15.80) in MS media. Similar results were obtained by Ahmed *et al.* (2003) in peach.

In the present investigation, the number of roots produced per microshoot varied from 1.00 to 2.67. Hossain *et al.* (1993) could also obtain similar results in *Aegle marmelos*. The same trend was also reported by Arumugam and Rao (1996) and Hazeena (2001). Single root formation was observed in majority of the treatments. Usually single root formation in *in vitro* plants have been reported to occur in tree species (Mascarenhas *et al.*, 1981). In bael, similar results were obtained by Ajith kumar and Seeni (1998) and Hazeena (2001).

When the microshoots were cultured in the rooting medium supplemented with IAA, very high callusing was observed. Suppression of rooting in the presence of IAA was also reported by Batra *et al.* (2001) in *Salvadora persica* and Deepa (2004) in *Clitoria ternatea* and *Mucuna pruriens*.

The root induction was delayed in certain treatments with higher levels of BA that was optimized for shoot regeneration. This result is in conformity with the findings of Islam *et al.* (1996 a) in *Aegle marmelos*.

The basal media requirement depends upon the plant species and the purpose of cell, tissue and organ culture. MS was found to be the best basal medium for rooting when compared to half MS and WPM with respect to number of roots (2.67), length of roots (1.30 cm), percentage of root initiation (50.00) etc. Similar results were obtained by Hazeena (2001) in *Aegle marmelos*. Hwang (2004) in *Xanthoxylum piperitum* and Anis *et al.* (2004) in *Pterocarpus marsupium*.

In the present study, sucrose at 30.00 g l^{-1} showed 50.00 per cent root initiation, the least number of days (10.00) for root initiation and the highest value (50.00 per cent) with respect to the successful establishment of plantlets *ex vitro*. These results are in conformity with the findings of Hazeena (2001) in *Aegle marmelos*, Ramesh (1990) in jack and Hazarika *et al.* (2004) in citrus plantlets. Though, leaves of plants derived from sucrose free medium have a greater ability to photosynthesize, acclimatization environment may not permit appreciable photosynthesis to take place resulting in very high mortality when transferred to *in vivo* (Takayama and Misawa, 1980).

The different concentrations of agar tried in the present study failed to show any statistically significant difference with respect to rooting. Among the different concentrations of agar tried , 6.00 g l^{-1} was found to be superior to others with respect to root initiation percentage (50.00), number of roots (1.67) and length of roots (1.20 cm) which in turn affects the *ex vitro* establishment phases. Increased absorption of nutrients from the media due to the lowered osmotic potential may be the reason .The results are in agreement with the findings of Ramesh (1990) in jack plantlets, Phillip *et al.* (2001) in paper shell almond, Leshem (1983) in carnation, Marin and Gella (1987) in cherry and Short *et al.* (1987) in cauliflower and chrysanthemum plantlets. The optimum agar concentration creates an osmotic potential favourable for the uptake of nutrients.

In the present study, 1.0 per cent activated charcoal in the rooting medium was found to be superior with respect to root initiation percentage (60.00), early initiation of roots (11.50 days), number of roots (2.00) and length of roots (2.10

cm). Improved rooting response has been observed in many instances when AC was included in the medium (Ziv (1979) and Takayama and Misawa (1980)). This might be because activated charcoal absorbed the toxic substances and cytokinins. inhibitory to rooting. The average number of days for root initiation was more (4.00) at higher concentrations of AC. With increase in concentration of AC, rooting response was found to be less. Similar results were obtained by Sanchez *et al.* (1996) in *Quercus robur*.

The length of shoots used for rooting influenced the rooting success. Healthy shoots of 3.00 to 5.00 cm with 3.00 to 4.00 leaves gave the highest *ex vitro* survival percentage. Similar findings were made by Ramesh (1990) in jack plantlets. Higher food reserves and increased auxin production in the lengthy shoots might be the reason for the survival.

The light and dark culture conditions also influenced the rooting of microshoots in the present study. The shoots kept in rooting medium were placed under dark for a period of seven days and then transferred to hormone free medium and later transferred to light when root initials were observed. Similar results were obtained by Ramesh (1990) in jack plantlets, Phillip *et al.* (2001) in almond, Sanchez *et al.* (1996) in *Quercus robur* and Tonon *et al.* (2001) in *Fraxinus angustifolia*. Under dark, all the nutrients may be used entirely for the rooting process. In the present study, just before planting out, high light intensity was given. Similar findings were reported by Murashige (1978). According to Hazarika *et al.* (2000), any treatment before or after transfer would increase the photosynthetic capacity during acclimatization and improve plant quality and establishment. In the present study, no rooting was observed in cultures covered with aluminium foil at the basal portion and kept in light. The results are in agreement with the findings of Sanchez *et al.* (1996) in *Quercus robur*.

In this study. no significant difference could be observed among solid and liquid medium for the various characters studied with respect to rooting. Solid media was found to be better with respect to percentage root initiation (50.00) and number of roots (2.67).

EX VITRO ROOTING

The major cost involved in the production of *in vitro* plants is for rooting and hardening. The process of rooting *in vitro* has been estimated to account for approximately 30.00 to 75.00 per cent of the total cost of micropropagation. *Ex vitro* rooting is preferred in many crops with a view to save time and resources (Maene and Debergh, 1983). Labour cost can be dropped considerably if rooting happens *ex vitro* (Hazarika, 2003) .

Among the pre-treatments with IBA, pulse treatment with 1000.00 mg l^{-1} for 20 s and 50.00 mg l^{-1} overnight was found to show highest survival rate (66.67 and 33.33 per cent), higher number of roots (1.67 and 1.00) and length of roots (0.63 and 0.23 cm) respectively, one month after planting. Similar results were obtained by Hazarika (2003) in *Aegle marmelos*, Deepa (2004) in *Clitoria ternatea* and *Indigofera tinctoria*, John (1996) in *Holostemma annulare* and Kannan and Jasrai (1998) in *Vitex negundo*.

EX VITRO ESTABLISHEMENT

A substantial number of micropropagated plants do not survive transfer from *in vitro* conditions to green house or field environment. The green house and field have substantialy lower RH, higher light levels and septic environment that are stressful to micropropagated plants compared to *in vitro* conditions (Hazarika, 2003). During acclimatization, the mode of nutrition is switched from hetrotrophic to photo autotrophic growth. A proper hardening method is a major factor, which determines plantlet survival and establishment in the field.

