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Studies on in Vitro Propagation of Aegle Marmelos. Corr.

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STUDIES ON *IN VITRO* PROPAGATION OF *AEGLE MARMELOS*, Corr.

By

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A THESIS
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BANGLADESH

Dedicated

to

my beloved mother

DECLARATION

I hereby declare that the entire work submitted as a thesis for the degree of Master of Philosophy at the Institute of Biological Sciences, University of Rajshahi is the result of my own investigation.

10.10.96

(Professor O.I. Joarder)
Supervisor

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(Md. Razaul Karim)
Candidate

CERTIFICATE

I hereby certify that the work embodied in this thesis has not already been submitted in substance for any degree and has not been concurrently submitted in candidature for any degree.

20/10/15

(Md. Razaul Karim)
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ABSTRACT

The aim of the present investigation was to standardize *in vitro* culture techniques for plant regeneration and root induction from the different explants such as, embryo, cotyledon, hypocotyl, leaf and nucellar tissue of the different 5 trees of *Aegle marmelos*. The morphogenic competence of these explants and trees were not same. Among these explants the morphogenic potentiality of the embryo explant was the highest and the leaf explant was the lowest. The morphogenic competency of Tree 5 was found to be higher than other trees.

The embryo explant produced large number of adventitious buds when cultured in MS medium containing 1.0 mgl^{-1} BA + 0.5 mgl^{-1} GA_3 without intermediate callus formation. The cytokinins BA, Kn or 2ip alone was less effective. Lower concentrations of auxins or GA_3 incombination with cytokinins increased direct shoot proliferation but supported callus growth. Occasional shoot proliferation was observed from root tip and leaf while they were attached with parent shoot. The morphogenic potentiality of the immature embryo was higher than the mature embryo.

Higher concentrations of auxins (5.0 mgl^{-1}) incombination with lower concentrations of cytokinins (1.0-2.0 mgl^{-1}) suppressed adventitious bud induction but influenced callus proliferation from embryo explant.

The cotyledon explant induced multiple shoot formation directly or by passing callus stage when incubated in MS medium containing 1.0 mgl^{-1} BA + 0.5 mgl^{-1} GA_3. BA, Kn or 2ip with higher concentrations of auxins promoted callusing rather than direct shoot proliferation. Kn 2.0 mgl^{-1} + 2,4-D 5.0 mgl^{-1} was the optimum growth regulator supplement for callusing from the cotyledon explant.

Adventitious shoot regeneration directly or through organogenesis from the callus was obtained from the hypocotyl explant after culturing onto MS medium supplemented with different growth regulations formulations. Among the various growth regulators formulations, 2.0 mgl^{-1} BA + 0.2 mgl^{-1} NAA + 1.0 mgl^{-1} 1.0 was found more effective

combination for direct shoot regeneration and 2.0 mgl^{-1} BA + 0.5 mgl^{-1} GA_3 was found to be the best for shoots induction from hypocotyl callus. Growth regulator combination 2.0 mgl^{-1} Kn + 5.0 mgl^{-1} 2,4-D was proved to be the best for long term maintenance of callus culture.

The leaves of *in vitro* grown shoots showed potentiality to regenerate shoot. BA 2.0 mgl^{-1} + NAA 0.2 mgl^{-1} and BA 0.2 mgl^{-1} + IAA 0.2 mgl^{-1} were the optimum growth regulator supplements for direct shoot regeneration. Frequent shoot regeneration was observed from primary callus of the leaf explant. The morphogenic potentiality of more recently formed leaves was higher than the older ones.

The nucellus of *A. marmelos* showed potentiality to regenerate shoots. BA 1.0 mgl^{-1} + NAA 0.1 mgl^{-1} was the optimum growth regulator supplement for the induction of direct shoot regeneration from the nucellar tissue. On the other hand, the nucellar explant induced to develop callus in 5.0 mgl^{-1} NAA + 2.0 mgl^{-1} Kn. Multiple shoot regeneration from the callus was obtained after the subsequent subculture onto 1.0 mgl^{-1} BA + 0.5 mgl^{-1} GA_3 supplemented medium.

The rooting potentiality of the microcuttings varied greatly with their sources of explant and different other factors. IBA 30 mgl^{-1} in MS medium showed more effective media formulation for the maximum frequency of root initiation and root growth. Among the different agar, sucrose, and pH levels in the rooting medium 7.5 gl^{-1}, 30 gl^{-1} and 7.0 respectively, the best for root initiation and root length. In general, the rooting potentiality of microcuttings derived juvenile tissues was higher than those derived from nucellar tissues. Rooted microcuttings were successfully acclimatized in non-sterile natural environment.

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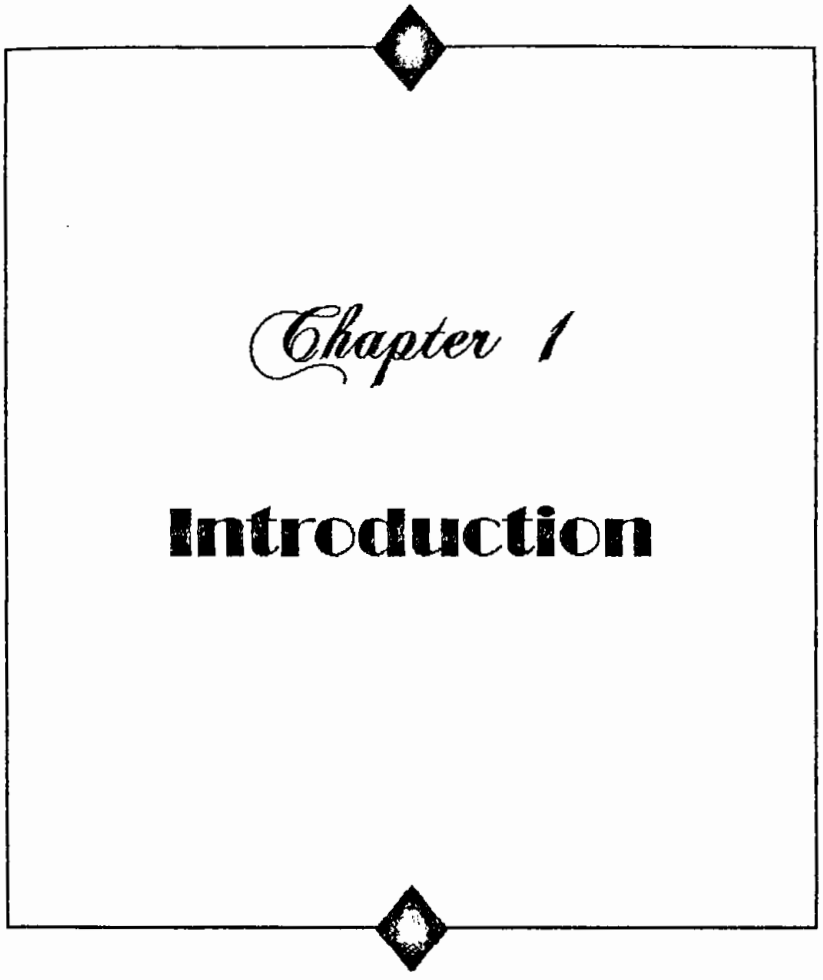
ABBREVIATIONS

BA	6-benzyl adenine
B_5	Gamborg *et al.* (1968) medium
cm	Centimeter(s)
°C	Celsius
2,4-D	2,4-Dichlorophenoxy acetic acid
dw	Distilled water
et al.	Et alli = Other people
Fig.	Figure(s)
GA_3	Gibberellic acid
g	Gram
g^{l-1}	Gram per litre
h	Hour
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
2ip	2-isopentenyl adenine
Kg	Kilogram(s)
Kn	6-furfuryl amino purine
l	Litre
LS	Linsmaier and Skoog
lb	Pounds
mgl^{-1}	Miligram per litre
mg	Miligram
ml	Mililitre(s)
mm	Milimeter(s)
MS	Murashige and Skoog (1962) medium
N_6	Chu *et al.* (1975) medium
N	Normal
NAA	α-naphthalene acetic acid
p^H	Negative logarithm of hydrogen ion (H^-) concentration
Soln.	Solution
sp/spp.	Species
T	Tree
viz.	Namely
%	Percentage



Chapter 1

Introduction

INTRODUCTION

The increased emphasis on sustainable agriculture and an increasing world population (now at 5.25 billion), coupled with the continued loss of prime agricultural land to housing and industry, signify that will be have to fed, clothe and house more people than ever existed in the history of mankind. There are basically two ways to increase the world's food supply : one can cultivate 'new' land or increase per unit crop yields. On a world wide basis, about 1.4 billion hectares of land are cultivated and an additional 1.7 billion ha. of reasonably good land could be brought under cultivation. These areas are mainly in Africa and in Latin America. However, to bring such land into cultivation would require large-scale population shifts, with all the associated social and economic problems. Clearly this is not feasible. The alternative is to increase productivity and to do so major requirements and approaches have been suggested. Biotechnology has a role to play in increasing per unit crop yields and in many research areas, the promise of tissue culture technology is about to be realized.

Plant tissue culture (PTC), an essential component of plant biotechnology, offers novel approaches to plant production, propagation and preservation. During the past decade or so, major advances have been made in this field and from being an art it has become an industrial technology. It is being used for large-scale multiplication of ornamentals and some fruit tree species. The potential impact of these novel and powerful biotechnologies on the genetic improvement of crop plants has generated considerable interest, enthusiasm, and optimism in the scientific community and is in part responsible for the rapidly expanding biotechnology industry.

The technology owes its origin to the ideas of the German plant physiologist, Haberlandt, who in his famous address to the German Academy in 1902 suggested it should be possible to cultivate artificial embryo from vegetative cells (Krikorian and Berquam, 1969). He introduced the concept of totipotency, viz, that all living cell

containing a normal complement of chromosomes should be capable of regenerating the entire plant.

Although Haberlandt was not successful in proving his ideas, his early studies, together with those of his students and contemporaries (Gautheret, 1985), culminated in the successful and indefinite culture of excised roots of tomato (White, 1934). Further studies led to the independent demonstrations by Gautheret (1939), Nobécourt (1939) and White (1939) that cells in culture can be made to proliferation continuously and also undergo differentiation. These findings set the stage for the large increase in research undertaken during the 1940s, 1950s and 1960s. From this period, advances such as the eradication of viruses through meristem culture (Morel and Martin, 1952), the cultivation of single cells and suspension cultures (Muir *et al.*, 1954), the auxin-cytokinin basis of organogenesis (Skoog and Miller, 1957), somatic embryogenesis (Reinert, 1959), the large scale culture of cells, large scale production of protoplasts (Cocking, 1960), anther culture (Guha and Maheswari, 1964), the regeneration of plants from single cells (Vasil and Hildebrandt, 1965).

The use of tissue culture technique in vegetative propagation of plants has become the most widely used technology in agriculture, horticulture and forestry. During the last 25 years it has become possible to regenerate plantlets from explants and/or callus from ornamental plants, food crops, vegetable and condiment plants, fruit and nut crops, medicinal plants and forest trees (Murashige, 1978; Conger, 1981). Several problems such as internal infestations (Murashige, 1978), vitrification (Debergh *et al.*, 1981; Gasper *et al.*, 1987), volatile emissions (Thomas and Murashige, 1979) and production of aberrant plants need to be resolved. In addition, some problems are specific to woody plants. These include phenolic secretions (Chalupa, 1977; Murashige, 1978) and the need for some type of rejuvenation or reactivation treatment for mature explants (Bonga, 1987; Franklet

et al., 1987). However, in spite of these problems lab-scale micro-propagation protocols exist for over 1000 plant species (Brown and Thorpe, 1980).

Protocols for the large-scale mass clonal propagation of several of heterogenous groups such as apple and pears has already been established (Zimmerman, 1986). Scion cultivars propagated on their own roots are also available. Unit costs are considerable less than that of the conventionally grafted trees and are allowing for the development of mechanized high density plantings. Similar progress is being made with some of the stone fruit, namely, rootstock clones of peach. Several tropical fruit trees can be regenerated via somatic embryogenesis using nucellar tissue or by organogenesis using shoot tips and axillary buds (Litz, 1987). However, only banana is being exploited commercially (Hwang *et al.*, 1984). *In vitro* propagation techniques have also been successfully developed for oil, date, coconut, some ornamental palms and peach palm.

Micropropagation techniques have been developed for several berry crops and grape (Zimmerman, 1986). Grapes can be regenerated from axillary shoots, adventitious budding and via somatic embryogenesis but none of these methods as yet allow for mass clonal propagation.

Successful micropropagation of forest trees is a relatively recent phenomenon (Mott, 1981). Plantlets can be produced via organogenesis and somatic embryogenesis in both hardwoods and softwoods and at present, protocols exist over 70 angiosperms and 30 gymnosperms. Several woody species such as wild cherry, eucalyptus, redwood and reliata pine are now commercially micropropagated (Haissing *et al.*, 1987).

In recent years the technique of micropropagation has been used for the rapid clonal multiplication of many fruit plants (Zimmermann and Broome, 1980a; 1980b; Anderson, 1980b; Hutchinson, 1981; Zimmermann, 1981; Jones *et al.*, 1985; Zimmermann, 1985). *In vitro* plant regeneration and subsequent

establishment in soil have been reported for an Indian cultivar of guava (Jaiswal and Amin, 1986; Amin and Jaiswal, 1987). Mango (*Mangifera indica*), the most important fruit tree of the tropics, was initially exposed to *in vitro* culture by Rao *et al.* (1981a), Litz *et al.* (1982) demonstrated somatic embryogenesis from nucellar tissue of five polyembryonic cultivars of mango. They also reported (1984) somatic embryogenesis in other two cultivars 'ono' and 'chino', but in both the works they failed to develop plantlet. Initial success on *in vitro* cloning of jackfruit (*Artocarpus heterophyllus*) was achieved by Rao *et al.* (1981a). Later plant regeneration using explant from mature tree through precocious axillary bud proliferation and organogenesis have been reported by different workers (Amin, 1987; Rahman and Blake, 1988; Roy and De; 1990). Rao *et al.* (1981) started the work on *in vitro* culture of mangosteen. Much later, Goh *et al.* (1988) reported that plantlets were obtained from aseptically germinated seedlings. Normah (1990); Normah and Siti Maisarah (1990) were successful in obtaining multiple shoots from mangoesteen seeds which were segmented and cultured *in vitro*.

In vitro plant regeneration has been reported through embryogenesis from nucellar or integumental tissues of tropical fruits *Syzgium spp.*, *Myrciaria cauliflora* and *Eriobotrya japonica*. A complete protocol of microcloning has been demonstrated by Yadav *et al.* (1990). *In vitro* regeneration though cotyledon culture of some tropical fruit namely, *Averrhoa carambola*, *Citrus microcarpa*, *Cucumis melo* and *Litchi chinensis* were reported by Rao *et al.* (1981a). They found, callus development at various degree in all of the species but organogenesis was sporadic. Root and shoot development occurred only in *C. melo*. Regeneration of plant *in vitro* from somatic tissues of *Averrhoa carambola* was first attempted by Litz and Conover (1980) who observed best callus growth and shoot differentiation from seedling leaf explants. Rao *et al.* (1982) described callus formation associated with occasional organogenesis from cotyledon culture. More recently, Litz and Griffis (1989) reported regeneration of shoot without roots from cultured leaf segments of a mature carambola tree selection.

Considerable work has been done in Bangladesh in few years on *in vitro* propagation of *Morus nigra* (Yadav *et al.*, 1990), *M. indica* (Patel *et al.*, 1983), *M. alba* (Hossain *et al.*, 1990; Zaman *et al.*, 1991; Islam *et al.*, 1993; Zaman *et al.*, 1992; 1993), *M. laevigata* (Islam *et al.*, 1992; Hossain *et al.*, 1992). Papaya plants have been regenerated from seedlings apices (Rahman *et al.*, 1992; Islam *et al.*, 1993) petiole explants (Rahman *et al.*, 1992; Hossain *et al.*, 1993) and shoot tips (Hossain *et al.*, 1991) and lateral buds (Islam *et al.*, 1993) of mature trees. In recent years, the technique of micropropagation has been used for rapid clonal propagation of many tree plants, namely, *Delonix regia* (Rahman *et al.*, 1992), *Caesalpinia pulcherrima* (Rahman *et al.*, 1993) and *Morus laevigata* (Islam *et al.*, 1992; Hossain *et al.*, 1992). *Aegle marmelos* plants have regenerated from hypocotyl (Arya *et al.*, 1981), leaf-derived callus (Islam *et al.*, 1992), leaf (Islam *et al.*, 1993), nucellar callus (Hossain *et al.*, 1994), nucellus (Hossain *et al.*, 1993), cotyledon derived callus (Islam *et al.*, 1993) and cotyledon (Hossain *et al.*, 1994).

Besides these facts many tropical and subtropical fruits are being exposed for tissue culture propagation to assess morphogenic potentialities and methods for large scale micropropagation for many of the fruit plants yet to be established. Although large number of fruit as well as forest tree species have been successfully cultured at the research level, only a few can be cultured well enough to justify the cost of tissue culture propagation on commercial level (Levin and Vasil, 1988) which in term limited the growth of the micropropagation industry (Hussey, 1983; 1986; Rowe *et al.*, 1987; Levin *et al.*, 1988).

Bangladesh is rich in fruit tree species and has been regarded as a gene pool of many fruit trees. *Aegle marmelos* corr. is one of the major indigenous fruit tree species that has been listed as an underexploited tropical plant (Purseglove, 1968) with promising economic value (Hossain, 1992). *A. marmelos* locally called bael or bael fruit (Beng.), bengal quince (Eng.), Vilva (Sanskrit), Shriphall (Hindi). The generic name, *Aegle* is latin origin from the name of a goddes of ancient Greek and the specific epithet *marmelos* is a Portuguese word. *A. marmelos* belongs to the

crassinucellate family 'Rutaceae', of which polyembryony is a common phenomenon (Melchior, 1964; Rangaswamy, 1981); having 130 genera and 1500 spp. (Persglove, 1968).

A. marmelos is a medium-spreading, deciduous spiny tree (Prain, 1963; Singh, 1985) and important due to its fruit and socio-cultural values. Moreover, the fruit plant can be adapted and grown in very wide range of has the capacity to adopt successfully to a wide range of habitats from arid, semi-arid, xerophytic to mesophytic soil having pH 6-10 and temperature from 7°c-45°c. It can grow up to 1200-1500m altitude. The fruit grows well and gives good yield in both humid and dry condition. It is native to Indo-Barma subcontinent (Hooker, 1975), grown well throughout the South East Asia, specially in India, Bangladesh, Pakistan, Sri-Lanka, Barma and Thailand either in wild or under cultivation (Singh et al. , 1976; Heywood and Chant, 1982; Zaman, 1988). Now a days, it has been introduced into other tropical countries of Western Africa and South America (Purseglove, 1968).

A. marmelos is a slow growing deciduous tree. The bark of the tree is thick, nearly smooth, soft and grayish-white in colour. The leaves are alternate, trifoliate, deep green in colour with pungent smell due to presence of a volatile oil secreted from numerous oil gland (Zaman, 1988) shed during winter and sprout during spring; membranous leaflet, subcrenulate, extipulate. The flowers are large (1-3″ in diameter), sweet scented, greenish white, pedicles and calyx pubescent, axillary in panicle; blossoms during spring. The sepals are small, 4-5, deciduous. Petals 4-5, imbricate, spreading. The stamens are numerous (30-60), inserted out side the minute disk; filament free, shoot sublate; anther elongate, erect. Ovary ovoid, 80-20 locular; loculi peripheral, round thick and broad axis; style stout, stigma capitate or oblong or fusiform, deciduous; ovules numerous, 2-serrate in each locule. Fruit large (2-12″ in diameter), globose or ovoid or pyriform, green when unripe and turns greenish yellow-brown when ripe. Each fruit was 8-15 crescent shaped seed sac filled with gum and seeds embedded in aromatic, sweet and thick pulp. Testa

mucilaginous and woody. The seeds are oblong, compressed and embryo with thick flesh cotyledon.

The best known practice for propagation of the *A. marmelos* is by seeds or vegetatively root sucker formation. Root cuttings with suckers are the most preferable to conventional method of vegetative propagation (Zaman, 1988). Suckers developed from the root can be separated from the mother plant. Proper pruning of the suckers before transplantation reduces mortality of the root cuttings. Other conventional methods of vegetative propagation are budding onto seedlings root stock and air layering.

The average time to fruit production is 6-7 years. It would of course be useful to develop a technique to shorten this period. Bael produces single crop per year. It takes 10-12 months from flowering to fruit ripening. Fruits of early trees begin to be available in the market during January and fruits of late tree are found upto July. A fully developed plant can give as many as 300-400 fruits per season. But production capacity is varied with size of the fruit and age of the plant. There is a good deal of variation in shape and size of trees as well as in fruit. Fruit size may vary from 150g - 15 kg. Our survey concerning with size and shape of fruits and trees made as preparatory work pertaining with this investigtion revealed that, size of the tree also varied with the fruit size (Hossain *et al.*, 1994). As for example, there is a tree of bael (consisting only 4-5 plants) located at Chapainawabganj, 60 Km North West to the Rajshahi University campus. The fruits of this tree are extremely large ranges 10-15 Kg. The height is about 50-60 ft. and stem girth is about 5-6 ft. in diameter which are the largest among the trees surveyed (Hossain, 1992). *A. marmelos* is a multipurpose fruit tree with considerable traditional socio-cultural values.

The fruit is the most important part of the plant. Nutritionally it contains 56-77% water, 2.6% protein, 0.39% fat, 1.7% minerals, 31.8% starch per 100 gm eatable pulp contains 65 mg carotene, 0.13 mg thiamine, 1.19 mg riboflavin, 1.6 mg niacin, 8.0 mg vitamin C, 35 mg calcium and 5.3 mg iron (Khan and Haq, 1975).

In vitro studies of *A. marmelos* were first started by Singh (1963), who obtained nucellus tissue but failed to get any results because the nucelli did not survive. Arya *et al.* (1981) demonstrated plant regeneration through organogenesis from hypocotyl and cotyledon derived callus. Not much progress was obtained in *in vitro* of *A. marmelos* until recently. Hossain (1992) made systematic efforts to culture *in vitro* regenerate plant from mature and juvenile tissues of this plant. However, he did not obtain satisfactory results since rooting was poor. The aim of the present investigation was to standardize techniques for *in vitro* culture of *A. marmelos* tissues. However, success of these approaches depend on the ease of *in vitro* culturing of *A. marmelos* tissues and their penultimate organogenesis to yield fertile trees. It has been established that overall response in tissue culture of *A. marmelos* is genetypically oriented, as various cultivars under the same *in vitro* culture conditions exhibit different responses (Hossain, 1992), The present study was, therefore, primarily directed at;

1. Determination of optimal growth regulator concentration for the most efficient production of clones either through precocious axillary and adventitious bud proliferation directly or through indirect regeneration via intermediate callus stage from different explant sources.

2. Optimization of culture condition for adventitious root induction on *in vitro* propagated shoots.

3. Investigation on *in vitro* responses of different genotypes of *A. marmelos* on multiple shoot induction from different explants and on adventitious rooting of micropropagated shoots.

4. Establishment of *in vitro* grown plants on the soil under natural environment.

Chapter II

Materials & Methods

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Plant Material

Five different trees of *Aegle marmelos* Corr. were used as experimental material for the *in vitro* investigation. The following parts of the plants were used as explants viz., immature embryo, mature embryo, cotyledon, hypocotyl, leaf and nucellus. Hypocotyl and leaf explants were collected from *in vitro* grown seedlings. Embryo, cotyledon & nucellar explants were collected from mature field grown plant. Plants of five trees which are located at the Rajshahi University campus and its near superb were the sources of these explants. The tree were considered on the basis of ripe fruits and other characters which are given in the table.

Characters of five trees which were considered as the source of plant materials.

Geno-type	[4] Fruit shape	[1] Fruit diameter, cm, $\bar{x}$	[1] Fruit weight, g, $\bar{x}$	[2] Pulp weight, g	[3] Number of fruits per plant, $\bar{x}$	Approximate age of the plants (years)
T-1	Round at the base, oval at the apex	2.5-5.0	50-125	25-80	450-600	30
T-2	Pear shaped	5.5-10.5	175-400	145-375	400-500	40
T-3	Round	8.0-14.0	550-850	400-750	300-450	20
T-4	Round at the base, oval at the apex	13.0-17.0	1000-1500	950-3000	150-250	25
T-5	Round at the base, gradually oval at the apex	>25	6500-10000	6000-9000	50-100	45

[1] Diameter and weight of fruits were measured from 10 randomly selected mature fruit of three plants for each genotype.

[2] Pulp weight was taken at least from 15 randomly selected fruits after removing hard testa.

[3] Number of fruits per plant was taken at least from 10 plants of each genotype while the fruit began to ripe.

[4] Ripe fruits of T-1, T-2, T-3, T-4 and T-5 are shown in the table.

2.1.2. [a] Nutrition Media

Unless otherwise mentioned MS revised basal salt formulation (Murashige and Skoog, 1962) was used to conduct most of the experiments. Other media composition employed in the present study were : B_5 (Gamborg *et al.*, 1968) formulation, White's (White, 1943), LS (Linsmaier and Skoog, 1965) and N6 (Chu *et al.*, 1975) formulations. The media were used either as original formulations or as in modified forms to fulfil the special need of the experiment, which are mentioned in appropriate places.

2.1.3 [b] Plant Growth Regulators

The following plant growth regulators were employed for the present investigation :

Auxins :

Indole-3-acetic acid (IAA)

Indole-3-butyric acid (IBA)

a-naphthalene acetic acid (NAA)

2,4-dichlorophenoxy acetic acid (2,4-D)

Cytokinins :

6-Benzylaminopurine (BAP)

6-Furfurylaminopurine (Kn)

2-Isopentenyl adenine (2ip)

Gibberellic acid (GA_3)

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[a] For detail chemical compositions of the media formulations, *Appendix* may be seen.

[b] Source of the chemicals : Carolina Biological Supply Co., California, U.S.A.

2.1.4. Sterilizing Agents

In the present investigation murcuric chloride ($HgCl_2$) used as sterilizing agents. Tween-80 and [c] Salvon were used as detergent and surficant.

2.2. METHODS

The methods involved in this study were described below under separate heads.

2.2.1. Preparation of Stock Solution

Different constituents of different culture media formulations were prepared into stock solution as macro-nutrients, micro-nutrients, organic components and growth regulators separately for ready use during the preparation of the culture media.

2.2.1.1. Stock solution of macro-nutrients (soln. A) : Stock solution of macro-salts were prepared at 10X that of the required concentration. Required amount of all the macro-salt components presceibed for a particular medium formulation were weighed with electronic balance and dissolved separately in substantial volume of double distilled water (DW). The solutions were sequentially poured into a 1 litre volumetric flask. Final volume of the solution was made upto 1 litre by adding sufficient amount of DW. Special care was taken during dissolving calcium chloride ($CaCl_2$). The solution after filtering through Whatman No. 1 filter paper, was poured into clean plastic bottle and stored into refrigerator at -18°C.

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[c] Commercial product of 20% cetrimide is used as antiseptic as well as detergent; ICI, U.K. marketed in Bangladesh by the ICI Bangladesh Ltd.

2.2.1.2. Stock solution of micro-nutrients : Two separate stock solution of micro-salts were prepared as follows :

(a) Stock solution of $FeSO_4$ and Na-EDTA (soln. B) : This solution as prepared at 100X of that of required concentration. Requisite amount of $FeSO_4$ and Na-EDTA were taken and dissolved separately into clean glass beakers containing 22 hours (h) at 58°C by placing in an incubator. p^H of the solution was adjusted at 5.6 and after filtering it was stored at 4°C in refrigerator.

(b) Stock Solution of rest of the micro-nutrients (soln. C) : This was made at 100X in 500 ml DW as described for solution A. All components were weighed (except $CaCl_2$) separately and dissolved in 400 ml of DW. $CaCl_2$ was dissolved separately and added to the solution. Finally, the volume of the solution was adjusted upto 500 ml and after filtering was stored at -18°C in a plastic bottle.

2.2.1.3. Stock solution of organic components (soln. D) : This stock solution was also prepared at 100X, dissolved in 100 ml DW as described for the stock solution A. The solution was stored at -18°C in a plastic bottle.

Glutamine and tyrosine were directly added to the medium whenever necessary.

2.2.1.4. Stock solution of growth regulators : Stock solution of different phytohormones was prepared separately. Details of the methods of preparation of stock solution are given in the table.

Different plant growth regulators and their solution.

Growth regulators	Amount taken (mg^{l-1})	Dissolving solvent (ml)	Final volume of the stock solution with DW (ml)	Strength of the stock solution
IAA	10	80% ethanol 1 ml	10	1
IBA	10	70% ethanol 1 ml	10	1
NAA	10	0.1N KOH 70% ethanol 0.5 ml	10	1
2,4-D	10	70% ethanol 0.5 ml	10	1
BAP	10	0.1N KOH 1 ml	10	1
Kn	10	0.1N HCl 0.5 ml	10	1
2ip	10	70% ethanol 1 ml	10	1
GA_3	10	0.1N HCl 0.5 ml	10	1

To prepare stock solution, 10 mg of any of the growth regulators was taken in a clear test tube and dissolved in required volume of appropriate solvent. Final volume of the solution was made upto 10 ml by adding DW. Thus stock solutions of all growth regulators were prepared and stored at 4°C.

2.2.2. Sterilent Solution

$HgCl_2$ solution at various concentrations generally 0.1% was used for surface sterilization of plant materials. To prepare 0.1% solution, 1 gm of $HgCl_2$ was taken in a 1 litre bottle and dissolved in 1000 ml sterilized DW. Freshly prepared $HgCl_2$ was always used. Generally $HgCl_2$ solution was prepared 1 hour before use.

2.2.3. Preparation of Culture Medium

To prepare 1 litre of any of the above mentioned culture medium the following steps were involved :

i) 100 ml of stock solution A and 5 ml of each of the stock solution B, C and D were taken in 1 litre volumetric flask containing 750 ml DW.

ii) Different concentration of hormonal supplements as required was added singly or in combination to the solution and thoroughly mixed. Since 1 ml of each of the hormonal stock solution contained 1 mg solute, therefore, addition of 1 ml stock solution of any of the hormone of 1 litre medium resulted 1 mgl^{-1} concentration. Hormonal concentration was made different by varying the volume of the stock solution as per requirement.

iii) Other supplements such as glutamine, tyrosine, etc. if required, were added.

iv) Unless stated otherwise, the p^H of the medium was adjusted with a p^H meter using 0.1N KOH or 0.1N HCl whichever necessary.

v) Unless otherwise mentioned, 6 gl^{-1} phytoagar and 30 gl^{-1} sucrose were added to the medium. With the special need of the experiment any of these supplement condition was varied keeping other unchanged.

vi) Final volume of the medium was made 1 litre by adding DW.

vii) The medium was then transferred to 1 litre bottle and heated under low pressure in an autoclave for 5 minutes (min) to melt the agar. The medium was shaked well to ensure uniform distribution of agar throughout the medium.

viii) Requisite volume of the medium (while still hot) was then dispensed into cultue vessel (test tube/conical flask) of varying size.

ix) The culture vessels were then plugged with non-absorbent cotton wrapped in cheese-cloth or heavy duty alumnium foil. Then the vessels were marked properly by glass marker to indicate the specificity of the medium.

x) Finally, the culture vessels containing medium were sterilized by

autoclaving at 120°C for 15 min at 1.1 kg/cm pressure and stored in the culture room (not more than a week) for ready use.

As GA_3 is degraded at higher temperature and by autoclaving, this hormone was filter sterilized and added to cool autoclaved medium using microfilter of pore size 0.2 mm.

2.2.4. Collection, Surface Sterilization and Preparation of Explant

Different explants used in this investigation, their sources and process of preparation are summarized in the table.

Explant types, sources and methods of surface sterilization.

Plant Materials	Source of Plant Materials	Method of Washing	Surface Sterilization	
			$HgCl_2$	
			Conc.	Duration (Min.)
Embryo cotyledon	Seeds of ripe and various stages of developing fruits	Washed in 1% Savlon + 4 drops Tween 80 for 10 mins followed by 3 rinses with DW	0.1	1.0
Nucellus	Seeds of 30-130 days old developing fruits	Washed in 1% Savlon + 1 drops Tween 80 for 10 mins followed by 3 rinses with DW	0.1	10
Hypocotyl	Asceptically grown seedlings	—	—	—
Leaf	*In vitro* regenerated shoots from explants	—	—	—

2.2.4.1 Embryo and cotyledon : The ripe fruits and fruits at different stages of development were collected from mature plants and brought to laboratory. The fruits were washed in running tap water for 30 min. The fruits were cut into pieces and seeds were collected carefully with the help of a pair of forceps and dissecting blade. The seeds were then washed with DW containing 1% Savlon (v/v) and 4

drops of Tween 80 for 10 mins to remove gummy substance. This was followed by successive 3 wasing with DW to make the material free from savlon. Subsequently the materials were transferred to running laminar-flow hood. Surface sterilization was carried out in 0.1% $HgCl_2$. The sterilent with explants was constantly shaked while sterilization to increase the release of free Cl_2. To remove every trace of the sterilent, the material was then washed at least six times with sterile DW. The sterilized seeds were then taken in a sterilized petridish and seed coats were carefully removed with the help of a sterilized surgical blade, forceps and needle. After removing seed coat from the seeds, embryo and cotyledon were isolated carefully under the dissecting microscope. The cotyledons either in intact condition on cut into pieces and incubated in the culture vessels containing media. The embryos were also incubated in the culture media. For future use, the fruits were stored in refrigerator at 4°C.

*2.2.4.2. **Nucellar tissue*** : Developing seeds from 90-130-day-old fruits were collected and surface sterilized as mentioned earlier (2.2.4.1). To collect nucellus tissues, the sterilized seeds were placed under dissecting microscope and seed coat was removed very carefully. Precaution was taken to avoid any injury to the tissue. Developing cotyledon with embryo was removed from the exposed nucellus tissue by careful excision and pressing at a micropiler end with the help of a curve needle. Precaution was also taken to ensure complete removal of embryonic tissues from the nucelli. The nucelli were then transformed to the culture media.

*2.2.4.3. **Hypocotyl explant*** : For this purpose, seeds from the ripe fruits were collected and sterilized as mentioned earlier (2.2.4.1). The sterilized seeds were incubated (16h light/8h dark) in culture vessel containing MS medium, 2.0% sucrose, 0.6% agar having p^H 5.8. Hypocotyl explants (area 0.7-1.0 cm were in length) dissected from the 14-20 days old aseptically grown seedlings were used as inoculum.

2.2.4.4. Leaf explant : *In vitro* regenerated shoots were aseptically taken out from culture vessel and leaves excluding petiole were dissected. These explants were cultured singly in 30 ′ 150 mm culture tubes or 250 ml flask containing culture media.