In the present study, when sand and soilrite was used as the potting media, the plantlets showed the highest survival rate (50.00 per cent) one month after planting. In sand they also produced more number of leaves (4.67), thirty days after planting. The results are in agreement with the findings of Hazeena (2001) in *Aegle marmelos*, Deepa (2004) in *Clitoria ternatea, Mucuna pruriens* and *Indigofera tinctoria*, Ramesh (1990) in jack and John (1996) in *Holostemma*

annulare. Sand might be an ideal potting medium for maintaining an optimum moisture level and sufficient aeration to the root zone of the plantlets.

A higher or lower water content in sand was proved to be detrimental. At higher water content, rotting was observed. Ramesh (1990) also observed rotting in jack plantlets with increased water content. In the present study, media with two or more components showed relatively low survival rate (16.67 per cent). This is in agreement with the results of Bilderbach *et al.* (1982) who obtained higher plant growth in a media composed of single component when compared to that of blended media.

In the present study, plantlets in sand recorded the highest fresh weight (0.6501 g) and dry weight (0.2500 g), one month after planting. Dry weight was found to increase from 0.0773 to 0.2500 g. Similar results were obtained by Watson (1971). According to him dry matter accumulation increases with age.

Stomatal conductance is a measure of the rate of passage of either water vapour or CO_2 through the stomata, this plays a vital role in the acclimatization of micro propagated plantlets. In the study, stomatal conductance was found to be higher (0.7965 cm S^{-1}) in the first fortnight and it gradually decreased (0.1165 cm S^{-1}) by the end of second fortnight. This might be due to the fact that the leaves of *in vitro* grown plants showed open stomata and collapsed guard cells, while acclimatized leaves presented closed stomata as well as decreased stomatal density and aperture (Romano and Martins, 2004). Low stomatal conductance indicates reduced water loss and it is important in the maintenance of plant water status.

In the present study, the values obtained for LAI and CGR were not significantly different. This may be due to the reduced number of leaves, plant height etc. However, the higher LAI (0.1370) for plantlets grown in sand compared to that of other treatments might be due to the optimum plant growth regulating conditions of this medium.

MYCORRHIZA

The plantlets were inoculated with two AMF (*Glomus etunicatum* and *Glomus fasciculatum)* during the *ex vitro* establishment stage. The inoculated plantlets recorded a maximum survival rate of 66.67 per cent in sand, one month after planting. Various workers have reported the improved survival of micropropagated crops during the *ex vitro* establishment stage due to the inoculation of AMF in several crops (Puthur *et al.*, 1988; Ramesh, 1990; Sreelatha, 1992; Vidal *et al.*, 1992; Wang *et al.*, 1993; Schultz *et al.*, 1998 and Sato *et al.*, 1999). Inoculation of AMF during the *ex vitro* establishment stage of micropropagated plantlets significantly improved their survival and growth due to improved absorption of water and nutrients.

The highest mycorrhizal colonization (94.40 per cent) was observed for plantlets grown in sand compared to other media. The substrate used for *ex vitro* establishment is important for the growth of plants and development of AMF. This is in agreement with the findings of You-Shan *et al.* (2001). There is a positive role of mycorrhizae in the establishment of tissue culture raised plants by alleviating the transplantation shock. In the present study, VAM fungi produced hardy plants compared to control plants. This has been supported by the findings of Singh *et al.* (2004).

Mycorrhizae inoculated plants showed higher leaf number (6.00), plant height (4.30 cm), number of roots (2.33), fresh weight (0.6908 g) and dry weight (0.2847 g) compared to control plants. Similar results were obtained by Sivaprasad and Rai (1984), Varshey *et al.* (2002), Singh and Singh (2004), St.John (1980) and Nowak (2004). The higher vegetative growth of VAM treated plants may be due to the growth promotory effect of VAM that improves the phosphorus availability and there by causing more protein synthesis resulting in better morphological growth. The increased nutrient content in mycorrhizal plants has been attributed to the greater solubilisation, increased root surface to volume and permeation of hyphal pads beyond that explored by root hairs (Chang, 1992).

Stomatal conductance was found to be higher (0.0383 cm S^{-1}) for mycorrhizae inoculated plantlets grown in sand thirty days after planting. Similar results were obtained by Auge *et al.* (2004).

The values for CGR (0.9921 mg cm^{-2} day^{-1}) and LAI (0.1478) of mycorrhizal plants were found to be better than control plants. This has been supported by the findings of Setua *et al.* (1999) in mulberry, Yano-Melo *et al.* (1999) in banana, Estrada- Luna (2000) in guava and Varsheny *et al.* (2002) in *Lilium* spp. The benefits of AMF are attributed to the development of extensive network of hyphae around the root which acts as an extension of the root surface and supplies more nutrients to the plant. This might be due to the improved penetration of plant roots in the potting media due to the prolific mycelium of AMF which increased nutrient uptake and in turn improves the plant growth (Thaker and Jasrai, 2003).

In the present investigation, protocols were evolved for the *in vitro* rooting of microshoots of bael (*Aegle marmelos* L. (Corr.). But *ex vitro* rooting and *ex vitro* establishment gave only limited success. However, few plantlets could be acclimatized and they were transferred to mud pots filled with a potting mixture containing sand, soil and cowdung in the ratio 1:1:1 (Plate 10). These plants are being maintained for further observation . But to standardise the protocol, further studies on *ex vitro* establishment need be done by trying different potting media like vermicompost, vermiwash, phosphobacteria, mycorrhiza, triazoles etc. Refinement of media is thus necessary for the *ex vitro* rooting and *ex vitro* establishment of plantlets of bael (*Aegle marmelos* L. Corr.).

Summary

6. SUMMARY

Attempts were made in the Department of Plantation Crops and Spices and Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2003-2005 for evolving techniques for the rooting of microshoots and *ex vitro* establishment of plantlets of bael (*Aegle marmelos* (L.) Corr).

Standardisation of basal media, plant growth substances, sucrose, agar, activated charcoal and mode of culture for *in vitro* rooting was attempted. Standardisation of plant growth substances for *ex vitro* rooting was tried. The effects of different potting media and arbuscular mycorrhizal fungi on *ex vitro* establishment of bael plantlets were studied.

The salient findings of the studies are summarized below.

1. Microshoots for the present study were obtained from the cultures of previous study maintained in the Plant Biotechnology Laboratory, College of Agriculture, Vellayani.. Microshoots of 3.00 to 5.00 cm length and 3.00 to 4.00 leaves were selected for various treatments.

2. Among the different plant growth substances tried for *in vitro* rooting, the highest percentage of root initiation was obtained with IBA 2.50 mg l^{-1} and NAA 1.00 mg l^{-1}. Hence these were used for standardisation of other media components.