2.2.5. Culture Environment :

Unless metioned specially, all cultures were grown in an air conditioned culture room illuminated by 40w white fluorescent tubes fitted at a distance of 30 cm from culture shelves. The culture room temperature was 25 ±2°C and light intensity was 35.2 ±5 E/m/sec. Except special need most of the cultures were incubated at a photoperiod of 16h light and 8h dark. For dark treatment, the cultures were raised in an incubator and maintained at desired temperature or the culture vessels were placed in a cardboard box in the same room.

2.2.6. Setting and Designing of Experiments :

Different experiments were conducted on various chemical and physical conditions (factors) of the medium for evaluating proper culture requirements for the optimum morphogenic potentialities of different explants. Generally cultures were grown onto culture media supplemented with 3% sucrose, 0.8% agar and p^H at 5.8. In order to determine the optimum condition of a specific factor, experiments were conducted with various condition of a specific factor, experiments were conducted with various degree modification of these factors keeping other constant. Most of the treatment in an experiment had at least 20 replicate cultures and data were collected from 10 randomly selected cultures. All the experiments were repeated three times. The cultures were grown for 2-3 consecutive passages at 4-5 week interval of each and data on different parameters were recorded at the end of 2nd or 3rd passage.

2.2.7. Subculture :

Different processes were followed for maintaining the proliferating cultures through subcultures and are discussed under separate heads.

2.2.7.1. Adventitious bud proliferating cultures : The explants when cultured on suitable medium were induced to proliferate adventitious buds within 4-5 weeks. At this stage, the proliferating cultures were subcultured again in the same initial medium in order to increase budding frequency. After another 4 week incubation the proliferating cultures were transferred to different media for bud elongation. The elongated shoots (usable shoots) were excised from the proliferated cultures and transferred individually to the rooting media. Some of the shoots after isolating leaves, were cut into pieces and cultured individually or in groups for further adventitious and axillary regeneration. The stock cultures, after excising usable shoots were transferred to the initial medium and then to the shoot elongation medium for further adventitious regeneration. The process had been repeated for seveal times in order to establish continuous production of shoot.

2.2.7.2. Callus culture : The calli derived from embryo cotyledon, leaf, hypocotyl and nucellus explants were cut into pieces of 0.5 to 1 cm in diameter. These pieces were subcultured onto media with different concentrations and types of growth regulators and additives for observing morphogenic potentialities regarding shoot differentiation or further callus proliferation.

2.2.7.3 Nucellus culture : The nucellar tissues when incubated in the proper media formulation induced to develop adventitious shoots. The processes followed for maintaining nucellus culture were same as mentioned for adventitious regeneration from embryo and cotyledon (2.2.7.1).

2.2.8. Precaution

The dissecting instruments such as surgical blades, knives, needles, forceps etc. were sterilized by autoclaving. These instruments were dipped in 70% ethanol

and flamed over a spirit lamp before each time of use. Accessory glass wares such as petridishes, bottles, conical flask etc, were hot air sterilized in an oven at 150°C for 1 hour. Other tissue culture kits such as filter paper, cotton, thread etc. were also sterilized by autoclaving before use. The floor of the laminar-flow cabinet and hand were washed with 70% ethanol before starting aseptic procedure.

2.2.9. Microcutting Preparation and their Rooting :

The usuable shoots (one or more than one cm in length) were collected aseptically from proliferating culture of different types of explants at different stages of subculture. Until transferred to the rooting media the shoots were kept in a sterilized glass beaker lined with moistened filter paper to avoid desiccation. The cuttings were prepared from these shoots by snaping off the basal leaves and cultured individually in 25 × 150 mm tubes containing 15-20 ml rooting media having different strength of basic salt composition and different auxin concentration. The cultures were incubated under different physical factors.

2.2.10. Transplantation of Plantlets Under *In vivo* Environment :

Rooted clones were taken out from the culture tubes and washed carefully under running tap water for complete removal of remains of the medium. Polythene bags (9× 15 cm) were kept ready filled with garden soil, organic manure and sand in the proportion of 2:1:1 respectively. The soil in the polythene bags was moistured uniformly and treated with agroson (250 mgl^{-1}, w/v) two days before transplantation. The clones were then transplanted in the bag's soil (one in each bag) with special care then made sealed with the help of a pieces thread for providing high humidity to the clones. The bags containing transplanted clones were ekpt in the culture room under artificial illumination. On the 6/7th day after transplantation the polythene bags near proximity were perforated to allow ventilation to the clones. The polythene bags were finally reopened on the 15th day.

After a month, the transplanted plantlets were subjected to sunlight periodically and progressively. The practice facilitated the transplants for gradual acclimatization in *ex vitro* stress environment. After 2-3 months, the plants were transferred to bigger pots or to the soil. The plants were watered periodically and upper layer of the soil mulched occasionally whenever necessary.

2.2.11. Method of Scoring Growth Response :

Different growth parameters were considered to record data on morphogenic responses of various explants while under different culture conditions. The parameters are :

i) Percentage of shoots (adventitious/axillary) regenerating explants : Explants those induced adventitious buds were recorded 5 weeks after culture whereas % of axillary shoot regenerating explants (mature explants) were recorded 8-12 weeks after culture.

ii) Percentage of callus proliferating explants : Explants those induced callus development were expressed as % and data was recorded 4 weeks after culture.

iii) Percentage of explants induced root development : this parameter was used for rooting experiment and data was recorded 5 weeks after culture.

iv) Number of shoot (adventitious/axillary) per explant : Data on this character were recorded 4-18 weeks after culture.

v) Number of roots per clones : It was recorded 6 weeks after culture.

vi) Root length : Length of the longest root was measured in cm while the transplantation of clones.

vii) Fresh weight of callus : Each culture vessel containing proper amount of culture media was weighed carefully with an electronic balance before

placing explants in it. Each culture vessel was weighed again after placing explant (single explant/culture vessel) and weight of the explant was found out as follows :

Wt. of explant = Wt. of culture vessel + explant
- Wt. of culture vessel.

After 5 weeks of culture, each explant with proliferating callus was taken out and weighted. Entire process was operated in front of running laminar flow cabinet and sterilized petridishes were used to avoid contamination of the calli. Fresh weight of the callus was found out as follows :

Fresh weight of callus = Wt. of explant with callus - Wt. of explant.

Weight of explants and calli were taken in g.

2.2.12. Statistical Analysis :

All experiments were consisted of at least 20 replicate cultures and each of the experiments was repeated thrice. Data were recorded at least from 10 randomly selected cultures and mean values were calculated separately for each replication.

For evaluating and interpreting the results, collected data were analysed following the biometrical methods developed by Mather (1949) and Allard (1960) based on the mathematical models of Fisher *et al.* (1932). Statistical analysis of data given as percentage was carried out from angular transformed values.

2.2.12.1. Mean and standard error of mean **:** The mean of different batch of culture of different replications were worked out by taking arithmetic mean using the following formula :

$$\bar{Y} = \frac{1}{n} \sum_{i=1}^{n} Y_i$$

Where, $\bar{Y}$ = arithmatic mean, n = number of observation and

$\sum Y_i$ = Summation of variable

Standard error of mean (S.E.) were calculated as follows

$$\text{S.E. or } \delta\bar{x} = \sqrt{S^2 / n}$$

Where $\bar{x}$ = Sample mean

S^2 = Sample variance

n = Sample size.

2.2.12.2. Variance analysis : The analysis of variance was done for sorting out the variance due to different sources and for testing the significance among the sources. Variance analysis for each character was carried out separately on mean value of ten cultures in each of the repeated experiment.

Mean square in the analysis of variance were calculated as follows :

$$\text{Sum of square (SS)} = \sum x^2 - \frac{(\sum X)^2}{n}$$

$$\text{Mean squares (MS)} = \frac{\sum x^2 - (\sum x)^2 / n}{n-1}$$

where,

$\sum x$ = Sum of X.

n = Sample size.

2.2.12.3. Variance ratio test (F-test) : F-test was carried out for test of significance among the variance within a source.

$$\text{F value} = \frac{MS}{EMS}$$

Where, MS = Mean square value of specific source of variation

EMS = Error mean square.

*2.2.12.4. **Regression analysis*** : The statistical tool with the help of which are in a position to estimate (or predict) the unknown values of one variable from known values of another variable is called regression. With the help of regression analysis, we are in a position to find out the average probable change in one variable given a certain amount of change in another.

Analysis of regression were calculated as follows :

$$\text{Regression value (b)} = \frac{\Sigma xy - \frac{\Sigma x.\Sigma y}{n}}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}$$

where, Σ= Summation

Σx = Sum of X

Σy = Sum of Y

n = Sample Size.

*2.2.12.5. **Regression lines*** : The regression line were calculated using following formula.

$$X = \bar{X} - (\bar{Y} - Y)\, b$$

where, $\bar{X}$ = Average value of X

$\bar{Y}$ = Average value of Y.

Chapter III

Results

RESULTS

The present experiments were conducted with different types of explants viz., immature embryo, mature embryo, cotyledon, nucellus and the tissues such as hypocotyl and leaf of *in vitro* grown shoots which were collected from morphogenic competence of five trees. The explants were used for the induction of shoot regeneration, induction of callus, shoot regeneration from callus and the induction of roots to the *in vitro* regenerated shoots. The investigations deal with the studies of tree x environment interaction shown by five characters (tree) and their different environment (treatments). Details of the results so far obtained from each of the experiments are described under following heads.

3.1 EXPLANTS CULTURE FOR DIRECT SHOOT REGENERATION

The experiments were conducted to investigate direct regeneration ability of the different explants of five trees. Data on percentage of shoot forming explants and number of shoots per explant after eight weeks of culture were collected. The results are discussed according to types of explants under separate heads.

3.1.1 Embryo Culture for Shoot Regeneration:

Results regarding with the response of immature embryo (from the 5 month old developing seeds of five trees) and mature embryo (from mature seeds of same trees) after culturing on the MS (Murashige and Skoog, 1962) agar gelled media supplemented with 9 different concentrations of cytokinins (0.5, 1.0 & 2.0 mgl^{-1} BA, 1.0, 2.0 & 5.0 mgl^{-1} Kn, 1.0, 2.0 & 5.0 mgl^{-1} 2ip) are given in Tables 1, 2, 5 & 6. The shoot regeneration ability of the explants were markedly influenced by the source of explant (tree) as well as by the concentrations of BA, Kn and 2ip present in

the culture media. In all treatments lower frequency explants of all trees induced shoot regeneration during initial 3 weeks culture. However, it increased with the increase of culture period and became maximum at the end of 8 weeks. The enlargement of size of the explants was the first visible morphological changes occured within 5-7 days of culture. First visible morphological changes of cultured explants were enlargement of size within 5-7 days of culture. Further morphogenic differentiation varied with media composition as well as with explant type. In all the media formulations at least a number of mature and immature embryos differentiated into normal plant (plate 1A). Both types of embryos cultured onto media with the low (0.5-1.0 mgl^{-1}) or high concentration (5.0 mgl^{-1}) of cytokinins were found to be more prone to normal plantlet differentiation.

The differentiation of the explant other than normal shoot also varied with media formulations. A number of immature embryos induced to developed callus when cultured at 2.0 mgl^{-1} BA, 5.0 mgl^{-1} Kn and 5.0 mgl^{-1} 2ip. Similar response was also observed for mature embryos at very low frequency than those of immature embryo. In few cases, the calli of both types of embryos futher differentiated to shoot but (plate 1B).

Multiple shoot differentiation occured from the both types of explants when cultured media content 1.0 mgl^{-1} BA or 2.0 mgl^{-1} Kn or 2.0 mgl^{-1} 2ip. However, the morphogenic differentiation potentiality of the explants which induced adventitious buds was different than those formed normal plantlet or callus. The explants at initial stage of differentiation produced shoot buds and hypocotyl. The hypocotyl portion of the explant instead of growing to normal root, swelled up through lateral growth. Initially, adventitious buds started to visible as a very small nodule like protrusion coming out from the hypocotyl surface, within 10-15 days of culture (plate 1C). These nodule like structures subsequently developed into adventitious buds within 5 weeks of culture (plate 1D & 3C). In most of the cases the growth of the plumule of

the embryos that developed multiple adventitious buds ceased and ultimately senesced. These explants did not developed root. Morphogenic differentiation during adventitious bud regeneration was same irrespective of media formulation and nature of explant. Adventitious bud proliferating explants were subcultured onto MS media with 1.0 mgl^{-1} BA for development of bud to shoot.

The frequency of adventitious bud regeneration varied with the concentration of BA, Kn and 2ip present in the media and the nature and sources (table 1 and 2) of explants. The highest frequency of explants showing adventitious bud regeneration was recorded for immature embryo at 1.0 mgl^{-1} BA (tree 1, tree 2, tree 5) and 2.0 mgl^{-1} BA (tree 5) and for mature embryo recorded at 2.0 mgl^{-1} BA (tree 5). The frequency of shoot regeneration was the lowest at 1.0 mgl^{-1} Kn (43.3%) for tree 2 (table 1) and 1.0 mgl^{-1} 2ip (30%) for tree 2 (table 2).

The highest number of shoots per explant was 146.6 from immature embryo recorded at 1.0 mgl^{-1} BA of tree 1 (table 5; plate 2A and 2B) and for mature embryo recorded at 2.0 mgl^{-1} BA (79.4) of tree 5 (table 6; plate 2D)). Number of shoots per explant was the lowest at 1.0 mgl^{-1} Kn (11.1) of tree 3 (table 5) and 5.0 2ip mgl^{-1} (2.1) of tree 2 (table 6).

The mean performance of different characters under various environments (treatments) are given in Tables 1, 2, 5 & 6. Explants (trees) and culture environment (treatment) showed pronounced effect on the morphogenic differentiation of the embryo. Population means for five trees tested varied within treatments and among the treatment as well.

The analysis of environmental means (tables 1, 2, 5 & 6) reveals that the explants in general gave better performance in 1.0 mgl^{-1} BA (table 1 & 5) and the performance significantly decreased in other environments. The lowest performance was observed in 5.0 mgl^{-1} 2ip (table 2 & 6). Thus the cytokinins types might be arranged in order of importance as BA, Kn & 2ip. From the population means, it was

PLATE 1 (A-G)

PLATE 1 (A-D)

Multiple shoot proliferation and callus induction from immature and mature embryo explants

Plate 1A: A plantlet developed with multiple shoot from embryo in low (0.5-1.0 mgl^{-1}) or high concentration (5.0 mgl^{-1}) of cytokinins after 25 days of culture.

Plate 1B: A shoot proliferating callus of immature embryo in 1.0 mgl^{-1} BA after 35 days of culture.

Plate 1C: A very small nodule like protrusion coming out from the hypocotyl surface in 5.0 mgl^{-1} BA within 15-25 days of culture

Plate 1D: Nodule like structures developed into adventitious buds from immature embryo, after 35 days of culture.

PLATE 1 (A - D)

PLATE 2(A-D)

Multiple shoots formation from embryo explants.

Plate 2A: Multiple shoot proliferation from immature embryo in 1.0 mgl^{-1} BA after 5 weeks of culture

Plate 2B: Multiple shoots developed from immature embryo explant, after subculture in 1.0 mgl^{-1} BA (5 weeks after subculture)

Plate 2C: Multiple shoots developed from immature embryo in 1.0 mgl-1 BA + 0.5 mgl^{-1} GA_3 after 5 weeks of culture.

Plate 2D: Multiple shoots developed from mature embryo in 2.0 mgl-1 BA after 8 weeks of culture.

PLATE 2 (A - D)

observed that tree 5 gave the highest frequency shoot formation explants and number of shoots per explant (table 1, 2, 5 & 6).

The results of the analysis of variance are shown in Tables 1, 2, 5 & 6. In the analysis of variance the main items (tree and treatment) were significant against experimental error whereas the items replication, BA, Kn, 2ip and difference between hormones were non-significant (table 1 and table 2). The regression analysis showed that trees interacted significantly with the environment (Figs. 1 & 2). The nine different treatments i.e., the concentrations and combinations of BA, Kn & 2ip were treated as different environment.

3.1.2 Cotyledon Culture for Shoot Regeneration:

Cotyledon explants collected 5 different trees were cultured on to 9 different concentrations of BA, Kn and 2ip and the results concerning with this experiment are given in Tables 9 & 10. Pattern of morphogenic differentiation of the cultured explant markedly varied with the concentrations and types of cytokinins present in the culture media. First visible change after transferring of the explant in the culture media occurred by the enlargement of size. The explants were white in colour before culturing to the media but became green within 7-10 days of culture through chlorophyll synthesis. During resuming new growth most of the explants in all treatments induced to develop trace of callus at the cut surface of the explant.

In most of the media formulations, a number of explant showed adventitious bud proliferation. Initially adventitious buds were visible as tiny nodule like structures within 10-15 days of culture that subsequently developed to adventitious buds within next 15-20 days of culture (plate 3A). In majority cases bud proliferation occurred throughout the surface of explant. In a few cases adventitious bud regeneration occurred only from cut surface of the explant. There was no visible difference between dorsal and ventral surface of the explant regarding adventitious bud regeneration. After 4 weeks of culture in the initial medium, the explants with

proliferating buds were transferred to the bud elongation medium fortified with 1.0 mgl^{-1} BA + 0.1 mgl^{-1} IAA. The adventitious buds grew to shoot after 5 weeks of subculture onto this medium.

In most of the media formulations, number of explants showed adventitious bud proliferation. The highest 50.77% of explants induced to develop adventitious bud was recorded when cultured with 2.0 mgl^{-1} BA of tree 5. The lowest 25.56% of explants developed to adventitious bud for those cultured with 1.0 mgl^{-1} Kn, 2.0 mgl^{-1} Kn, 1.0 mgl^{-1} 2ip and 5.0 mgl^{-1} 2ip of tree 1, tree 4 and tree 3 respectively (table 9). Tree 5 developed maximum 12.3 shoots per explant at 2.0 mgl^{-1} BA (plate 3A)and tree 2 minimum 1.8 shoots per explant at 1.0 mgl^{-1} Kn (table 10). Analysis of variance reveals that most of the items were significant against the experimental error indicating that a real difference existed among trees and treatments (table 3).

3.1.3 Hypocotyl Culture for Shoot Regeneration

Hypocotyl explants of 5 trees were cultured onto MS media supplemented with 9 different concentration of BA, Kn & 2ip. Effect of these treatment concentrations on days to shoot initiation, percentage of explants formed multiple shoots and medium suitability rating (shoot growth) are presented in Tables 13 & 14. Initially shoot primordia started to develop at the cut surface of the distal region then from all over the surfaces of the explant. Adventitious buds initially grew as a globular embryoid like structure from the surface of hypocotyl (plate 3C). These embryoid like protrusions eventually grew to adventitious buds and formed multiple shoots during later period of culture. The result shows that maximum 90% of hypocotyl explants produced shoots at 1.0 mgl^{-1} BA and 2.0 mgl^{-1} BA for tree 1, tree and tree 5, respectively. Shoot regenerating explants were minimum (31.05%) at 5.0 mgl^{-1} Kn for tree 3 (table 13). The highest number of shoots per culture was 14.2

PLATE 3(A-D)

Adventitious shoot proliferation from cotyledon and hypocotyl explants.

Plate 3A: Adventitious buds proliferated from cotyledon explant in 2.0 mgl-1 BA, after 5 weeks of culture.

Plate 3B: Multiple shoots developed from cotyledon in 2.0 mgl-1 BA + 2.0 mgl^{-1} NAA + 1.0 mgl-1 GA_3, after 8 weeks of culture.

Plate 3C: A nodule like structures developed into adventitious buds from hypocotyl, after 3 weeks of culture.

Plate 3D: Multiple shoots developed from hypocotyl in 1.0 mgl^{-1} BA, after 8 weeks of culture.

PLATE 3 (A - D)

recorded at 1.0 mgl^{-1} BA for tree 5 (plate 3D) and the lowest was 1.8, recorded at 5.0 mgl^{-1} Kn for tree 4 (table 14). In respect of medium quality rating 1.0 mgl^{-1} BA and 2.0 mgl^{-1} BA gave a very good shoot growth. It is indicated from the result that the explants of tree 5 showed better response and BA showed better shoot yield.

The results of the analysis of variance are shown in Tables 13 & 14 reveal that the items tree & treatment were significant against the experimental error.

3.1.4 Leaf Culture for Shoot Regeneration

First 4-5 distended apical leaves of *in vitro* grown shoots (originated through embryo or cotyledon culture of 5 trees) were cultured onto MS medium supplemented with 9 different concentrations of BA, Kn & 2ip and the results are given in Tables 17 & 18. First visible change of cultured leaves was observed within first week of culture through increase in thickness and callus proliferation at the cut surface of the explant. The degree of callus proliferation varied with media formulations. Adventitious buds mainly developed on callus (plate 4B) that developed on the distal side of the cuts midrib of the leaf explants, but sometimes directly developed from leaf tissue (plate 4A). Adventitious shoot production capacity was greatly influenced by the growth regulator formulation and explants types (trees). The frequency of buds producing explants increased with the increase of culture period and became optimum at the end of eight weeks of culture. The number of regenerating explants as well as number of shoots per explant decreased with the increase of hormonal concentration (table 18). The maximum frequency of shoot regenerating explants (49.78%) and the highest number of shoots per explant (6.7) were recorded for tree 5 at 2.0 mgl^{-1} BA (plate 4B).

Analysis of variance reveals that morphogenic differentiation to adventitious bud regeneration of the explants of 5 trees were significantly different. The variable effect of different cytokinin concentrations on frequency of shoot regenerating explants and number of shoots per culture was also significant at 0.1% level of probability.

PLATE 4(A-E)

Adventitious shoot regeneration from leaf explant.

Plate 4A: Shoot proliferated from midrib of the leaf or directly leaf tissue in 2.0 mgl^{-1} BA, after 5 weeks of culture.

Plate 4B: Shoot proliferated from leaf callus in 2.0 mgl^{-1} BA, after 5 weeks of culture.

Plate 4C: Multiple shoot regeneration from leaf callus in 2.0 mgl^{-1} BA + 0.1 mgl^{-1} or 2.0 mgl-1 BA + 0.1 mgl-1 IAA, after 5 weeks of culture.

Plate 4D: Multiple shoot regeneration from leaf callus in 2.0 mgl^{-1} BA + 0.2 mgl^{-1} NAA, after 8 weeks of culture.

Plate 4E: Multiple shoot regeneration from leaf callus in 2.0 mgl^{-1} Kn, after 8 weeks of culture.

PLATE 4 (A - E)

3.1.5 Nucellus Culture for Shoot Regeneration

Morphogenic changes of nucellar explants were noticed within 2 weeks of culture. At the initial stage the explants increased in size and became green in colour. In most of the cases callogenesis and adventitious bud formation took place simultaneously. The percentage of regenerating explants and number of shoots varied greatly in MS medium supplemented with 9 different concentrations of BA, Kn & 2ip (tables 21 & 22). The adventitious buds at the initial stage appeared as tiny tube-like structures that resembled embryoids (plate 6A and 6B). At a later stage the shoot buds were recognizable monopolar structures with a maximum diameter of 0.5 mm. Later, morphologically significant changes were observed after 4 weeks of culture when maximum increase in length occurred. Among the different treatments 1.0 mgl^{-1} BA was found to be the best formulation where maximum frequency of regenerating explants and adventitious buds were recorded. The explants that developed shoot buds were transferred to medium containing a lower concentration of BA (0.01 - 0.1 mgl^{-1}) for development and elongation of shoots. When the explants with adventitious buds were cultured on elongation medium, more than 70% of shoot buds developed into shoots within 2-3 weeks of culture. After 5 weeks of incubation these shoots reached a height of 4-5 cm. After excision of the elongated shoots, the remaining part of explant with small shoots attached to it could be recultured in fresh medium for further elongation. Following this procedure, 100% of the shoots from an explant could be recovered from each treatment used in this study (plate 6D and 6E).

Like other explants, morphogenic from the nucellar explants of 5 trees differed significantly. Variation in growth regulators concentrations for shoot proliferation was also significant.

3.1.1.1 Effect of BA alone or in combination with NAA, IAA, GA_3 or NAA+GA_3 on embryo explants

Embryo explants of 5 trees were cultured onto MS medium supplemented with different concentrations of (0.5, 1.0 & 2.0 mgl^{-1}) BA in combination with (0.1 & 0.2 mgl^{-1}) NAA and IAA (0.5 & 1.0 mgl^{-1}), GA_3 (0.1 + 0.5 mgl^{-1}, 0.1 + 1.0 mgl^{-1}) and NAA+GA_3. Potentialities of explants including shoot regeneration are summarized in Tables 3, 4, 7 & 8. In all of the media formulations at least certain number of both types of embryos differentiated into normal plantlets. In most of these cases presence of NAA, IAA & GA_3 even at very low concentrations decreased the rate of normal plantlet differentiation and simultaneously increased the rate of callusing. In all cases differentiation was higher in immature embryo than mature embryo. The frequency of shoot forming immature embryo was the highest (85.71%) at 1.0 mg/l BA with low concentration of NAA (0.1 mgl^{-1}) however, decreased with the low concentration of IAA, GA_3 & NAA+GA_3 (table 3). The highest number of adventitious shoot regeneration was 164.20 recorded at 1.0 mgl^{-1} BA + 0.5 mgl^{-1} GA_3 (Table 7; plate 2C). Percentage of regenerating explants (84.18 & 77.42) and number of shoots per culture (175.28 & 73.65) were maximum for tree 5 for both immature and mature embryo explants respectively.

From the analysis of variance (Table 3, 4, 7 & 8), it is observed that morphogenic potentialities of 5 trees and different treatment significantly differed.

3.1.2.1 Effect of BA alone or in combination with NAA, IAA, GA_3 or NAA-GA_3 on cotyledon explants

Cotyledons explants of 5 different Trees were cultured onto MS medium supplemented with different concentrations and combinations of growth regulators. Results on morphogenic potentialities of the explant at different concentrations of BA in combinations with NAA, IAA, GA_3 & NAA+GA_3 are given in Tables 11 & 12.

The frequency of shoot forming cotyledon explants and number of shoots per explants were increased further addition of lower concentrations of NAA and IAA (0.1 & 0.2 mgl^{-1}) than BA alone. The cotyledons of 5 trees develop bud and callus simultaneously proliferation when cultured onto media having 2.0 mgl^{-1} BA in combination with 0.5 & 1.0 mgl^{-1} GA_3. The degree of bud induction and callus proliferation varied greatly with the growth regulator composition of the medium. In most of the cases callogenesis and adventitious bud initiation took place simultaneously. No correlation between amount of callus and number of shoot buds per culture was observed. Among the two treatment combinations 2.0 mgl^{-1} BA + 0.5 mgl^{-1} GA_3 was found to be the best formulation where maximum (52.40%) frequency of adventitious bud proliferating cultures and the highest number of shoot buds per culture (15.92) were recorded followed by 2.0 mgl^{-1} BA + 1.0 mgl^{-1} GA_3 (plate 3D). This frequency and number of shoots per culture increased after adding of lower concentrations of (0.2 mgl^{-1}) NAA. Higher frequency of regenerating cotyledon (56.50%) was also recorded at 2.0 mgl^{-1} BA + 0.2 mgl^{-1} NAA + 0.5 mgl^{-1} GA_3. The cotyledon of all trees cultured with 2.0 mgl^{-1} BA + 0.2 mgl^{-1} NAA + 1.0 mgl^{-1} GA_3 showed higher (plate 3B) number of shoots per culture (16.30).

Analysis of variance reveals that significant differences existed among 5 trees and among different growth regulators treatments. However, the items BA, BA+NAA, BA+IAA, BA+GA_3 & BA+NAA+GA_3 were non-significant against experimental error (table 11 & 12)

3.1.3.1 Effect of BA alone or in combination with NAA, IAA, GA_3 or NAA, GA_3 on hypocotyl explants

Hypocotyl explants of five trees viz., tree 1, tree 2, tree 3, tree 4 & tree 5 were cultured onto MS medium supplemented with 3 different concentrations (0.5, 1.0 & 2.0 mgl^{-1}) of BA in combinations with NAA (0.1 & 0.2 mgl^{-1}), IAA (0.1 & 0.2

mgl^{-1}), GA_3 (0.5 & 1.0 mgl^{-1}) and NAA+GA_3 (0.2 + 0.5 mgl^{-1} & 0.2 + 1.0 mgl^{-1}). Results on percentage of shoot regenerating hypocotyl explants and number of shoots per culture were evaluated after 3 weeks of culture which are presented in Tables 15 & 16. The adventitious shoot regeneration ability of the hypocotyl explants was markedly influenced by the source of explant (trees) as well as by the concentrations & in combinations of growth regulators present in the culture media.

Among the 11 growth regulators concentrations and combinations, 2.0 mgl^{-1} BA + 0.2 mgl^{-1} NAA, showed the best culturability regarding the percentage of shoot regeneration. The maximum 80.26% of shoot regenerating explants was recorded for this growth regulator combination. The minimum percentage of shoot regenerating explants (40.46%) was recorded at 2.0 mgl^{-1} BA + 1.0 mgl^{-1} GA_3. The highest number of shoots per culture (13.9) was observed in medium with 2.0 mgl^{-1} BA + 0.2 mgl^{-1} NAA + 1.0 mgl^{-1} GA_3 (plate 3D) & the lowest (6.54) was observed in 0.5 mgl^{-1} BA. Analysis of variance reveals that variation in morphogenic potentialities due to tree and due to growth regulators were significant.

3.1.4.1 Effect of BA alone or in combination with NAA, IAA, GA_3 or NAA-GA_3 on leaf explants

Leaf explants of 5 trees were cultured onto MS medium employed with 11 concentrations and combinations of BA, NAA, IAA & GA_3. Results concerning with shoot regeneration ability of the explant of 5 trees at 11 BA, BA+NAA, BA+IAA, BA+GA_3 and BA+NAA+GA_3 formulations are presented in Tables 19 & 20. The shoot regeneration ability of the explants was found to vary with trees and with media composition. The analysis of means shows that optimum adventitious regeneration frequency and number of shoots per culture of tree 5 were 47.23% and 7.27, respectively. The lowest percentage of bud regenerating explant for tree 3 was 36.91 and minimum number of shoots per explant was 4.0 recorded from Tables 19 & 20.

The treatment concentration and combination not only produced pronounced variation in days to shoot initiation, frequency of shoot proliferation and shoot growth but also had a great effect on number of shoots per culture. Effect of 11 concentrations and combinations of growth regulators on frequency of shoot regeneration and number of shoots per culture of the regenerated shoot from leaf explants of 5 trees of *A. marmelos* are shown in Tables 19 & 20. In respect of medium quality rating 2.0 mgl^{-1} BA + 0.1 mgl^{-1} NAA and 2.0 mgl^{-1} BA + 0.1 mgl^{-1} IAA gave a very good percentage of shoot regeneration (51.85%) and number of shoots (plate 4C) per culture (8.36). It is indicated from the result that BA with NAA and IAA showed better response than other treatments. Analysis of variance was calculated to quantify the results and results are shown in Tables 19 & 20. The items tree & treatment in the analysis of variance were highly significant against the experimental error indicating that a real difference existed among the trees and treatments.

3.1.5.1 Effect of BA alone or in combination with NAA, IAA, GA_3 or NAA-GA_3 on nucellar explants

Nucellar explants of 5 trees were cultured onto MS medium employed with 11 concentrations and combinations of BA, NAA, IAA & GA_3. Potentialities of the explants including shoot regeneration are summarized in Tables 23 & 24). When the medium was supplemented with 0.5, 1.0 & 2.0 mgl^{-1} BA, the explants produced shoot and frequency of shoot forming explant was low. Therefore, a low concentration (01. & 0.2 mgl^{-1}) of NAA was added to increase the frequency of shoot forming explants and number of shoots per culture. BA+NAA combinations were found to be more efficient that BA+IAA, BA+GA_3 & BA+NAA+GA_3 combinations for shoot induction. The best frequency of shoot formation & number

Table 1: Effect of cytokinins on percentage of shoot formation from immature embryo explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with five culture tubes per treatment.

Cytokinins (mg/l)	Percentage of shoot forming explants					Mean
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	
BA						
0.5	72.29	59.22	54.99	63.44	81.15	66.22
1.0	90.00	90.00	72.29	81.15	90.00	84.69
2.0	81.15	63.44	59.22	72.29	90.00	73.22
Mean	**81.15**	**70.89**	**62.29**	**72.29**	**87.05**	
Kn						
1.0	43.08	43.03	50.77	54.99	59.22	50.23
2.0	59.22	46.92	59.22	63.44	81.15	61.99
5.0	50.77	50.77	63.44	63.44	72.29	60.14
Mean	**51.02**	**46.92**	**57.81**	**60.62**	**70.89**	
2ip						
1.0	54.99	50.77	46.92	54.99	59.22	53.38
2.0	59.22	50.77	59.22	63.44	81.15	62.76
5.0	68.07	46.92	51.15	59.22	76.92	60.46
Mean	**60.76**	**49.49**	**52.43**	**59.22**	**72.43**	
GRAND MEAN	64.31	55.77	57.47	64.04	76.79	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	649.56	2.50^{NS}	Tree 1 0.842 ±0.009
Tree (T)	4	615.29	2.52*	Tree 2 0.666 ±0.011
Environment (E)	8	532.81	2.11*	Tree 3 1.104 ±0.087
BA	2	434.74	1.68^{NS}	Tree 4 1.221 ±0.008
Kn	2	200.04	-	Tree 5 0.801 ±0.025
2ip	2	119.53	-	
Diff. Betwn. Hormones	2	1376.92	5.32**	
T × E	32	38.36	-	
Error	88	258.96		

* Significant at 5% level of probability
** Significant at 1% level of probability
NS Non-significant

Table 2: Effect of cytokinins on number of shoots per culture from immature embryo explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with five culture tubes per treatment.

Cytokinins (mg/l)	Number of shoots per culture					Mean
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	
BA						
0.5	60.2	69.8	99.3	100.8	109.8	87.98
1.0	89.5	105.4	117.7	125.4	146.6	116.92
2.0	78.2	75.6	110.8	118.6	125.2	101.68
Mean	**75.97**	**83.60**	**109.27**	**114.93**	**127.20**	
Kn						
1.0	15.3	12.8	11.1	12.2	22.8	14.84
2.0	18.1	16.3	15.4	18.6	29.6	19.6
5.0	23.4	20.6	18.7	24.8	36.5	24.8
Mean	**18.93**	**16.57**	**15.07**	**18.53**	**29.63**	
2ip						
1.0	19.9	18.5	18.2	17.4	29.8	20.76
2.0	26.5	23.2	19.6	27.7	32.4	25.88
5.0	17.3	14.4	11.2	19.5	25.6	17.6
Mean	**21.23**	**18.70**	**16.33**	**21.53**	**29.27**	
GRAND MEAN	38.71	39.62	46.89	51.67	62.03	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	90.40	4.26*	Tree 1 1.42 ±0.289
Tree (T)	4	17286.69	814.64***	Tree 2 1.19 ±0.205
Environment (E)	8	413.83	19.50***	Tree 3 0.88 ±0.110
BA	2	1047.89	49.38***	Tree 4 0.86 ±0.106
Kn	2	124.09	5.85**	Tree 5 0.83 ±0.099
2ip	2	87.30	4.11*	
Diff. Betwn. Hormones	2	396.06	18.66***	
T × E	32	102.61	4.83***	
Error	88	21.22		

* Significant at 5% level of probability
** Significant at 1% level of probability
*** Significant at 0.1% level of probability

Table 3: Effect of BA either singly or incombination with NAA, IAA or GA_3 on percentage of shoot formation from immature embryo explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with ten culture tubes per treatment.