3. IBA 0.50 mg l^{-1} recorded the least number of days (8.00) for root initiation in MS medium.

4. Out of the various levels of auxin tried, IBA 2.50 mg l^{-1} recorded the highest number of roots (2.67) in MS medium.

5. Among the different basal media tried for *in vitro* rooting, the earliest root initiation (10.00 days) took place in full strength MS.

6. WPM registered the highest root initiation percentage (60.00) and length of roots (2.27), whereas MS registered the highest value (2.67) for number of roots.

7. Among the different levels of sucrose tried, the highest root initiation percentage (50.00), the least number of days for root initiation (10.00) and highest number of roots (2.67) was recorded by sucrose at 30.00 g l^{-1} in MS medium supplemented with IBA 2.50 mg l^{-1} whereas sucrose at 20.00 g l^{-1} produced lengthy roots (1.57 cm).

8. In MS media supplemented with NAA 1.00 mg l^{-1} also, sucrose at 30.00 g l^{-1} was found to be superior to that of other concentrations.

9. Agar at 6.00 g l^{-1}, recorded the highest root initiation percentage (50.00) in MS basal media supplemented with IBA 2.50 mg l^{-1} and NAA 1.00 mg l^{-1}.

10. Addition of activated charcoal did not evoke any significant difference on rooting. Charcoal at 1.00 g l^{-1} was found to be superior to that of others with respect to root initiation percentage (60.00). It also recorded the least number of days for root initiation (11.50), more number of roots (2.00) and the longest roots (2.10 cm).

11. Solid medium was found to be superior to liquid medium with respect to root initiation, number of roots as well as length of roots.

12. Among the different levels of auxins tried for pre-treatments of microshoots planted out for *ex vitro* rooting, IBA at 1000.00 mg l^{-1} for 20 s (quick dip) was found to be the best with respect to survival rate fifteen (100.00 per cent) and thirty days (66.67 per cent) after planting.

13. Quick dip of shoots in IBA 1000.00 mg l^{-1} before planting out produced the highest number of roots (1.67) having length of 0.63 cm.

14. Among the auxins tried for pre-treatments of shoots planted out for *ex vitro* rooting, IBA at 50.00 mg l^{-1} kept overnight (slow dip) was found to be the best with respect to survival rate fifteen (66.67 per cent) and thirty days (33.33 per cent) after planting.

15. Slow dip of shoots with IBA 50.00 mg l^{-1} showed the highest root length (0.63 cm). Whereas, IBA at 50.00,100.00 and 150.00 mg l^{-1} produced same number of roots (1.00) per shoot.

16. Among the different potting media tried for *ex vitro* establishment, sand recorded the highest survival percentage fifteen (83.30) and thirty days (50.00) after planting.

17. Sand and sand, soil and leaf mould (1:1:1) recorded the highest leaf number (3.67) fifteen days after planting .Whereas sand alone recorded the highest leaf number (4.67) one month after planting.

18. Out of the various potting media tried, the plantlets in sand were the highest (3.67 cm) with a fresh weight of 0.6501 g and dry weight 0.2500 g, one month after planting. Whereas sand, soil and leaf mould in the ratio 1:1:1 produced the highest number of roots (2.67) one month after planting.

19. Among the different potting media tried, stomatal conductance (0.1165 cm S^{-1}), leaf area index (0.137) and crop growth rate (0.0291 mg cm^{-2} day^{-1}) was also found to be the highest for plantlets grown in sand, thirty days after planting.

20. Mycorrhizal colonization was found to be the highest (94.40 per cent) for plantlets in sand and the least (75.00 per cent) for plantlets in sand, soil and leaf mould (1:1:1). Survival rate was also found to be the highest (66.67 per cent) in sand one month after planting.

21. Mycorrhiza inoculated plantlets in sand recorded the highest leaf number (6.00), plant height (4.30 cm) and number of roots (2.33) one month after planting.

22. The highest fresh weight (0.6908 g) and dry weight (0.2841 g) were recorded by mycorrhiza inoculated plantlets in sand.

23. Out of the various potting media tried, mycorrhiza inoculated plantlets in sand registered the highest stomatal conductance (0.0383 cm S^{-1}), leaf area index (0.1478) and crop growth rate (0.9921 mg cm^{-2} day^{-1}), thirty days after planting.

References

7. REFERENCES

Aggarwal, D. and Barna, K. S. 2004. Tissue Culture Propagation of elite plants of *Aloe vera* Linn. *J. Pl. Biochem. Biotech.*10: 53-55

Ahmed, M. S., Abbasi, N. A. and Amer, M. 2003. Effects of IBA on Hard Wood Cuttings of Peach Rootstocks under green house Conditions. *Asian J. Pl. Sci.* 2 (3): 265-269

Ahuja, A., Sambyal, M. and Koul, S. 2002. *In Vitro* Propagation and Conservation of *Atropa acuminata* Royle ex Lindl - An Indigenous Threatened Medicinal Plant. *J. Pl. Biochem. Biotech.* 11: 121-124

Ajithkumar, D. and Seeni, S. 1998. Rapid clonal multiplication through *in vitro* axillary shoot proliferation of *Aegle marmelos* (L.) Corr., a medicinal plant. *Pl. Cell Rep.*17: 422-426

Amin, M. N. and Akhteruzzaman, M. 2001. Large Scale Plant Regeneration in *Ficus religiosa* L. through tissue culture. *Fourth int. Pl. Tissue Cult. Conf., November 1-3, 2001* (ed. Dey, P. P. and Gatton, T. M). Dhaka, pp: 9

Amin. M. N. and Jaiswal, V. S. 1987. Rapid Clonal Propagation of guava through *in vitro* shoot proliferation of nodal explants of mature trees. *Pl. Cell Tissue Organ Cult.* 9: 235-245

Anis, M., Hussain, M. K. and Shahzad, A. 2004. *In vitro* plantlet regeneration of *Pterocarpus marsupium* Roxb., an endangered leguminous tree. *Curr. Sci.* 88(6): 861-863

Annamaria, M., Andrea, B., Pinter, E. and Gabor, M. 1999. Micropropagation of lemon balm. *Pl. Cell Tissue Organ Cult.* 57: 149-152.