Growth regulators (mg/l)	Percentage of shoot forming explants					Mean
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	
BA 0.5	65.88	65.88	67.21	77.08	79.37	71.08
BA 1.0	90.00	71.56	79.37	90.00	90.00	84.19
BA 2.0	90.00	68.53	75.00	82.50	90.00	81.21
Mean	**81.96**	**68.66**	**73.86**	**83.19**	**86.46**	
BA 1.0 + NAA 0.1	90.00	68.53	90.00	90.00	90.00	85.71
BA 1.0 + NAA 0.2	0.00	73.15	77.08	90.00	90.00	80.05
Mean	**80.00**	**70.84**	**83.54**	**90.00**	**90.00**	
BA 1.0 + IAA 0.1	77.08	67.21	70.00	68.53	79.37	72.44
BA 1.0 + IAA 0.2	64.60	62.24	73.15	62.24	70.00	66.45
Mean	**70.84**	**64.73**	**71.58**	**65.39**	**74.69**	
BA 1.0 + GA_3 0.5	73.15	64.60	55.73	77.08	90.00	72.11
BA 1.0 + GA_3 1.0	62.24	58.89	60.00	70.00	67.21	63.67
Mean	**67.69**	**61.75**	**57.87**	**73.54**	**78.61**	
BA 1.0 + NAA 0.1 + GA_3 0.5	90.00	77.08	70.00	90.00	90.0	83.42
BA 1.0 + NAA 0.1 + GA_3 1.0	73.15	62.24	60.00	71.56	90.00	71.39
Mean	**81.58**	**69.66**	**65.00**	**80.78**	**90.00**	
GRAND MEAN	76.92	67.26	70.69	78.99	84.18	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	213.03	2.18^{NS}	Tree 1 0.605 ±0.010
Tree (T)	4	1985.43	5.08**	Tree 2 1.213 ±0.63
Environment (E)	10	287.29	2.94**	Tree 3 0.549 ±0.032
BA	2	235.84	2.41^{NS}	Tree 4 0.680 ±0.011
BA + NAA	1	80.26	-	Tree 5 0.695 ±0.029
BA + IAA	1	89.76	-	
BA + GA_3	1	178.25	1.82^{NS}	
BA + NAA + GA_3	1	361.57	3.70^{NS}	
Diff. Betwn. Hormones	4	422.86	4.33**	
Tree × Treatment	40	36.03	-	
Error	108	97.72		

* Significant at 5% level of probability
** Significant at 1% level of probability
NS Non-significant.

Table 4: Effect of BA either singly or incombination with NAA, IAA or GA_3 on number of shoots per culture from immature embryo explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with 10 culture tubes per treatment.

Growth regulators (mg/l)	Number of shoots per culture					Mean
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	
BA 0.5	60.2	72.8	100.5	120.4	130.6	96.9
BA 1.0	80.2	113.2	132.2	145.3	153.5	126.48
BA 2.0	74.4	67.8	108.4	112.2	125.2	97.60
Mean	**71.60**	**84.60**	**113.70**	**125.97**	**136.43**	
BA 1.0 + NAA 0.1	118.2	120.3	178.2	188.2	194.1	159.80
BA 1.0 + NAA 0.2	99.3	118.2	165.5	178.4	179.9	148.26
Mean	**108.75**	**119.25**	**171.85**	**183.3**	**187.00**	
BA 1.0 + IAA 0.1	116.4	118.6	166.5	179.8	184.4	153.14
BA 1.0 + IAA 0.2	102.3	113.2	158.7	158.9	178.1	142.24
Mean	**109.35**	**115.90**	**162.60**	**19.35**	**181.25**	
BA 1.0 + GA_3 0.5	122.5	122.6	174.7	200.8	200.4	164.20
BA 1.0 + GA_3 1.0	105.4	120.7	168.1	180.9	192.2	153.40
Mean	**113.95**	**121.65**	**171.40**	**190.85**	**196.30**	
BA 1.0 + NAA 0.1 + GA_3 0.5	124.4	129.4	172.2	185.1	198.4	161.90
BA 1.0 + NAA 0.1 + GA_3 1.0	110.3	119.5	170.8	152.6	191.3	148.90
Mean	**117.35**	**124.45**	**171.50**	**168.85**	**194.85**	
GRAND MEAN	101.23	110.57	154.16	163.87	175.28	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	4185.28	3.10^{NS}	Tree 1 1.093 ±0.029
Tree	4	7233.32	5.36***	Tree 2 1.126 ±0.249
Treatment	10	4753.31	3.52**	Tree 3 0.863 ±0.099
BA	2	1424.6	1.06^{NS}	Tree 4 0.804 ±0.009
BA + NAA	1	332.93	-	Tree 5 0.890 ±0.026
BA + IAA	1	297.03	-	
BA + GA_3	1	288.37	-	
BA + NAA + GA_3	1	422.50	-	
Diff. Betwn. Hormones	4	10835.79	8.03***	
Tree × Treatment	40	1970.14	1.46*	
Error	108	1350.09		

* Significant at 5% level of probability
** Significant at 1% level of probability
*** Significant at 0.1% level of probability
NS Non-significant.

Table 5: Effect of cytokinins on percentage of shoot formation from mature embryo explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with four culture tubes per treatment.

Cytokinins (mg/l)	Percentage of shoot forming explants					Mean
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	
BA 0.5	45.00	54.70	60.00	65.88	65.88	58.29
BA 1.0	54.70	60.00	65.88	65.88	73.15	63.92
BA 2.0	60.00	65.88	73.15	73.15	90.00	72.44
Mean	**53.23**	**60.19**	**66.34**	**68.30**	**76.34**	
Kn 1.0	35.24	40.16	49.78	45.00	54.70	44.98
Kn 2.0	49.78	35.24	54.70	49.78	60.00	49.90
Kn 5.0	54.70	60.00	65.88	60.00	65.88	61.29
Mean	**46.57**	**45.13**	**56.79**	**51.59**	**60.19**	
2ip 1.0	49.78	30.00	49.78	45.00	54.70	45.85
2ip 2.0	60.00	40.16	54.70	49.78	65.88	54.10
2ip 5.0	45.00	35.24	45.00	40.16	49.78	43.04
Mean	**51.59**	**35.13**	**49.83**	**44.98**	**56.79**	
GRAND MEAN	50.47	46.82	57.65	54.96	64.44	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	246.05	3.3*	Tree 1 0.846 ±0.029
Tree	4	412.98	5.54***	Tree 2 0.696 ±0.249
Treatment	8	467.50	6.27***	Tree 3 0.498 ±0.099
BA	2	253.54	3.40*	Tree 4 0.827 ±0.009
Kn	2	350.19	4.69**	Tree 5 1.070 ±0.026
2ip	2	165.44	2.22^{NS}	
Diff. Betwn. Hormones	2	1100.83	14.76***	
Tree × Treatment	32	34.09	0.46^{NS}	
Error	88	74.56		

* Significant at 5% level of probability
** Significant at 1% level of probability
*** Significant at 0.1% level of probability
NS Non-significant.

Table 6: Effect of cytokinins on number of shoots per culture from mature embryo explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with four culture tubes per treatment.

Cytokinins (mg/l)	Number of shoots per culture					Mean
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	
BA 0.5	40.0	49.4	52.2	38.4	70.2	50.08
BA 1.0	50.4	52.4	60.2	31.5	68.2	52.54
BA 2.0	62.6	55.5	75.4	46.8	79.4	63.94
Mean	**51.0**	**52.43**	**62.60**	**38.90**	**72.60**	
Kn 1.0	5.6	3.4	4.4	4.0	8.5	5.18
Kn 2.0	6.5	4.2	6.2	3.9	7.2	5.60
Kn 5.0	11.2	8.6	9.1	7.9	10.2	9.40
Mean	**7.77**	**5.40**	**6.57**	**5.27**	**8.63**	
2ip 1.0	3.5	4.6	5.2	5.3	5.4	4.80
2ip 2.0	6.2	5.4	7.2	6.6	8.2	6.72
2ip 5.0	5.2	2.1	4.6	4.4	4.9	4.24
Mean	**4.97**	**4.03**	**5.67**	**5.43**	**6.17**	
GRAND MEAN	21.24	20.62	24.94	16.53	29.13	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	85.01	2.50^{NS}	Tree 1 1.006 ±0.010
Tree	4	204.03	6.00***	Tree 2 1.042 ±0.063
Treatment	8	2550.15	75.05***	Tree 3 0.866 ±0.032
BA	2	273.43	8.04***	Tree 4 0.882 ±0.099
Kn	2	27.02	-	Tree 5 0.763 ±0.037
2ip	2	8.46	-	
Diff. Betwn. Hormones	2	9891.73	290.89***	
Tree × Treatment	32	191.21	5.62***	
Error	88	34.005		

*** Significant at 0.1% level of probability

NS Non-significant.

Table 7: Effect of growth regulators on percentage of shoot formation from mature embryo explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with four culture tubes per treatment.

Growth regulators (mg/l)	Percentage of shoot forming explants					Mean
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	
BA 0.5	40.16	46.89	54.70	54.70	60.00	51.29
BA 1.0	54.70	50.94	55.75	73.15	90.00	64.91
BA 2.0	60.00	56.91	64.01	73.57	90.00	68.89
Mean	**51.62**	**51.58**	**58.15**	**67.14**	**80.00**	
BA 1.0 + NAA 0.1	73.15	60.00	73.15	90.00	73.1	73.89
BA 1.0 + NAA 0.2	73.15	65.88	90.00	73.15	90.00	78.44
Mean	**73.15**	**62.94**	**81.58**	**81.58**	**81.58**	
BA 1.0 + IAA 0.1	65.88	49.78	54.70	60.00	73.15	60.70
BA 1.0 + IAA 0.2	49.78	40.16	49.78	54.70	65.88	52.06
Mean	**57.83**	**44.97**	**52.24**	**57.35**	**69.52**	
BA 1.0 + GA_3 0.5	65.88	54.70	73.15	65.88	73.15	66.55
BA 1.0 + GA_3 1.0	60.00	45.00	60.00	60.00	73.15	59.63
Mean	**62.94**	**49.85**	**66.58**	**62.94**	**73.15**	
BA 1.0 + NAA 0.1 + GA_3 0.5	65.88	60.00	73.15	73.15	90.00	72.44
BA 1.0 + NAA 0.1 + GA_3 1.0	54.70	49.78	65.88	65.88	73.15	61.88
Mean	**60.29**	**54.89**	**69.52**	**69.52**	**81.58**	
GRAND MEAN	60.29	52.73	64.93	67.65	77.42	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	126.29	1.56^{NS}	Tree 1 0.742 ±0.249
Tree	4	916.27	11.32***	Tree 2 1.370 ±0.10
Treatment	10	371.62	4.59***	Tree 3 0.921 ±0.012
BA	2	426.18	5.26**	Tree 4 0.699 ±0.135
BA + NAA	1	51.66	-	Tree 5 0.918 ±0.035
BA + IAA	1	186.71	2.31^{NS}	
BA + GA_3	1	119.79	1.48^{NS}	
BA + NAA + GA_3	1	278.67	3.44^{NS}	
Diff. Betwn. Hormones	4	556.75	6.88***	
Tree × Treatment	40	37.11	-	
Error	108	80.96		

** Significant at 1% level of probability
*** Significant at 0.1% level of probability
NS Non-significant.

Table 8: Effect of growth regulators on number of shoots per culture from mature embryo explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with four culture tubes per treatment.

Growth regulators (mg/l)	Number of shoots per culture					Mean
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	
BA 0.5	83.3	43.4	46.4	27.8	62.6	52.70
BA 1.0	43.5	47.8	52.1	33.5	64.2	48.22
BA 2.0	54.2	50.9	61.8	46.8	69.4	56.62
Mean	**60.33**	**47.37**	**53.43**	**36.03**	**65.40**	
BA 1.0 + NAA 0.1	55.5	56.6	58.3	56.7	70.4	59.50
BA 1.0 + NAA 0.2	60.2	68.2	68.6	62.8	75.6	67.08
Mean	**57.85**	**62.40**	**63.45**	**69.75**	**73.00**	
BA 1.0 + IAA 0.1	60.0	52.8	54.3	57.8	70.2	59.02
BA 1.0 + IAA 0.2	52.8	47.7	43.2	50.2	61.5	51.08
Mean	**56.40**	**50.25**	**48.75**	**54.00**	**65.85**	
BA 1.0 + GA_3 0.5	59.3	61.2	62.8	60.2	73.2	63.34
BA 1.0 + GA_3 1.0	68.2	72.6	78.2	65.6	81.2	73.16
Mean	**63.75**	**66.90**	**70.50**	**62.90**	**77.20**	
BA 1.0 + NAA 0.1 + GA_3 0.5	68.5	65.2	70.4	68.2	83.5	71.16
BA 1.0 + NAA 0.1 + GA_3 1.0	58.2	76.2	84.2	75.6	98.4	78.52
Mean	**63.35**	**70.70**	**77.30**	**71.90**	**90.95**	
GRAND MEAN	60.34	58.42	61.86	55.02	73.65	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	152.79	4.86*	Tree 1 0.856 ±0.010
Tree	4	621.22	19.76***	Tree 2 0.852 ±0.063
Treatment	10	593.42	18.87***	Tree 3 0.720 ±0.32
BA	2	214.93	6.84***	Tree 4 0.799 ±0.011
BA + NAA	1	143.64	4.57*	Tree 5 0.854 ±0.055
BA + IAA	1	157.60	5.01*	
BA + GA_3	1	241.08	7.68*	
BA + NAA + GA_3	1	135.42	4.31*	
Diff. Betwn. Hormones	4	1206.66	34.38***	
Tree × Treatment	40	25.59	0.81^{NS}	
Error	108	31.44		

* Significant at 5% level of probability
** Significant at 1% level of probability
*** Significant at 0.1% level of probability
NS Non-significant.

Table 9: Effect of cytokinins on percentage of shoot formation from cotyledon explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with five culture tubes per treatment.

Cytokinins (mg/l)	Percentage of shoot forming explants					Mean
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	
BA 0.5	31.05	39.23	31.05	35.63	43.05	36.00
BA 1.0	39.23	35.63	35.63	46.89	46.89	40.85
BA 2.0	46.89	43.05	39.23	46.89	50.77	47.37
Mean	**39.06**	**39.30**	**35.30**	**43.13**	**46.90**	
Kn 1.0	26.56	31.05	31.05	26.56	31.05	29.25
Kn 2.0	26.56	35.63	39.23	31.05	35.63	33.62
Kn 5.0	31.05	39.23	35.63	35.63	39.23	36.15
Mean	**28.06**	**35.30**	**35.30**	**31.05**	**35.30**	
2ip 1.0	26.56	31.05	35.63	26.56	31.05	30.17
2ip 2.0	31.05	39.23	35.63	31.05	34.52	34.29
2ip 5.0	26.56	35.63	26.56	31.05	26.56	39.27
Mean	**28.06**	**35.30**	**32.60**	**29.55**	**31.05**	
GRAND MEAN	31.72	36.64	34.40	34.59	37.76	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	30.07	2.35^{NS}	Tree 1 0.816 ±0.010
Tree (T)	4	48.52	3.67*	Tree 2 1.136 ±0.063
Environment (E)	8	190.08	14.38***	Tree 3 0.849 0.132
BA	2	162.58	12.29***	Tree 4 0.718 ±0.039
Kn	2	60.91	4.61**	Tree 5 1.376 ±0.026
2ip	2	57.53	4.35**	
Diff. Betwn. Hormones	2	479.32	36.26***	
T × E	32	3.62	-	
Error	88	13.22		

* Significant at 5% level of probability
** Significant at 1% level of probability
*** Significant at 0.1% level of probability.
NS Non-significant

Tabel 10: Effect of cytokinins on number of shoots per culture from cotyledon explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with five culture tubes per treatment.

Cytokinins (mg/l)	Number of shoots per culture					Mean
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	
BA 0.5	5.8	6.5	6.8	8.8	9.2	7.42
BA 1.0	4.2	7.2	5.6	6.4	8.4	6.36
BA 2.0	6.2	8.5	8.3	10.2	12.3	9.10
Mean	**5.40**	**7.40**	**6.90**	**8.47**	**9.97**	
Kn 1.0	3.2	1.8	3.3	2.9	3.5	2.94
Kn 2.0	3.4	2.6	4.4	3.7	4.2	3.66
Kn 5.0	6.1	4.2	5.1	4.3	5.4	5.02
Mean	**4.23**	**2.87**	**4.27**	**3.63**	**4.37**	
2ip 1.0	2.8	3.1	2.9	2.6	3.2	2.92
2ip 2.0	3.9	4.0	4.2	3.9	4.8	4.16
2ip 5.0	3.0	2.9	3.1	3.2	3.9	3.22
Mean	**3.23**	**3.33**	**3.40**	**3.23**	**3.97**	
GRAND MEAN	4.28	4.53	4.86	5.11	6.10	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	-	-	Tree 1 1.350 ±0.249
Tree (T)	4	4.42	0.97^{NS}	Tree 2 0.911 ±0.10
Treatment (E)	8	24.22	5.31***	Tree 3 1.198 ±0.011
BA	2	9.55	2.09^{NS}	Tree 4 0.792 ±0.009
Kn	2	5.58	1.22^{NS}	Tree 5 0.695 ±0.026
2ip	2	2.09	-	
Diff. Betwn. Hormones	2	79.66	17.47***	
T × E	32	0.93	-	
Error	88	4.56		

*** Significant at 0.1% level of probability

NS Non-significant.

Table 11: Effect of growth regulators on percentage of shoot formation from cotyledon explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with five culture tubes per treatment.

Growth regulators (mg/l)	Percentage of shoot forming explants					Mean
	T_1	T_2	T_3	T_4	T_5	
BA 0.5	35.24	26.56	31.05	26.56	32.77	30.44
BA 1.0	39.23	31.05	46.89	35.24	32.77	37.04
BA 2.0	32.77	46.89	35.24	32.77	54.70	40.47
Mean	**35.75**	**34.83**	**37.73**	**31.52**	**40.08**	
BA 2.0 + NAA 0.1	46.89	50.77	32.77	32.77	50.77	42.79
BA 2.0 + NAA 0.2	50.77	54.70	46.89	50.77	58.89	52.40
Mean	**48.83**	**52.73**	**39.83**	**41.77**	**54.83**	
BA 2.0 + IAA 0.1	39.23	46.89	39.23	32.77	50.77	41.78
BA 2.0 + IAA 0.2	32.77	50.77	32.77	46.89	54.40	43.58
Mean	**36.00**	**48.83**	**36.00**	**39.83**	**52.74**	
BA 2.0 + GA_3 0.5	46.89	50.77	50.77	54.70	58.89	52.40
BA 2.0 + GA_3 1.0	35.24	46.89	32.77	46.89	54.70	43.29
Mean	**41.07**	**48.83**	**41.77**	**50.79**	**56.79**	
BA 2.0 + NAA 0.2 + GA_3 0.5	50.77	54.70	54.70	58.89	63.44	56.50
BA 2.0 + NAA 0.2 + GA_3 1.0	32.77	46.89	50.77	46.89	58.89	47.24
Mean	**41.77**	**50.79**	**52.74**	**52.89**	**61.17**	
GRAND MEAN	40.23	46.08	41.26	42.29	51.94	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	145.21	1.59^{NS}	Tree 1 0.668 ±0.249
Tree	4	243.84	2.67*	Tree 2 1.092 ±0.063
Treatment	10	279.29	3.06**	Tree 3 0.599 ±0.032
BA	2	130.02	1.42^{NS}	Tree 4 0.836 ±0.011
BA + NAA	1	230.88	2.53^{NS}	Tree 5 1.204 ±0.019
BA + IAA	1	8.12	-	
BA + GA_3	1	207.30	2.27^{NS}	
BA + NAA + GA_3	1	214.28	2.35^{NS}	
Diff. Betn. Hormones	4	468.06	5.12*	
Tree × Treatment	40	38.68	-	
Error	108	91.33		

* Significant at 5% level of probability
** Significant at 1% level of probability
NS Non-significant.

Table 12: Effect of growth regulators on number of shoots per culture from cotyledon explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with five culture tubes per treatment.

Growth regulators (mg/l)	Number of shoots per culture					Mean
	T_1	T_2	T_3	T_4	T_5	
BA 0.5	4.4	5.6	6.2	8.1	8.3	6.52
BA 1.0	5.2	6.3	6.2	7.2	9.1	6.80
BA 2.0	7.5	7.2	8.5	9.1	11.8	8.82
Mean	**5.70**	**6.37**	**6.97**	**8.13**	**9.73**	
BA 2.0 + NAA 0.1	8.2	9.8	9.8	10.2	14.5	10.5
BA 2.0 + NAA 0.2	12.5	10.2	11.2	12.8	18.8	13.1
Mean	**10.35**	**10.00**	**10.50**	**11.50**	**16.65**	
BA 2.0 + IAA 0.1	8.6	7.7	6.7	11.2	8.7	8.58
BA 2.0 + IAA 0.2	11.4	9.8	8.8	10.4	12.8	10.64
Mean	**10.00**	**8.75**	**7.75**	**10.80**	**10.75**	
BA 2.0 + GA_3 0.5	9.4	9.2	12.7	12.6	16.4	12.06
BA 2.0 + GA_3 1.0	13.2	15.5	16.5	14.2	20.2	15.92
Mean	**11.30**	**12.35**	**14.60**	**13.40**	**18.30**	
BA 2.0 + NAA 0.2 + GA_3 0.5	10.2	11.2	14.5	13.4	17.1	13.28
BA 2.0 + NAA 0.2 + GA_3 1.0	14.3	15.0	15.3	15.2	21.7	16.30
Mean	**12.25**	**13.10**	**14.90**	**14.30**	**19.40**	
GRAND MEAN	9.54	9.77	10.58	11.31	14.49	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	28.59	1.65^{NS}	Tree 1 0.980 ±0.010
Tree	4	44.02	2.54*	Tree 2 1.002 ±0.029
Treatment	10	55.03	3.16**	Tree 3 0.860 ±0.157
BA	2	7.87	-	Tree 4 1.240 ±0.017
BA + NAA	1	16.90	-	Tree 5 0.686 ±0.111
BA + IAA	1	10.61	-	
BA + GA_3	1	37.25	2.16^{NS}	
BA + NAA + GA_3	1	22.80	1.32^{NS}	
Diff. Betn. Hormones	4	111.76	6.45***	
Tree × Treatment	40	1.57	-	
Error	108	17.33		

* Significant at 5% level of probability
** Significant at 1% level of probability
*** Significant at 0.1% level of probability.
NS Non-significant.

Table 13: Effect of cytokinins on percentage of shoot formation from hypocotyl explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with four culture tubes per treatment.

Cytokinins (mg/l)	Percentage of shoot forming explants					Mean
	T_1	T_2	T_3	T_4	T_5	
BA 0.5	60.00	63.44	60.00	50.77	85.20	63.88
BA 1.0	90.00	68.53	58.89	54.70	84.56	71.34
BA 2.0	68.53	58.89	90.00	85.20	90.00	78.52
Mean	**72.84**	**63.62**	**69.63**	**63.56**	**86.59**	
Kn 1.0	49.78	50.77	40.16	43.05	54.70	47.69
Kn 2.0	45.00	40.16	35.24	40.16	50.77	42.27
Kn 5.0	43.05	35.24	31.05	35.24	4689	38.29
Mean	**45.94**	**42.06**	**35.48**	**39.48**	**50.79**	
2ip 1.0	46.89	54.70	45.00	50.77	49.78	49.43
2ip 2.0	40.16	46.89	43.05	40.16	45.00	43.05
2ip 5.0	35.24	39.23	35.24	39.23	40.16	37.82
Mean	**40.76**	**46.94**	**41.09**	**43.39**	**44.98**	
GRAND MEAN	53.18	50.87	48.74	48.81	60.78	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	184.64	2.16*	Tree 1 1.349 ±0.009
Tree	4	223.95	2.62*	Tree 2 1.149 ±0.027
Treatment	8	1128.41	13.20***	Tree 3 0.765 ±0.055
BA	2	268.02	3.14^{NS}	Tree 4 0.880 ±0.022
Kn	2	111.29	1.30^{NS}	Tree 5 0.733 ±0.009
2ip	2	168.98	1.98^{NS}	
Diff. Betn. Hormones	2	3965.36	46.39***	
Tree × Treatment	32	64.93	-	
Error	88	85.48		

* Significant at 5% level of probability
*** Significant at 0.1% level of probability
NS Non-significant.

Table 14: Effect of cytokinins on number of shoots per culture from hypocotyl explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with four culture tubes per treatment.

Cytokinins (mg/l)	Number of shoots per culture					Mean
	T_1	T_2	T_3	T_4	T_5	
BA 0.5	9.6	6.0	8.6	7.2	12.6	8.80
BA 1.0	13.6	10.2	13.3	12.0	14.2	12.66
BA 2.0	11.5	7.4	12.8	9.8	13.3	10.96
Mean	**11.57**	**7.87**	**11.57**	**9.67**	**13.37**	
Kn 1.0	3.5	1.9	2.4	3.0	2.9	2.74
Kn 2.0	5.4	2.7	3.6	2.9	4.3	3.78
Kn 5.0	2.2	2.0	2.6	1.8	3.1	2.34
Mean	**3.70**	**2.20**	**2.87**	**2.57**	**3.43**	
2ip 1.0	2.7	3.7	3.5	3.4	5.6	3.78
2ip 2.0	3.9	4.4	4.4	5.3	6.2	4.84
2ip 5.0	4.0	3.2	4.0	2.9	4.4	3.70
Mean	**3.53**	**3.77**	**3.97**	**3.87**	**5.40**	
GRAND MEAN	6.26	4.61	6.13	5.36	7.4	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	5.56	2.09^{NS}	Tree 1 0.89 ±0.005
Tree	4	9.83	3.69*	Tree 2 1.34 ±0.012
Treatment	8	73.31	27.56***	Tree 3 0.88 ±0.022
BA	2	18.72	7.04**	Tree 4 1.06 ±0.004
Kn	2	2.77	1.04^{NS}	Tree 5 0.82 ±0.004
2ip	2	2.03	-	
Diff. Betn. Hormones	2	269.74	101.41***	
Tree × Treatment	32	1.14	-	
Error	88	2.66		

* Significant at 5% level of probability
** Significant at 1% level of probability
*** Significant at 0.1% level of probability
NS Non-significant.

Table 15: Effect of growth regulators on percentage of shoot formation from hypocotyl explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with four culture tubes per treatments.

Growth regulators (mg/l)	Percentage of shoot forming explants					Mean
	T_1	T_2	T_3	T_4	T_5	
BA 0.5	43.05	43.05	60.00	54.70	90.00	58.16
BA 1.0	65.88	54.70	73.15	58.89	75.00	65.52
BA 2.0	90.00	60.00	65.88	73.15	73.15	72.44
Mean	**66.31**	**52.58**	**66.34**	**62.25**	**79.38**	
BA 2.0 + NAA 0.1	90.00	65.88	60.00	58.89	73.15	69.58
BA 2.0 + NAA 0.2	75.00	73.15	73.15	90.00	90.00	80.26
Mean	**82.50**	**69.52**	**66.58**	**74.45**	**81.58**	
BA 2.0 + IAA 0.1	54.70	54.70	65.88	60.00	63.44	59.74
BA 2.0 + IAA 0.2	65.88	60.00	63.44	75.00	73.15	67.49
Mean	**6029**	**57.35**	**64.66**	**67.50**		
BA 2.0 + GA_3 0.5	46.89	45.00	40.16	35.24	49.78	43.41
BA 2.0 + GA_3 1.0	43.05	40.16	31.05	43.05	45.00	40.46
Mean	**44.97**	**42.58**	**50.13**	**39.15**	**68.30**	
BA 2.0 + NAA 0.2 + GA_3 0.5	45.00	49.78	43.05	45.00	50.77	46.72
BA 2.0 + NAA 0.2 + GA_3 1.0	43.05	50.77	35.24	40.16	49.78	43.80
Mean	**44.03**	**50.28**	**39.15**	**42.58**	**50.28**	
GRAND MEAN	60.23	54.29	55.55	57.64	66.66	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	61.24	1.19^{NS}	Tree 1 0.621 ±0.0.10
Tree	4	264.01	5.13**	Tree 2 1.176±0.063
Treatment	10	914.37	17.77***	Tree 3 1.226 ±0.032
BA	2	254.84	4.95*	Tree 4 0.739 ±0.011
BA + NAA	1	284.94	5.54*	Tree 5 0.717 ±0.029
BA + IAA	1	150.16	2.92^{NS}	
BA + GA_3	1	21.78	-	
BA + NAA + GA_3	1	21.31	-	
Diff. Betn. Hormones	4	2038.97	39.62***	
Tree × Treatment	40	73.79	1.43*	
Error	108	51.46		

* Significant at 5% level of probability
** Significant at 1% level of probability
*** Significant at 0.1% level of probability.
NS Non-significant.

Table 16: Effect of growth regulators on number of shoots per culture from hypocotyl explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with four culture tubes per treatments.

Growth regulators (mg/l)	Number of shoots per culture					Mean
	T_1	T_2	T_3	T_4	T_5	
BA 0.5	4.2	6.5	6.8	5.3	9.9	6.54
BA 1.0	8.6	9.4	7.2	8.2	11.4	8.96
BA 2.0	9.1	8.2	10.3	7.2	12.8	9.52
Mean	**7.3**	**7.67**	**7.81**	**7.58**	**8.34**	
BA 2.0 + NAA 0.1	7.3	7.5	11.2	8.2	13.5	9.52
BA 2.0 + NAA 0.2	12.8	10.0	12.3	9.5	12.9	11.5
Mean	**8.54**	**8.56**	**11.75**	**8.87**	**12.48**	
BA 2.0 + IAA 0.1	6.5	9.6	8.3	8.6	11.2	8.84
BA 2.0 + IAA 0.2	8.2	10.2	9.5	10.3	10.6	9.76
Mean	**7.35**	**8.63**	**8.72**	**9.45**	**10.90**	
BA 2.0 + GA_3 0.5	12.6	15.2	13.6	8.9	13.8	12.82
BA 2.0 + GA_3 1.0	10.8	13.2	12.2	10.2	12.4	11.76
Mean	**11.70**	**12.95**	**12.90**	**9.55**	**13.10**	
BA 2.0 + NAA 0.2 + GA_3 0.5	9.1	12.2	11.4	9.8	10.0	10.5
BA 2.0 + NAA 0.2 + GA_3 1.0	14.3	15.4	14.3	12.0	13.5	13.9
Mean	**11.70**	**13.80**	**12.85**	**10.90**	**11.75**	
GRAND MEAN	9.40	10.67	10.64	8.93	12.0	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	11.04	3.21*	Tree 1 0.892 ±0.249
Tree	4	16.01	4.65**	Tree 2 0.623 ±0.063
Treatment	10	21.29	6.19***	Tree 3 0.777 ±0.099
BA	2	12.54	3.65*	Tree 4 1.021 ±0.009
BA + NAA	1	9.80	2.85^{NS}	Tree 5 0.967 0.100
BA + IAA	1	2.12	-	
BA + GA_3	1	2.81	-	
BA + NAA + GA_3	1	28.9	-	
Diff. Betn. Hormones	4	36.05	10.48***	
Tree × treatment	40	1.98	-	
Error	108	3.44		

* Significant at 5% level of probability
** Significant at 1% level of probability
*** Significant at 0.1% level of probability.
NS Non-significant.

Table 17: Effect of cytokinins on percentage of shoot formation from leaf explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with four culture tubes per treatments.

Cytokinins (mg/l)	Percentage of shoot forming explants					Mean
	T_1	T_2	T_3	T_4	T_5	
BA 0.5	35.24	30.00	30.00	35.24	40.16	34.13
BA 1.0	40.16	35.24	35.24	40.16	45.00	39.16
BA 2.0	49.78	45.00	40.16	45.00	49.78	45.94
Mean	**41.73**	**36.75**	**35.13**	**40.13**	**44.98**	
Kn 1.0	30.00	20.70	14.42	20.70	30.00	23.16
Kn 2.0	30.00	35.24	20.70	30.00	35.24	30.24
Kn 5.0	40.16	40.16	35.24	35.24	40.16	38.19
Mean	**33.39**	**32.03**	**23.45**	**28.65**	**35.13**	
2ip 1.0	20.70	14.42	14.42	20.70	30.00	20.05
2ip 2.0	30.00	20.70	20.70	20.70	30.00	24.42
2ip 5.0	35.24	30.00	20.70	30.00	35.24	30.24
Mean	**28.65**	**21.71**	**18.61**	**23.80**	**31.75**	
GRAND MEAN	34.59	30.16	25.73	30.86	37.29	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	39.70	2.0^{NS}	Tree 1 0.975 ±0.249
Tree	4	176.04	8.87***	Tree 2 0.801 ±0.010
Treatment	8	357.81	18.03***	Tree 3 0.850 ±0.011
BA	2	175.81	8.86***	Tree 4 0.930 ±0.031
Kn	2	282.63	14.24***	Tree 5 1.169 ±0.26
2ip	2	130.61	6.58***	
Diff. Betn. Hormones	2	842.19	42.43***	
Tree × Treatment	32	8.15	-	
Error	88	19.85		

*** Significant at 0.1% level of probability

NS Non-significant

Table 18: Effect of cytokinins on number of shoots per culture from leaf explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with four culture tubes per treatments.

Cytokinins (mg/l)	Number of shoots per culture					Mean
	T_1	T_2	T_3	T_4	T_5	
BA 0.5	3.6	3.5	2.6	2.2	3.5	3.08
BA 1.0	4.3	3.7	2.8	3.9	5.2	3.98
BA 2.0	5.2	4.6	3.5	4.2	6.7	4.84
Mean	**4.37**	**3.93**	**2.97**	**3.43**	**5.13**	
Kn 1.0	1.7	2.2	1.3	1.5	2.4	1.82
Kn 2.0	2.3	3.3	2.5	3.1	3.7	2.98
Kn 5.0	2.5	2.4	2.1	2.5	3.2	2.54
Mean	**2.17**	**2.63**	**1.97**	**2.37**	**3.10**	
2ip 1.0	1.7	2.3	1.8	2.2	2.5	2.1
2ip 2.0	3.3	3.2	4.2	3.5	4.2	3.68
2ip 5.0	2.7	2.6	2.1	2.7	3.8	2.78
Mean	**2.57**	**2.70**	**2.70**	**2.80**	**3.50**	
GRAND MEAN	3.03	3.09	2.54	2.87	3.91	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	3.71	3.34*	Tree 1 0.766 ±0.020
Tree	4	2.56	2.43*	Tree 2 1.131 ±0.186
Treatment	8	4.53	4.08***	Tree 3 0.897 ±0.015
BA	2	3.87	3.49*	Tree 4 1.006 ±0.170
Kn	2	1.72	1.55^{NS}	Tree 5 0.606 ±0.025
2ip	2	3.14	2.83^{NS}	
Diff. Betn. Hormones	2	9.39	8.46***	
Tree × Treatment	32	0.21	-	
Error	88	1.11		

* Significant at 5% level of probability
*** Significant at 0.1% level of probability
NS Non-significant.