Anu, A., Nirmalbabu, K., John, C. Z. and Peter, K. V. 2001. *In vitro* clonal multiplication of *Acorus calamus* L. *J. Pl. Biochem. Biotech.* 10: 53-55

Arumugam, S. and Rao, M. V. 1996. *In vitro* production of plantlets from cotyledonary node cultures of *Aegle marmelos* (L.) Corr. *Adv. Pl. Sci.* 9: 181-186

Arumugam, S. and Rao, M. V. 2000. *In vitro* somatic embryogenesis in *Aegle marmelos*. *J. Med. Arom. Pl. Sci.* 22: 9-10

Arumugam, S., Alagamanian, S. and Rao, M. V. 1997. Shoot differentiation and development from cotyledon and hypocotyl explants of *Aegle marmelos* (L.) Corr. via organogenesis. *J. Swamy. Bot. Cl.* 14: 108-112

Arya, H. C., Ramawat, K. G. and Suthar, K. C. 1981. Culture and differentiation of plants of economic importance. *J. Indian Bot. Soc.* 60: 134-137

Arya, V., Sekhawat, N. S. and Singh, R. P. 2003. Micropropagation of *Leptadenia reticulata*-a medicinal plant. *In vitro Cell. Dev. Biol. - Pl.* 39 (2): 180-185

Auge, R. M., Moore, J. L., Sylvia, D. M. and Cho, K. 2004. Mycorrhizal promotion of host stomatal conductance in relation to irradiance and temperature. *Mycorrhiza* 14: 85-92

Azad, M. A. K., Amin, M. N. and Begum, F. 2003. Rapid Clonal Propagation of a Medicinal plant-*Adhatoda vasica* Nees. Using Tissue Culture Technique. *On Line J. Biol. Sci.* 3 (2): 172-182

Batra, A., Mathur, S. and Shekhawat, G. S. 2001. Clonal Propagation of *Salvadora persica* L., An important medicinal plant of arid zone. *J. Pl. Biol.* 28 (3): 277-281

Bertsoukhi, K., Papafotion, M. and Balotis, G. 2003. Effect of medium on *in vitro* growth and *ex vitro* establishment of *Globularia alypum* L. *Acta Hort.* 616 : 177-180

Bhuyan, P., Choudhary, S.S. and Handique, P. J. 2000. *In vitro* regeneration of an important medicinal plant, *Wedelia chinensis* Merill. *J. Phytol. Res.* 13: 57-62

Bilderbach, T. E., Fonteno, W. C. and Johnson, D. R. 1982. Physical properties of media composed of pea nut hulls, pine bark and peat moss and their effects on azalea growth. *J. Am. Soc. Hort. Sci.*107: 522-525

Chang, D. C. N. 1992. Studies and prospects of horticultural vesicular arbuscular mycorrhizae in Taiwan. *Sci. Agric.* 40: 45-52

Chattopadhyay, S., Datta, S. K. and Mahato, S. B. 1995. Rapid micropropagation for *Mucuna pruriens* f. pruriens. *Pl. Cell. Rep.* 15: 271-273

CSIR.1985. *Wealth of India* Vol A. (ed. Chadha, Y. R.). Council of Scientific and Industrial Research, New Delhi, 530 p.

Daniel. B., John, S., Soniya, E. V. and Nair, G.M. 1999. Micropropagation of *Naregamia alata* W & A – An important medicinal plant. *J. Pl. Biochem. Biotech.* 8: 105-107

*Das, S.C., Baeman, T.S. and Singh, R. 1990. Plant regeneration and establishment in the nursery. *Assam Rev. Tea News.* 79: 24-27

Das, G. and Rout, G. R. 2002. Direct plant regeneration from leaf explants of *Plumbago* species. *Pl. Cell Tissue Organ Cult.* 68: 311-317

Deepa, S. N. 2004. Rapid propagation and conservation of selected leguminous medicinal plants using *in vitro* techniques. Ph.D. thesis, Kerala Agricultural University, Thrissur, 182 p.

Desjardins, Y. and Tiessen, H. 1985. The effect of sucrose concentration and ancymidol on the rooting of asparagus *in vitro*. *Proc. Sixth int. asparagus Symp., August 17-21, 1985* (eds. Lougheed, E. C. and Tiessen, H.). Canada, pp. 287-297

Dixon, R. K., Garrett, H. E. and Cox, G. S. 1988. Cytokinin in the root pressure exudates of *Citrus jambhiri* Lush. colonized by vesicular arbuscular mycorrhizae. *Tree Physiol.* 4: 9-18

Earanna, N., Tanuja, B. P., Bhagyaraj, D. J. and Suresh, C. K. 2002. Vesicular arbuscular mycorrhizal selection for increasing the growth of *Rauvolfia serpentina*. *J. Med. Arom. Pl. Sci.* 24: 695-697

Estrada-Luna, A. A., Davis, F. T. Jr. and Egilla, J. N. 2000. Mycorrhizal fungi enhancement of growth and gas exchange of micropropagated guava plantlets (*Psidium guajava* L.) during *ex vitro* acclimatization and plant establishment. *Mycorrhiza* 10: 1-8

Gomes, G. A. C., Paiva, R., Duarte, P. and Jose, E. 2003. Plant regeneration from callus cultures of *Maclura tinctoria*, an endangered woody species. *In vitro Cell. Dev. Biol. - Pl.* 39 (3): 293-295

Gonclaves, J. C., Diogo, G., Coelho., M. T. and Amancio, S. 2001. Effect of Rooting Condition on survival and growth during acclimatization of micropropagated chestnut plants (*Castanea sativa* × *Castanea cernata*). *Acta Hort.* 494: 241-245

Goodwin, P. B. 1966. An improved medium for rapid growth of isolated potato buds. *J. Exp. Bot.* 17: 590-595

Hainwright, H. and Scrace, J. 1989. Influence of *in vitro* preconditioning with carbohydrates during the rooting of microcuttings on *in vivo* establishment. *Sci. Hort.* 38: 261-267

Hatzilazarou, S., Kotis, M., Economou, A. S. and Rifaki, N. 2003a. Rooting of microcuttings and acclimatization of Bogainvillea. *Acta Hort.* 616: 265-269

Hatzilazarou, S., Ttooulos, C., Economou, A. S., Rifaki, N. and Ralli, P. 2003b. *In vitro* and *ex vitro* rooting and plantlet acclimatization in *Nerium oleander*. *Acta Hort.* 616: 221-225

Hazarika, B. N., Parthasarathy, V. A., Nagaraju, V. and Bhowmik, G. 2000. Sucrose induced biochemical changes in *in vitro* microshoots of Citrus species. *Indian J. Hort.* 57 (1): 27-31

Hazarika, B. N. 2003. Acclimatization of tissue cultured plants. *Curr. Sci.* 85 (2): 1704-1712

Hazarika, B. N., Parthasarathy, V. A. and Nagaraju, V. 2004. Influence of *in vitro* pre-conditioning of *Citrus* sp. microshoots with sucrose on their *ex vitro* establishment. *Indian J. Hort.* 61 (1): 29-31

Hazeena, M. S. 2001. Standardisation of *in vitro* techniques for the rapid clonal propagation of *Aegle marmelos* (L.) Corr. M.Sc. (Hort.) thesis, Kerala Agricultural University, Thrissur, 92 p.