Table 19: Effect of growth regulators on percentage of shoot formation from leaf explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with four culture tubes per treatments.

Growth regulators (mg/l)	Percentage of shoot forming explants					Mean
	T_1	T_2	T_3	T_4	T_5	
BA 0.5	35.24	30.00	30.00	35.24	40.16	35.13
BA 1.0	40.16	35.24	35.24	40.16	45.00	39.16
BA 2.0	43.05	45.00	40.16	45.00	49.78	44.60
Mean	**39.48**	**36.75**	**35.13**	**40.13**	**44.98**	
BA 2.0 + NAA 0.1	49.78	45.00	49.78	54.70	60.00	51.85
BA 2.0 + NAA 0.2	45.00	54.70	45.00	49.78	54.70	49.84
Mean	**47.39**	**49.85**	**47.39**	**52.24**	**57.35**	
BA 2.0 + IAA 0.1	45.00	49.78	40.16	45.00	49.78	45.94
BA 2.0 + IAA 0.2	35.24	40.16	35.24	40.16	45.00	39.16
Mean	**40.12**	**44.97**	**37.70**	**42.58**	**44.99**	
BA 2.0 + GA_3 0.5	40.16	30.00	30.00	35.24	40.16	35.11
BA 2.0 + GA_3 1.0	43.05	40.16	35.24	45.00	49.78	42.65
Mean	**41.61**	**35.08**	**32.62**	**40.12**	**44.97**	
BA 2.0 + NAA 0.2 + GA_3 0.5	35.24	40.16	30.00	30.00	40.16	35.11
BA 2.0 + NAA 0.2 + GA_3 1.0	43.05	45.00	35.24	40.16	45.00	41.69
Mean	**39.15**	**42.58**	**32.62**	**35.08**	**42.58**	
GRAND MEAN	41.36	41.38	36.91	41.86	47.23	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	68.94	3.86*	Tree 1 1.103 ±0.053
Tree	4	147.68	8.27***	Tree 2 0.823 ±0.211
Treatment	10	176.79	9.90***	Tree 3 0.975 ±0.142
BA	2	137.10	7.68**	Tree 4 0.898 ±0.011
BA + NAA	1	10.16	-	Tree 5 0.906 0.003
BA + IAA	1	115.05	6.44*	
BA + GA_3	1	141.90	7.95**	
BA + NAA + GA_3	1	108.17	6.06*	
Diff. Betn. Hormones	4	279.61	15.66***	
Tree × Treatment	40	70.64	3.99*	
Error	108	17.86		

* Significant at 5% level of probability
** Significant at 1% level of probability
*** Significant at 0.1% level of probability.

Table 20: Effect of growth regulators on number of shoots per culture from leaf explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with four culture tubes per treatments.

Growth regulators (mg/l)	Number of shoots per culture					Mean
	T_1	T_2	T_3	T_4	T_5	
BA 0.5	2.7	2.5	2.6	2.4	3.5	2.74
BA 1.0	3.5	2.8	2.8	3.9	4.9	3.58
BA 2.0	5.2	4.5	3.5	4.4	5.8	4.68
Mean	**3.48**	**3.27**	**2.97**	**3.57**	**4.73**	
BA 2.0 + NAA 0.1	8.9	7.3	5.4	6.4	10.6	7.72
BA 2.0 + NAA 0.2	6.5	5.8	4.2	5.2	8.2	5.98
Mean	**7.70**	**6.55**	**4.80**	**5.80**	**9.40**	
BA 2.0 + IAA 0.1	8.9	7.9	6.6	8.6	9.8	8.36
BA 2.0 + IAA 0.2	6.3	6.2	4.9	5.8	7.6	6.16
Mean	**7.60**	**7.05**	**5.75**	**7.20**	**8.70**	
BA 2.0 + GA_3 0.5	3.8	3.3	2.1	4.3	4.9	3.68
BA 2.0 + GA_3 1.0	4.3	4.9	3.9	5.5	6.6	5.04
Mean	**4.05**	**4.10**	**3.0**	**4.9**	**5.75**	
BA 2.0 + NAA 0.2 + GA_3 0.5	5.1	4.9	3.9	4.7	8.3	5.38
BA 2.0 + NAA 0.2 + GA_3 1.0	7.8	6.6	4.2	5.6	9.8	6.80
Mean	**6.45**	**5.75**	**4.05**	**5.15**	**9.05**	
GRAND MEAN	5.72	5.15	4.0	5.16	7.27	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	6.53	3.25*	Tree 1 0.803 ±0.013
Tree	4	38.71	19.26***	Tree 2 0.971 ±0.020
Treatment	10	6.21	3.09**	Tree 3 1.278 ±0.278
BA	2	4.73	2.35^{NS}	Tree 4 1.036 ±0.036
BA + NAA	1	7.56	3.76^{NS}	Tree 5 0.727 ±0.033
BA + IAA	1	12.10	6.02*	
BA + GA_3	1	4.62	2.29^{NS}	
BA + NAA + GA_3	1	5.04	2.51^{NS}	
Diff. Betn. Hormones	4	5.83	2.90^{NS}	
Tree × Treatment	40	0.48	-	
Error	108	2.01		

* Significant at 5% level of probability
** Significant at 1% level of probability
*** Significant at 0.1% level of probability.
NS Non-significant.

Table 21: Effect of cytokinins on percentage of shoot formation from nucellar explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with four culture tubes per treatments.

Cytokinins (mg/l)	Percentage of shoot forming explants					Mean
	T_1	T_2	T_3	T_4	T_5	
BA 0.5	10.47	18.44	14.89	10.47	14.89	13.83
BA 1.0	21.39	22.79	18.44	21.39	24.04	21.61
BA 2.0	18.44	14.89	10.47	18.44	22.79	17.01
Mean	**16.77**	**18.71**	**14.60**	**16.77**	**20.57**	
Kn 1.0	10.47	14.89	10.47	10.47	14.89	21.24
Kn 2.0	21.39	18.44	14.89	18.44	21.39	18.21
Kn 5.0	14.89	18.44	10.47	14.89	18.44	15.43
Mean	**15.58**	**17.26**	**11.94**	**14.60**	**18.24**	
2ip 1.0	10.47	10.47	14.89	10.47	14.89	12.24
2ip 2.0	14.89	18.44	10.47	18.44	18.44	16.16
2ip 5.0	14.89	14.89	10.47	14.89	14.89	14.01
Mean	**13.42**	**14.60**	**11.94**	**14.60**	**16.07**	
GRAND MEAN	15.26	16.85	12.83	15.32	18.30	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	14.07	3.18^{NS}	Tree 1 0.665 ±0.011
Tree	4	37.47	8.26***	Tree 2 0.687 ±0.029
Treatment	8	48.46	10.67***	Tree 3 0.520 ±0.010
BA	2	76.48	16.85***	Tree 4 0.692 ±0.249
Kn	2	55.68	12.26***	Tree 5 0.783 ±0.63
2ip	2	19.50	4.30*	
Diff. Betn. Hormones	2	42.63	9.39***	
Tree × Treatment	32	5.71	1.26^{NS}	
Error	88	4.54		

* Significant at 5% level of probability
*** Significant at 0.1% level of probability
NS Non-significant.

Table 22: Effect of cytokinins on number of shoots per culture from nucellar explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with four culture tubes per treatments.

Cytokinins (mg/l)	Number of shoots per culture					Mean
	T_1	T_2	T_3	T_4	T_5	
BA 0.5	2.1	2.8	2.1	2.6	4.3	2.78
BA 1.0	3.4	3.4	2.8	4.2	4.5	3.66
BA 2.0	3.8	2.5	1.7	3.4	3.7	3.02
Mean	**3.10**	**2.90**	**2.20**	**3.40**	**4.17**	
Kn 1.0	1.4	2.3	1.3	1.9	2.3	1.84
Kn 2.0	2.8	2.8	2.4	2.1	2.6	2.54
Kn 5.0	1.5	2.2	2.7	1.8	3.8	2.40
Mean	**1.90**	**2.43**	**2.13**	**1.93**	**2.90**	
2ip 1.0	2.1	1.8	1.7	1.8	2.7	2.02
2ip 2.0	2.7	3.5	2.1	2.6	3.2	2.82
2ip 5.0	2.9	2.1	1.4	2.1	2.8	2.26
Mean	**2.57**	**2.47**	**1.73**	**2.17**	**2.90**	
GRAND MEAN	2.52	2.60	2.02	2.50	3.32	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	0.84	1.16^{NS}	Tree 1 0.486 ±0.249
Tree	4	1.96	2.72*	Tree 2 0.735 ±0.029
Treatment	8	1.54	2.13*	Tree 3 0.645 ±0.111
BA	2	0.86	1.19^{NS}	Tree 4 0.629 ±0.022
Kn	2	0.69	-	Tree 5 0.568 ±0.009
2ip	2	0.84	1.17^{NS}	
Diff. Betn. Hormones	2	3.77	5.24**	
Tree × Treatment	32	0.26	-	
Error	88	0.72		

* Significant at 5% level of probability
** Significant at 1% level of probability
NS Non-significant.

Table 23: Effect of growth regulators on percentage of shoot formation from nucellar explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with four culture tubes per treatments.

Growth regulators (mg/l)	Percentage of shoot forming explants					Mean
	T_1	T_2	T_3	T_4	T_5	
BA 0.5	10.47	18.44	14.89	10.47	14.89	13.83
BA 1.0	21.39	22.79	18.44	21.39	24.04	21.61
BA 2.0	18.44	14.89	10.47	18.44	22.79	17.01
Mean	**16.77**	**18.71**	**14.60**	**16.77**	**20.57**	
BA 2.0 + NAA 0.1	31.05	28.86	27.69	30.00	32.14	29.95
BA 2.0 + NAA 0.2	32.14	30.00	28.86	31.05	34.20	31.25
Mean	**31.60**	**29.43**	**28.28**	**30.53**	**33.17**	
BA 2.0 + IAA 0.1	27.69	25.33	22.79	25.33	28.86	26.00
BA 2.0 + IAA 0.2	28.86	26.56	24.04	27.69	31.05	27.64
Mean	**28.28**	**25.95**	**23.42**	**26.51**	**29.96**	
BA 2.0 + GA_3 0.5	22.79	21.39	18.44	22.79	25.33	22.15
BA 2.0 + GA_3 1.0	25.33	22.79	21.39	25.33	27.69	24.51
Mean	**24.06**	**22.09**	**19.92**	**24.06**	**26.51**	
BA 2.0 + NAA 0.2 + GA_3 0.5	27.69	26.56	25.33	28.86	31.05	27.90
BA 2.0 + NAA 0.2 + GA_3 1.0	31.05	28.86	27.69	30.00	32.14	29.95
Mean	**29.37**	**27.71**	**26.51**	**29.43**	**31.60**	
GRAND MEAN	25.17	24.22	21.82	24.67	27.65	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	15.89	1.08^{NS}	Tree 1 0.844 ±0.008
Tree	4	48.25	3.28*	Tree 2 1.124 ±0.028
Treatment	10	155.98	10.60***	Tree 3 0.917 ±0.040
BA	2	76.48	5.20**	Tree 4 0.889 ±0.020
BA + NAA	1	4.24	-	Tree 5 0.978 ±0.031
BA + IAA	1	6.63	-	
BA + GA_3	1	13.90	-	
BA + NAA + GA_3	1	13.90	-	
Diff. Betn. Hormones	4	342.88	23.31***	
Tree × Treatment	40	2.87	-	
Error	108	14.71		

* Significant at 5% level of probability
** Significant at 1% level of probability
*** Significant at 0.1% level of probability.
NS Non-significant.

Table 24: Effect of growth regulators on number of shoots per culture from nucellar explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with four culture tubes per treatments.

Growth regulators (mg/l)	Number of shoots per culture					Mean
	T_1	T_2	T_3	T_4	T_5	
BA 0.5	2.1	2.8	1.8	2.6	4.3	2.72
BA 1.0	4.4	3.4	2.8	4.2	6.5	4.26
BA 2.0	3.8	2.6	2.1	3.0	4.7	3.24
Mean	**3.43**	**2.93**	**2.23**	**3.27**	**5.17**	
BA 1.0 + NAA 0.1	24.4	18.6	15.0	23.2	29.7	22.18
BA 1.0 + NAA 0.2	21.5	17.8	16.2	18.4	24.8	19.74
Mean	**22.95**	**18.20**	**15.60**	**20.80**	**27.25**	
BA 1.0 + IAA 0.1	22.6	19.7	17.9	18.8	23.0	20.40
BA 1.0 + IAA 0.2	17.2	15.6	14.9	16.6	19.7	16.80
Mean	**19.90**	**17.65**	**16.40**	**17.70**	**21.35**	
BA 1.0 + GA_3 0.5	12.9	12.4	11.8	12.1	13.4	12.52
BA 1.0 + GA_3 1.0	15.6	14.5	14.5	15.2	16.3	15.22
Mean	**14.25**	**13.45**	**13.15**	**13.65**	**14.85**	
BA 1.0 + NAA 0.2 + GA_3 0.5	16.5	15.6	14.3	15.2	27.4	17.80
BA 1.0 + NAA 0.2 + GA_3 1.0	12.0	19.7	18.4	20.4	24.6	19.02
Mean	**14.25**	**17.65**	**16.35**	**17.80**	**26.0**	
GRAND MEAN	13.91	12.97	11.79	13.61	17.67	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	20.76	0.96^{NS}	Tree 1 0.887 ±0.125
Tree	4	53.86	2.49*	Tree 2 1.055 ±0.026
Treatment	10	264.93	12.25***	Tree 3 1.101 ±0.249
BA	2	3.07	-	Tree 4 0.991 ±0.011
BA + NAA	1	14.88-	-	Tree 5 0.759 0.178
BA + IAA	1	32.40	1.50^{NS}	
BA + GA_3	1	18.22	-	
BA + NAA + GA_3	1	3.72	-	
Diff. Betn. Hormones	4	643.49	29.75***	
Tree × Treatment	40	5.34	-	
Error	108	21.63		

* Significant at 5% level of probability
*** Significant at 0.1% level of probability.
NS Non-significant.

of shoots per culture was obtained using 2.0 mgl^{-1} BA and 0.1 or 0.2 mgl^{-1} NAA with or without 1.0 mgl^{-1} GA_3. However, better shoot growth (healthy green and long) was obtained when 1.0 mgl^{-1} GA_3 was added.

In the analysis of variance, the items tree and treatment (E) were significant against the experimental error indicating that a real difference existed among trees and treatments.

3.2 INDUCTION OF ORGANOGENIC CALLUS

The growth of a callus culture over a period of time is characterized by an increase in cell number, an increase in volume or mass and changes in biochemistry and cellular complexity. The growth rates of callus cultures were highly influenced by different treatment concentrations and combinations of growth regulators. The quantitative measurement of callus growth was estimated in terms of percentage of callus forming explant and fresh weight (g) of calli per culture obtained from different treatment concentrations and combinations after 8 weeks of incubation. The results of effect of MS media and different growth regulators on percentage of callus forming explant and fresh weight of calli per culture derived from embryo, cotyledon, hypocotyl, leaf and nucellus explants of 5 trees. The results are discussed according to type of explants under separate heads.

3.2.1 Effect of Auxins either Singly or in Combination with Cytokinin on Callus Formation from Embryo Explants

Embryo explants of 5 trees were cultured onto MS medium supplemented with 11 different concentrations and combinations of 2,4-D, NAA, IAA, BA, Kn & 2ip. Results obtained on callus differentiation of the cultured explants are shown in Tables 25 & 26.

Callus proliferation was noticed in all media formulations, however, there was a wide range of variation among them. First visible changes of the cultured explant occurred through lateral swelling. However, localized callus proliferation was also noticed at the end of 6 weeks of culture (plate 5A). Colour and texture of the callus was found to vary with hormonal supplement. Texture of the calli was hard, spongy and friable and colour was green, white, greenish white, greenish yellow and yellowish white.

Among the three auxin concentrations, 5.0 mgl^{-1} 2,4-D induced the highest percentage of explant to form callus and fresh weight of calli per culture. Calli proliferated in 5.0 mgl^{-1} 2,4-D were spongy to hard and yellowish white. This calli failed to show any organogenesis. Calli proliferated with NAA or IAA from 5.0 mgl^{-1} were spongy, greenish-white and composed of rapidly growing large vacuolated cells. This type of calli did not show organogenesis during 8 weeks of culture. Lowest frequency of callus formation (46.14%) and lowest fresh weight of callus (0.55 g) were recorded at 5.0 mgl^{-1} IAA.

Frequency of callus forming explant and fresh weight of callus were more when 2,4-D was used with 1.0 & 2.0 mgl^{-1} BA, Kn or 2ip. The highest frequency of explants formed calli in media having 5.0 mgl^{-1} 2,4-D + 2.0 mgl^{-1} Kn and the calli were hard, green to greenish yellow and composed of small compact cells. High concentration (5.0 gml^{-1}) of 2,4-D in combinations with (1.0 and 2.0 mgl^{-1}) BA, Kn or 2ip showed promotive effect on callus induction.

The analysis of variance reveals that morphogenic differentiation to frequency of callusing of 5 trees were significantly different. Variable effect of different treatment concentrations and combinations on frequency of callusing was also highly significant. Nevertheless, T×E interaction concerning with callusing was non-significant.