Heslin, M. C. and Douglas, G. C. 1986. Effects of ectomycorrhizal fungi on growth and development of popular plants derived from Tissue Culture. *Sci. Hort.* 30: 143-149

Hoque, A., Rahman, S. M., Arima, S. and Takagi, Y. 2001. Efficient *in vitro* germination and shoot proliferation of chilling treated water chestnut (*Trapa japonica* Flerov.) embryonal explants. *In vitro Cell. Dev. Biol. Pl.* 37 (3): 369-374

Hossain, M., Karim, M. R., Islam, R. and Joarder O. I. 1993. Plant regeneration from nucellar tissues of *Aegle marmelos* through organogenesis. *Pl. Cell Tissue Organ Cult.* 34: 199-203

Hossain, M., Islam, R., Karim, M. R., Joarder. O.I. and Biswas, B. K. 1994a. Regeneration of plantlets from *in vitro* cultured cotyledons of *Aegle marmelos* Corr. *Sci. Hort.* 57: 315-321

Hossain, M., Islam, R., Karim, M. R., Rahman, S. M. and Joarder.O. I. 1994b. Prduction of Plantlets from *Aegle marmelos* nucellar callus. *Pl. Cell Rep.* 13 (10): 570-573

Hossain, M., Islam, R., Islam, A. and Joarder. O.I. 1995.Direct organogenesis in cultured hypocotyls explants of *Aegle marmelos* Corr. *Pl. Tissue Cult.* 5: 21-25

Hutchinson, J.F. 1981. Tissue Culture Propagation of fruit trees. *Proc. int. tissue culture of economically important Pl. Symp., April 28-30, 1981* (ed. Rao, A. N.). Singapore, pp. 113-120

Hwang, S. J. 2004. An efficient *in vitro* propagation of *Xanthoxylum piperitum*. *Acta Hort.* 676: 124-126

Indrani, C. and Bhanja, P. 2002. Study of organogenesis *in vitro* from callus tissue of *Flacourtia jangomas* (Lour.) Raeusch through scanning electron microscopy. *Curr. Sci.* 83 (4): 476-479

Islam, R., Hossain, M., Joarder, O. I. and Karim, M. R. 1993. Adventitious shoot formation on excised leaf explants of *in vitro* grown seedlings of *Aegle marmelos* Corr. *J. Hort. Sci.* 68: 495-498

Islam, R., Ahad, A., Rahman, M. H., Hossain, M. and Joarder, O. I. 1996a. High Frequency Adventitious Plant Regeneration from Radicle Explants of *Aegle marmelos* Corr. *Pak. J. Bot.* 28 (2): 203-206

Islam, R., Hossain, M., Haque, A. and Joarder, O. I. 1996b. Somatic embryogenesis and plant regeneration in *Aegle marmelos*-a multipurpose social tree. *Curr. Sci.* 71: 259-260

Islam, R., Karim, M. R., Rahman, S. M., Hossain, M. and Joarder, O. I. 1994. Plant Regeneration from Excised Cotyledon of *Aegle marmelos* Corr. *Pak. J. Bot.* 26 (2): 393-396

Islam, R., Karim, M. R., Hossain, M. and Joarder, O. I. 1992. Plant Regeneration from leaf- derived callus in *Aegle marmelos* Corr. *Pl. Tissue Cult.* 2 (2): 55-59

John, J. A. 1996. Standardisation of *in vitro* techniques for rapid multiplication of *Holostemma annulare* K. Schum. M.Sc. (Hort.) thesis, Kerala Agricultural University, Thrissur, 136 p.

Kannan, V. R. and Jasrai, V.T. 1998. Micropropagation of medicinal plant *Vitex negundo*. *J. Med. Arom. Pl. Sci.* 20: 693-696

Kim, Y. S., Hahn, E. J., Yeung, E. C. and Park, K. Y. 2003. Lateral Root Development and Saponin Accumulation as affected by IBA or NAA in Adventitious Root Cultures of Panax Ginseng C.A. Meyer. *In vitro Cell. Dev. Biol. - Pl.* 39 (2): 245-249

Komalavalli, N. and Rao, M.V. 2000. *In vitro* micropropagation of *Gymnema sylvestre* – A multipurpose medicinal plant. *Pl. Cell Tissue Organ Cult.* 61: 97-105

Kool, L. F., Keng, C. and Hoe, C. T. 1999. *In vitro* rooting of Sentang roots (*Azadirachta excelsa* L.) and Acclimatization of plantlets. *In vitro Cell. Dev. Biol. - Pl.* 35: 396-400

Kothari, S. K., Singh, S., Singh, U. B. and Kumar, S. 1999. Response of bergomot mint (*Mentha citrata*) to vesicular arbuscular mycorrhizal fungi and phosphorus supply. *J. Med. Arom Pl. Sci.* 21: 990-995

Kumar, P. K., Sonuja, E. V., Lawrence, B. and Nair, G. M. 1993. Micropropagation of *Clitoria ternatea* L. through callus regeneration and shoot tip multiplication. *J. Spices Arom. Pl.* 2 (1&2): 41-46

Kunneman, B. P. A. M. and Albers, M. R. J. 2003. Effects of Tissue Culture and Acclimatization conditions on the survival and growth of rooted and unrooted *Malus* and *Prunus* microcuttings. *Acta Hort.* 314: 18

Lane, W. D. 1978. Regeneration of apple plants from shoot meristem tips. *Pl. Sci. Lett.* 13: 281-285

Leshem, B. 1983. The Carnation succulent plantlet –A stable tetralogical growth. *Ann. Bot.* 52: 873-876

Longford, P. J. and Wainright, H. 1987. Effect of sucrose concentration on photosynthetic ability of rose shoots *in vitro. Ann. Bot.* 62: 633-640

Maene, L. M. and Debergh, P. C. 1983. Rooting of tissue culture plants under *in vitro* conditions. *Acta Hort.* 131: 201-208

Marin, J. A. and Gella, R. 1987. Acclimatization of the micropropagated cherry root stock "Masto de montanana" (*Prunus cerasus* L.). *Acta Hort.* 212: 603-606

Martin, L.P., Beena, M. R. and Joseph, D. 2003. High frequency axillary bud multiplication and *ex vitro* rooting of *Wedelia chinensis* (Osbeck) Marr. - A medicinal plant. Indian *J. Exp. Biol.* 41: 262-266

Mascarenhas, A. F., Gupta, P. K., Kulkarni, V. M., Urmil, M., Iyer, R. S., Khuspe, S. S. and Jagannathan, V. 1981. Propagation of trees by tissue culture. *Proc. COSTED Symp. Tissue Culture of Economically Important plants. April 28-30, 1981* (ed. Rao, A. N.). Singapore, pp.175-179

Mathur, S., Sekhawat, G. S. and Batra, A. 2002. An Efficient *In Vitro* Method for mass Propagation of *Salvadora persica* via apical meristem. *J. Pl. Biochem. Biotech.* 11: 125-127

Misra, P., Datta, S. K., Johri, J. K.,Singh, H. B. and Srivastava, A. 2004. An Improved Method for *In vitro* Large Scale Propagation of *Piper betle* L. *J. Pl. Biochem. Biotech.* 13: 161-164

Mumtaz, N., Choudhary, Q. F. and Quraishi, A. 1990. Tissue culture studies of *Catharanthus roseus. J. Agric.* 6: 465-470

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

Murashige, T. 1974. Plant propagation through tissue culture. *Ann. Rev. Pl. Physiol.* 25: 135-166

Murashige, T. 1977. Clonal propagation through tissue culture. *Plant Tissue and its Biochemical Applications.* (eds. Barz, W., Reinhard, E. and Zenk, M. H.). Sprigler-Verlag, Berlin, pp. 392-403

Murashige, T. 1978. Principles of rapid propagation. In: *Propagatipon of higher plants through tissue culture, a bridge between research and application* (eds. Hughes, K., Henke, R. and Constantine, M.). Tech. Inf. Centre, U. S. Dept.of Energy, Oak Ridge, pp. 14-24

Nambiar, K., Jayanthi, V. P. and Sabu, T. K. 2000. Pharmacognostical studies on vilva-*Aegle marmelos* (Linn.) Corr. *Aryavaidyan*. 30: 73-96

Naomita, V. D and Rai, R. V. 2001. Rapid Clonal Propagation of *Ochreinauclea missionis* (Wall. Ex G. Don), an ethnomedical endemic and threatened tree. *In vitro Cell. Dev. Biol. - Pl.* 37(6): 820-823

Nath, S. and Buragohain, A. K. 2003. *In vitro* Method for Propagation of *Centelle asiatica* (L.) Urban by Shoot Tip Culture.*J. Pl. Biochem. Biotech.* 12: 167-169

*Nowak, J. 2004. The effect of rooting media, CO_2 enrichment, P nutrition and mycorrhizal inoculation on rooting and growth of Oesteospermum. *Acta Hort.* 644: 589-593

Obul Reddy, B., Giridhar, P. and Ravishankar, G. A. 2001. *In vitro* rooting of *Decalepis hamiltonii* Wight & Arn., an endangered shrub, by auxins and root promoting agents. *Curr. Sci.* 81(11): 1479-1481

Panse, V. G. and Sukhatme, P. V. 1985. *Statistical methods for agricultural workers,* ICAR, New Delhi, 135 p.

Pateli, P., Papafotion, M. and Chronopoulos, J. 2003. Influence of *in vitro* culture medium on *Epidendrum radicans* seed germination, seedling growth and *ex vitro* establishment. *Acta Hort.* 616: 189-192

Phillips, J. M. and Hayman.D. S. 1970. Improved procedure for clearing roots and staining parasitic and vesicular arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Br. Mycol. Soc.* 55: 158-161

Phillip, J. A., Graham, G. C. and Margaret, S. 2001. *In vitro* rooting of Almond (*Prunus dulcis* Mill.). *In vitro Cell. Dev. Biol. - Pl.* 37(6): 778-785

* Plastira, V. and Karetsos, G. 2003. Effect of auxin treatment and substrate nature on the *in vitro* rooting and establishment in soil of citrus plantlets. *Acta Hort.* 616: 245-249

Podwyszynska, M. and Hempel, M. 1988. The factors influencing acclimatization of *rosa hybrida* plants multiplied *in vitro* to green house conditions. *Acta Hort.* 226: 639-642

Praveen, S., Pawar, V. and Ahlawat, Y. S. 2003. Somotic Embryogenesis and Plant Regeneration in Kinnow Mandarin. *J. Pl. Biochem. Biotech.* 12: 163-165

Puthur, T., Prasad, K. V. S. K., Sharmila, P. and Saradhi, P. P. 1988. Vesicular arbuscular mycorrhizal fungi improves establishment of micropropagated *Leucaena leucocephala* plantlets. *Pl. Cell Tissue Organ Cult.* 53: 41-47

Radha, S. and Chandra Roy, S. 2003. Efficient Plant Regeneration in *Holarrhena antidysenterica* Wall., from Shoot Segment derived callus. *In vitro Cell. Dev. Biol. - Pl.* 39(2): 151-155

Rahman, M. A. and Blake, J. 1998. The effect of medium composition and culture condition on *in vitro* rooting and *ex vitro* establishment of jack fruit (*Atrocarpus heterophyllus* Lam.). *Pl. Cell Tissue Organ Cult.* 13 : 189-200

Rahman, M. M., Amin, M. N., Azad, M. A. K., Begum, F. and Karim, M. Z. 2004. *In vitro* Rapid Regeneration of Plantlets from Leaf Explants of Native Olive *(Elaeocarpus robustus* Roxb.) *On Line J. Biol. Sci.* 3(8): 718-725

Rai, R. V. 2002. Rapid Clonal Propagation of *Nothapodytes foetida* (Wight) Sleumer-A threatened medicinal tree. *In vitro Cell. Dev. Biol. Pl.* 38 (4): 347-351

Ramesh, B. 1990. *Ex vitro* establishment of jack (*Atrocarpus heterophyllus* Lam.) plantlets. M. Sc. (Hort.) thesis, Kerala Agricultural University, Thrissur, 95 p.

Ranjit, M. and Kester, D. E. 1988. Micropropagation of cherry rootstocks II invigoration and enhanced rooting by Co culture with Colt. *J. Am. Soc. Hort. Sci.* 113(1): 152

Rani, J., Gita, V., Virk, G. S., Nagpal, B. and Avinash, J. 2002. Callus Induction and Plantlet regeneration in *Withania somnifera* (L.) Dunal. *In vitro Cell. Dev. Biol .-Pl.* 39 (5): 468-474

Rawat, R. B. S. and Uniyal, R. C. 2003. National Medicinal Plants Board Committed for Overall Development of the sector. *Agrobios Newsl.* 1: 12-17

Razdan, M. K. 2003. *Introduction to Plant Tissue Culture*. Second edition. Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi, 375 p.

Rehman, R. Y., Israr, M., Srivastava, P. S., Bansal, K. C. and Abdin, M. Z. 2003. *In vitro* regeneration of Witloof Chicory (*Cichorium intybus* L.) from leaf explants and accumulation of esculin. *In vitro Cell. Dev. Biol. Pl.* 39(2): 142-146

Romano, A. and Martins, M. A. 2004. Strategies to improve rooting and acclimatization of Cork Oak. *Acta Hort.* 616: 275-278

Rout, G. R., Saxena, C., Samantaray, S. and Das, P. 1999. Rapid plant regeneration from callus cultures of *Plumbago zeylanica*. *Pl. Cell Tissue Organ Cult.* 56: 47-51

Saha, S., Mukhopadhyaya, M. J. and Mukhopadhyaya, S. 2003. *In vitro* clonal propagation through bud culture of *Hemidesmus indicus* (L.) R Br., an important medicinal plant. *J. Pl. Biochem. Biotech.* 12: 61-64

Sanchez, M. C., San-Jose, M. C., Ballester. A. and Vieitez, A. M. 1996. Requirements for *in vitro* rooting of *Quercus robur* and *Quercus rubra* shoots derived from mature trees. *Tree Physiol.* 16: 673-680

Sato, A. V., Nannetti, C., Pinto, J. E. B. P., Siqueira, J. O. and Blank, M. 1999. Application of arbuscular mycorrhiza to micropropagated heliconia and gerbera plants during acclimatization period. *Hort. Bras.* 17: 25-280

Schultz, C., Ginting, G., Moadwad, A., Mollers,C., Pamin,K., Tahardi, J. S and Vlek, P. L. G. 1998. The role of (vesicular) Arbuscular mycorrhiza in the weaning stage of micropropagated oil plams. *Proc. Fourth BTIG Workshop Oil Palm Improv. Biotech. April 16-17, 1998* (eds. Tahardi, J. S., Darmmo, T. W., Santoso, D. and Nataatmadju, R.). Indonesia, pp. 59-64

Setua, G. C., Kar, R., Satpathy, B., Das, N. K., Ghosh, J. K. and Saratchandra, S. 1999. Effect of vesicular arbuscular mycorrhiza on growth, leaf yield and phosphorus uptake in mulberry (*Morus alba*) under irrigated, alluvial soil conditions. *Indian J. agric. Sci.* 69: 833-861

Sharma, R. K., Wakhlu, A. K. and Boleria, M. 2004. Micropropagation of *Anethum graveolens* L. Through Axillary Shoot Proliferation. *J. Pl. Biochem. Biotech.* 13: 157-159

Short, K. C., Warburton, J. and Robert, A. V. 1987. *In vitro* hardening of cultured cauliflower and chrysanthemum plantlets to humidity. *Acta Hort.* 212: 329-334

Singh, A. and Singh, S. P. 2004. Response of Banana (*Musa* sp.) to vesicular arbuscular mycorrhizae and varied levels of inorganic fertilizers. *Indian J. Hort.* 61(2): 109-113

Singh, S. K., Patel, V. B., Saxena, A. K. and Khawale, R. N. 2004. Mycorrhizae induced hardening of tissue culture raised grape (*Vitis vinifera* L.) plantlets. *Indian J. Hort.* 61(1): 13-17

Sivaprasad, P. and Rai, P. V. 1984. Photosynthesis and competition for photosynthate in tripartite symbiosis. *Curr. Sci.* 54: 468-469

Sivaprasad, P., Ramesh, B., Mohanakumaran, N., Rajmohan, K. and Joseph, P. J. 1995. Vesicular arbuscular mycorrhizae for the *ex vitro* establishment of tissue culture plantlets. *Proc. Third Nat. Conf. Mycorrhiza, March 13-15, 1995* (eds. Adholeya, A. and Singh, S.). Tata Energy Research Institute, New Delhi, pp. 281-283

Sreelatha, U. 1992. Improvement of propagation efficiency of *Anthurium* species *in vitro*. Ph. D. thesis, Kerala Agricultural University, Thrissur, 219 p.

Sreeramulu, K. R. and Bhagyaraj, D. J. 1997. Selection of efficient arbuscular mycorrhizal fungi for Malabar cultivar of Cardamom. *J. Soil Biol. Ecol.* 17: 109-114

St. John, T. V. 1980. Root size and root hairs and mycorrhizal infection. A re-examination of Bayalis hypothesis with tropical trees. *New Phytol.* 84: 483-488

Sumana, K. R., Kaveriappa, K. M. and Bhat, H. K. 1999. *In vitro* multiplication of *Holarrhena pubescens*. *J. Med. Arom. Pl. Sci.* 21: 299-303

Takayama, S. and Misawa, M. 1980. Differentiation in *Lilium* bulbscales grown *in vitro*. Effects of activated charcoal, physiological age of bulbs and sucrose concentration on differentiation and scale leaf formation *in vitro*. *Physiol. Pl.* 48: 121-125

Tal, E., Stein-Seneider, R., Watad, A. A. and Ben-Jaacov, J. 2003. Rooting and weaning of micropropagated *Leucospermum cordifolium*. *Acta Hort.* 314: 19

Thaker, M. N. and Jasrai, Y. T. 2003. AMF based growth promotory effect on micropropagated banana. *Second int. Congr. Pl. Physiol. January 8-12, 2003.* Indian Council of Agricultural Reseach, New Delhi. *Abstract*: 574

Thorpe, T. A. 1980. Perspective in plant cell and tissue culture. *Int. Rev. Cytol. Sup.* II A. (ed. Vasil, I. K.). Acad Press, New York, pp. 71-111

Ticha, L., Cap, F., Pacovska, D., Hofinan, P., Heisel, D., Capkova, V. and Schafer, C. 1998. Culture on sugar medium enhances photosynthetic capacity and high light resistance of plantlets grown *in vitro*. *Physiol. Pl.* 102: 155-162

Tonon, G., Kevers, C. and Gaspar, T. 2001. Changes in polyamines, auxins and peroxidase activity during *in vitro* rooting of *Fraxinus angustifolia* shoots: an auxin independent rooting model. *Tree Physiol.* 21: 655-663

Varghese, S. K., Inamdar, J. A., Kalia, K., Subramaniam, R. B. and Natraj, M. 1993. Micropropagation of *Aegle marmelos* (L.). *Phytomorphology* 43: 87-92

Varsheny, A., Sharma, M. P., Adholeya, A., Dhavan, V. and Srivastava, P. S. 2002. Enhanced growth of micropropagated bulblets of *Lilium* sp. inoculated with arbuscular mycorrhizal fungi in different P fertility levels in an alfisol. *J. hort. Sci. Biotech.* 77: 258-263

Vengadesan, G., Ganapathi, A., Prem Anand, R., Ramesh, V. and Anbazhagam, K. 2002. *In vitro* propagation of *Acacia sinuate* (Lour.) Merr. via cotyledonary nodes. *Agro For. Systems* 55 (1): 9-15

Vidal, M. T., Azcon-Aguilar, C. and Barea, J. M. 1992. Mycorrhizal inoculation enhances growth and development of micropropagated plants of avocado. *Hort. Sci.* 27: 785-787

Wang, H., Parent, S., Gosselin, A. and Desjardins, Y. 1993. Vesicular arbuscular mycorrhizal peat based substrates enhance symbiosis, establishment and growth of three micropropagated species. *J. Am. Soc. Hort. Sci.* 118: 896-901

Watson, D. J. 1971. Size, structure and activity of the productive system of crops. *Potential Crop Production*. Educational Books, London, 271 p,

Williams, R. F. 1946. The phenology of plant growth with special reference to the concept of net assimilation rate. *Ann. Bot.* 10: 41-72

William, R. R. and Taji, M. A. 1989. Auxin type, gel concentration, rooting, survival of *Cheiranthera volubilis in vitro*. *Hort. Sci.* 24 (2): 305-307

Yang, J., Hu, Z., Guo, G. Q. and Zheng, G. C. 2001. *In vitro* plant regeneration from cotyledonary explants of *Swainsonia salsula* Taubert. *Pl. Cell TissueOrgan Cult*. 66: 35-40

Yano-Melo, A. M., Maia, L. C., Saggin, O. J. Jr., Lima-Filho, J. M. and Melo, N. F. 1999. Effect of arbuscular mycorrhizal fungi on the acclimatization of micropropagated banana plantlets. *Mycorrhiza* 9: 119-123

* You-Shan, W., XiangMei, L., MeiQuing, Z. and LiJun, X. 2001. Effects of substrate for pot culture and nutrient solution on reproduction of arbuscular mycorrhizal fungi inoculam. *Acta Agric. Boreali-Sin*. 16: 81-86

Yusuf, A. 2002. Clonal Propagation of *Anogeissus sericea* var.nummularia- A Rare Tree of Arid Forestry. J. Sustainable For. 20(1): 67-68

Ziv, M. 1979. Transplanting *Gladiolus* plants propagated *in vitro*. *Scient. Hort.* 11: 257-260

*Originals not seen

Appendix

APPENDIX – I

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

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

* in g l^{-1}

ROOTING OF MICROSHOOTS AND *EX VITRO* ESTABLISHMENT OF PLANTLETS OF BAEL [*Aegle marmelos* (L.) Corr.]

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Abstract of the
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ABSTRACT

Studies were conducted on "Rooting of microshoots and *ex vitro* establishment of plantlets of bael (*Aegle marmelos* (L.) Corr.)" for evolving techniques for *in vitro* rooting, *ex vitro* rooting and *ex vitro* establishment of plantlets of bael during 2003-2005 in the Department of Plantation Crops and Spices and Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram.

The *in vitro* propagation techniques in bael have already been standardised. The microshoots 3.00 to 5.00 cm long with 3.00 to 4.00 leaves were taken from the cultures of previous study maintained in the Plant Biotechnology Laboratory, College of Agriculture, Vellayani, Thiruvananthapuram.

The highest rooting of the microshoots (50.00 per cent) occurred in two treatments namely, MS basal medium supplemented with IBA 2.50 mg l^{-1}, sucrose 30.00 g l^{-1} and agar 6.00 g l^{-1} and on the same basal medium supplemented with NAA 1.00 mg l^{-1}, sucrose 30.00 g l^{-1} and agar 6.00 g l^{-1}.

The microshoots took the least number of days (8.00) for root initiation in full strength MS basal medium supplemented with IBA 0.50 mg l^{-1}, sucrose 30.00 g l^{-1} and agar 6.00 g l^{-1}. The highest number of roots (2.67) was obtained in full strength MS basal medium supplemented with IBA 2.50 mg l^{-1}, sucrose 30.00 g l^{-1} and agar 6.00 g l^{-1}.

The best pre-treatment identified for *ex vitro* rooting was IBA 1000.00 mg l^{-1} for 20 s (quick dip) which gave the highest survival rate (50.00 per cent), number of roots (1.67) and length of roots (0.63 cm), compared with IBA 50.00 mg l^{-1} (slow dip).

Different potting media like sand, soilrite, sand and soil (1:1), sand, soil and coirpith (1:1:1) and sand, soil and leafmould (1:1:1) were compared to study their effect on *ex vitro* establishment.. Sand was found

to be the ideal potting media for *ex vitro* establishment. The highest survival rate (50.00 per cent), leaf number (4.67), plant height (3.67 cm), fresh weight (0.6501 g), dry weight (0.25 g), stomatal conductance (0.1165 cm S^{-1}), leaf area index (0.1370) and crop growth rate (0.0291 mg cm^{-2} day^{-1}) were obtained when planted out in sand than in other potting media.

The effect of VAM (*Glomus etunicatum* and *Glomus fasciculatum*) in different potting media were also studied in the *ex vitro* establishment of plantlets. It was observed that mycorrhiza inoculated plants in a potting media of sand showed higher survival rate (66.67 per cent) than control (50.00 per cent). The highest values for number of leaves (6.00), plant height (4.30 cm), number of roots (2.33), fresh weight (0.6908 g), dry weight (0.2847 g), stomatal conductance (0.0383 cm S^{-1}), leaf area index (0.1478) and crop growth rate (0.9921 mg cm^{-2} day^{-1}) were obtained for the mycorrhiza treated plantlets in sand, compared to other mycorrhizal treated potting media.

In the present investigation, protocols were evolved for the *in vitro* rooting of microshoots of bael (*Aegle marmelos* (L.) Corr.). But *ex vitro* rooting and *ex vitro* establishment gave only limited success. However, few plantlets could be acclimatized and were transferred to mud pots containing sand, soil and cowdung in the ratio 1:1:1 for observing further growth and establishment.