3.2.2 Effect of Auxins alone or in combination with Cytokinins on Callus Development from Cotyledon Explants

The cotyledon explants of 5 different trees were cultured onto MS medium supplemented with different concentrations and combinations of auxins and cytokinins. Results on morphogenic potentialities of the explant at different concentrations and combinations of auxins and cytokinins are given in Tables 27 & 28. The response to callus greatly varied with types of explants (trees) and treatment concentrations and combinations. Usually, callus proliferation began from cut surface of the explant (plate 5A). Localized callus proliferation during later period of 8 weeks culture (plate 5B) was also recorded. Calli formed in media with higher concentration of auxins (5.0 mgl^{-1}) were spongy, white to yellowish white. Calli proliferated in auxins employed media did not show organogenic differentiation. Among the three auxins, 2,4-D, showed the best frequency of callusing and fresh weight of callus per culture and the optimum level was 5.0 mgl^{-1}.

Cytokinins with auxins (2,4-D) in culture media showed promotive effect on the frequency of callusing and fresh weight of callus. Calli proliferated in media having 2,4-D (5.0 mgl^{-1}) with BA, Kn and 2ip (1.0 & 2.0 mgl^{-1}) were soft, white or greenish white. Effect of Kn with 2,4-D on callus initiation was more pronounced than in combinations with BA and 2ip. The highest frequency of callusing (65.86%) and fresh weight of calli per culture (1.15 g) were recorded at 5.0 mgl^{-1} 2,4-D + 2.0 mgl^{-1} Kn (plate 5B). Frequency of callusing and fresh weight of calli were comparatively low when the explants were cultured in media with 5.0 mgl^{-1} NAA or IAA and 2.0 mgl^{-1} Kn.

The results of the analysis of variance (table 27 & 28) show that significant differences existed among trees and treatments. All the interaction items were significant in both the characters except that the item T×E was non-significant.

3.2.3 Effect of Auxins either singly or in combination with Cytokinins on Callusing of Hypocotyl Explant

The responses of the hypocotyl explants were found to vary with growth regulators formulations present in the culture media (table 29 & 30). Callus proliferation began at the cut surfaces of the explant. Colour and texture of the callus was found to vary with hormonal supplement. The texture of calli was hard, spongy and friable and colour was green, white, greenish white, greenish yellow and yellowish white. Among the three concentrations (5.0 mgl^{-1} 2,4-D, NAA & IAA) of auxins, highest frequency (60.10%) of callusing and fresh weight of calli per culture (0.89 g) were recorded at 5.0 mgl^{-1} 2,4-D. The other auxins NAA and IAA showed lower frequency of callusing and degree of callus proliferation. The calli in auxins employed media failed to show any organogenesis. Calli proliferated in auxins were spongy to hard and yellowish-white.

Degree of callusing considerably increased when an auxin was combined with 1.0 or 2.0 mgl^{-1} BA, Kn or 2ip. Among eight 2,4-D—BA, 2,4-D—Kn, 2,4-D—2ip, NAA—Kn and IAA—Kn formulations, maximum frequency of callusing and highest (plate 5B) fresh weight of callus (79.71% and 1.65 g) were record at 5.0 mgl^{-1} 2,4-D + 2.0 mgl^{-1} Kn, at the end of 8 weeks of culture. Calli developed in auxin-cytokinin fortified media were hard green or friable, white to greenish white. More or less these calli showed organogenesis potentiality.

In the analysis of variance, the main items were significant against experimental error except the interaction item (T×E). The regression analysis showed that the trees interacted significantly with the treatments (Figs. 5 & 6).

3.2.4 Effect of Auxins either singly or in combination with cytokinins on callusing of *in vitro* grown leaf explants

The leaf explants of 5 trees were cultured on MS medium supplemented with auxins at 5.0 mgl^{-1} level either singly or in combination with cytokinins at 1.0 or 2.0

mgl^{-1} levels for induction and proliferation of callus. Result concerning with effect of these cultured treatment on percentage of callus formation and fresh weight of callus are given in Tables 31 & 32.

The callus induction ability of the leaves of all trees was markedly influenced by the culture treatment. Callus proliferation started at the cut surface of the explant. The media with 2.0 mgl^{-1} Kn and 5.0 mgl^{-1} 2,4-D was more effective for callus induction that other concentrations and combinations (plate 4B) with 2,4-D— Kn, 2,4-D— BA, 2,4-D—2ip, NAA- Kn & IAA- Kn treatments.

The analysis of variance showed that a real difference existed among the trees and treatments but the item T×E was non-significant. The regression analysis showed that the trees interacted significantly with the treatments (Figs. 5 & 6).

3.2.5 Effect of Auxins alone or in combination with Cytokinins on Callusing of Nucellus Explants

Different concentrations of 2,4-D, NAA and IAA alone or in combination with BA, Kn and 2ip were used for callus induction when MS medium was supplemented with 5.0 mgl^{-1} of 2,4-D, NAA and IAA, the explants produced callus but the growth of callus was very poor and the frequency of callusing was low (table 33 & 34). Therefore, a low concentration (1.0 & 2.0 mgl^{-1}) of BA, Kn & 2ip were added to increase the frequency of callusing. Kn-NAA was found to be more efficient than other auxins-cytokinins combinations for callus induction. Maximum response was obtained in medium when 5.0 mgl^{-1} NAA and 2.0 mgl^{-1} Kn were added. The growing callus was transferred in same fresh medium at 15 days intervals.

This 2-month-old culture consisted of a hard, compact, irregular-shaped callus with fresh weight ranging from 0.32-0.72 g per culture.

Table 25: Effect of auxins either singly or incombination with cytokinins on percentage of callus formation from embryo explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with five culture tubes per treatment.

Growth regulators (mg/l)	Percentage of callus forming explant					Mean
	T_1	T_2	T_3	T_4	T_5	
5.0 2,4-D	54.70	63.44	46.89	54.70	58.89	55.72
5.0 NAA	50.77	46.89	43.05	50.77	54.70	49.24
5.0 IAA	46.89	50.77	39.23	43.05	50.77	46.14
5.0 2,4-D + 1.0 BA	54.70	58.89	50.77	46.89	58.89	54.03
5.0 2,4-D + 2.0 BA	63.44	68.53	54.70	50.77	68.53	61.19
5.0 2,4-D + 1.0 Kn	68.53	63.44	58.89	63.44	75.00	65.86
5.0 2,4-D + 2.0 Kn	75.00	68.53	63.44	68.53	90.00	73.10
5.0 2,4-D + 1.0 2ip	54.70	58.89	50.77	54.70	58.89	55.59
5.0 2,4-D + 2.0 2ip	58.89	63.44	54.70	58.89	63.44	59.87
5.0 NAA + 2.0 Kn	54.70	50.77	46.89	54.70	58.89	53.19
5.0 IAA + 2.0 Kn	50.77	46.89	43.05	50.77	54.70	49.24
Mean	**57.55**	**58.23**	**50.22**	**54.29**	**62.97**	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	143.89	3.05^{NS}	Tree 1 0.936 ±0.010
Tree	4	248.13	5.26**	Tree 2 0.823 ±0.032
Treatment	10	313.82	6.65***	Tree 3 1.056 ±0.099
Tree × Treatment	40	13.65	-	Tree 4 0.959 ±0.029
Error	108	47.17		Tree 5 1.005 ±0.037

** Significant at 1% level of probability
*** Significant at 0.1% level of probability
NS Non-significant.

Table 26: Effect of auxins either singly or incombination with cytokinins on fresh weight of calli per culture from embryo explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with five culture tubes per treatment.

Growth regulators (mg/l)	Fresh weight of calli per culture (g)					Mean
	T_1	T_2	T_3	T_4	T_5	
5.0 2,4-D	0.78	0.72	0.63	0.68	0.82	0.73
5.0 NAA	0.69	0.63	0.55	0.58	0.73	0.64
5.0 IAA	0.56	0.55	0.49	0.52	0.67	0.55
5.0 2,4-D + 1.0 BA	0.94	0.86	0.78	0.83	1.00	0.88
5.0 2,4-D + 2.0 BA	1.10	1.01	0.89	0.99	1.23	1.04
5.0 2,4-D + 1.0 Kn	1.31	1.25	1.21	1.36	1.62	1.35
5.0 2,4-D + 2.0 Kn	1.50	0.94	1.37	1.56	1.79	1.54
5.0 2,4-D + 1.0 2ip	0.99	1.23	0.81	0.97	1.07	0.97
5.0 2,4-D + 2.0 2ip	1.12	1.08	1.00	1.09	1.28	1.14
5.0 NAA + 2.0 Kn	1.22	0.99	1.13	1.20	1.33	1.19
5.0 IAA + 2.0 Kn	1.14	0.97	1.06	1.14	1.26	1.12
Mean	**1.03**	**0.93**	**0.90**	**0.99**	**1.16**	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	0.09	2.87^{NS}	Tree 1 1.079 ±0.104
Tree	4	0.103	3.43*	Tree 2 0.966 ±0.064
Treatment	10	0.45	16.09***	Tree 3 1.059 ±0.008
Tree × Treatment	40	0.003	-	Tree 4 0.926 ±0.028
Error	108	0.03		Tree 5 0.849 ±0.109

* Significant at 5% level of probability
*** Significant at 0.1% level of probability
NS Non-significant.

Table 27: Effect of auxins alone or incombinations with cytokinins on percentage of callus formation from cotyledon explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with five culture tubes per treatment.

Growth regulators (mg/l)	Percentage of callus forming explant					Mean
	T_1	T_2	T_3	T_4	T_5	
5.0 2,4-D	50.77	58.89	43.05	50.77	54.70	51.64
5.0 NAA	46.89	46.89	39.23	46.89	50.77	46.13
5.0 IAA	43.05	50.77	35.24	43.05	46.89	43.80
5.0 2,4-D + 1.0 BA	54.70	58.89	46.89	54.70	58.89	54.81
5.0 2,4-D + 2.0 BA	58.89	58.89	50.77	58.89	63.44	58.18
5.0 2,4-D + 1.0 Kn	58.89	63.44	54.70	58.89	68.53	60.89
5.0 2,4-D + 2.0 Kn	63.44	68.53	58.89	63.44	75.0	65.86
5.0 2,4-D + 1.0 2ip	50.77	54.70	46.89	50.77	54.70	51.57
5.0 2,4-D + 2.0 2ip	54.70	58.89	50.77	54.70	58.89	55.59
5.0 NAA + 2.0 Kn	50.77	46.89	43.05	50.77	54.70	49.24
5.0 IAA + 2.0 Kn	46.89	50.77	39.23	46.89	50.77	46.91
Mean	**52.71**	**56.14**	**46.25**	**52.71**	**57.93**	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	78.68	3.32*	Tree 1 1.088 ±0.044
Tree	4	219.71	9.27***	Tree 2 0.902 ±0.134
Treatment	10	225.56	9.52***	Tree 3 0.922 ±0.007
Tree × Treatment	40	4.01	-	Tree 4 1.088 ±0.031
Error	108	23.70		Tree 5 0.999 ±0.099

* Significant at 5% level of probability
*** Significant at 0.1% level of probability

Table 28: Effect of auxins alone or incombination with cytokinins on fresh weight of calli per culture from cotyledon explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with five culture tubes per treatment.

Growth regulators (mg/l)	Fresh weight of calli per culture (g)					Mean
	T_1	T_2	T_3	T_4	T_5	
5.0 2,4-D	0.58	0.55	0.44	0.50	0.60	0.53
5.0 NAA	0.50	0.47	0.39	0.46	0.54	0.47
5.0 IAA	0.42	0.43	0.37	0.41	0.46	0.42
5.0 2,4-D + 1.0 BA	0.82	0.75	0.65	0.74	0.88	0.77
5.0 2,4-D + 2.0 BA	0.91	0.86	0.73	0.83	0.94	0.85
5.0 2,4-D + 1.0 Kn	1.08	0.98	0.84	0.96	1.12	1.00
5.0 2,4-D + 2.0 Kn	1.22	1.09	1.0	1.14	1.30	1.15
5.0 2,4-D + 1.0 2ip	0.79	0.76	0.64	0.76	0.84	0.76
5.0 2,4-D + 2.0 2ip	0.92	0.91	0.68	0.81	0.90	0.84
5.0 NAA + 2.0 Kn	0.99	0.92	0.78	0.78	1.0	0.89
5.0 IAA + 2.0 Kn	0.87	0.86	0.65	0.72	0.94	0.81
Mean	**0.83**	**0.78**	**0.65**	**0.74**	**0.87**	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	0.017	0.96^{NS}	Tree 1 0.908 ±0.016
Tree	4	0.075	3.98**	Tree 2 1.027 ±0.170
Treatment	10	0.247	13.72***	Tree 3 1.145 ±0.008
Tree × Treatment	40	0.002	-	Tree 4 1.014 ±0.013
Error	108	0.018		Tree 5 0.886 ±0.211

** Significant at 1% level of probability
*** Significant at 0.1% level of probability
NS Non-significant

Table 29: Effect of auxins either singly or incombinations with cytokinins on percentage of callus formation from hypocotyl explants of five different trees of *Aegle marmelos*. Scoring was done after weeks of culture. Each value is an average of three replications with five culture tubes per treatment.

Growth regulators (mg/l)	Percentage of callus forming explant					Mean
	T_1	T_2	T_3	T_4	T_5	
5.0 2,4-D	58.89	68.53	50.77	58.89	63.44	60.10
5.0 NAA	54.70	50.77	46.89	54.70	58.89	53.19
5.0 IAA	50.77	54.70	43.05	46.89	54.70	50.02
5.0 2,4-D + 1.0 BA	58.89	68.53	54.70	50.77	63.44	59.27
5.0 2,4-D + 2.0 BA	68.53	75.00	58.89	54.70	75.00	66.42
5.0 2,4-D + 1.0 Kn	75.00	68.53	63.44	68.53	75.00	70.10
5.0 2,4-D + 2.0 Kn	90.00	75.00	68.53	75.00	90.00	79.71
5.0 2,4-D + 1.0 2ip	58.89	63.44	54.70	58.89	63.44	59.87
5.0 2,4-D + 2.0 2ip	63.44	68.53	58.89	63.44	68.53	64.57
5.0 NAA + 2.0 Kn	58.89	54.70	50.77	58.89	63.44	57.34
5.0 IAA + 2.0 Kn	54.70	50.77	46.89	54.70	58.89	53.19
Mean	**62.97**	**63.50**	**54.32**	**58.67**	**66.80**	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	169.64	3.34*	Tree 1 0.747 ±0.005
Tree	4	257.02	5.06**	Tree 2 1.076 ±0.220
Treatment	10	367.57	7.24***	Tree 3 1.045 ±0.018
Tree × Treatment	40	16.13	-	Tree 4 0.936 ±0.024
Error	108	50.79		Tree 5 1.199 ±0.341

* Significant at 5% level of probability
** Significant at 1% level of probability
*** Significant at 0.1% level of probability

Table 30: Effect of auxins either singly or incombination with cytokinins on fresh weight of calli per culture from hypocotyl explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with five culture tubes per treatment.

Growth regulators (mg/l)	Fresh weight of calli per culture (g)					Mean
	T_1	T_2	T_3	T_4	T_5	
5.0 2,4-D	0.94	0.86	0.79	0.87	0.97	0.89
5.0 NAA	0.90	0.82	0.73	0.82	0.89	0.83
5.0 IAA	0.86	0.76	0.67	0.78	0.80	0.77
5.0 2,4-D + 1.0 BA	1.07	1.00	0.83	0.96	1.13	0.99
5.0 2,4-D + 2.0 BA	1.14	1.06	0.94	1.09	1.17	1.08
5.0 2,4-D + 1.0 Kn	1.43	1.37	1.33	1.46	1.84	1.48
5.0 2,4-D + 2.0 Kn	1.58	1.53	1.46	1.67	2.02	1.65
5.0 2,4-D + 1.0 2ip	1.17	1.10	1.00	1.13	1.32	1.14
5.0 2,4-D + 2.0 2ip	1.23	1.18	1.09	1.26	1.47	1.25
5.0 NAA + 2.0 Kn	1.32	1.28	1.23	1.37	1.68	1.37
5.0 IAA + 2.0 Kn	1.20	1.17	1.09	1.29	1.57	1.26
Mean	**1.17**	**1.10**	**1.01**	**1.15**	**1.35**	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	0.102	3.2*	Tree 1 1.240 ±0.014
Tree	4	0.168	5.26**	Tree 2 1.67 ±0.049
Treatment	10	0.389	12.23***	Tree 3 1.091 ±0.111
Tree × Treatment	40	0.006	-	Tree 4 0.975 ±0.240
Error	108	0.032		Tree 5 0.692 ±0.041

* Significant at 5% level of probability
** Significant at 1% level of probability
*** Significant at 0.1% level of probability

Table 31: Effect of auxins either singly or incombinations with cytokinins on percentage of callus formation from *in vitro* grown leaf explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with five culture tubes per treatment.

Growth regulators (mg/l)	Percentage of callus forming explant					Mean
	T_1	T_2	T_3	T_4	T_5	
5.0 2,4-D	43.05	39.23	31.05	35.24	46.89	39.09
5.0 NAA	39.23	35.24	35.24	31.05	43.05	36.76
5.0 IAA	35.24	31.05	26.56	31.05	35.24	31.83
5.0 2,4-D + 1.0 BA	43.05	43.05	39.23	39.23	50.77	43.80
5.0 2,4-D + 2.0 BA	46.89	43.05	43.05	35.24	50.77	43.80
5.0 2,4-D + 1.0 Kn	46.89	39.23	43.05	39.23	50.77	43.83
5.0 2,4-D + 2.0 Kn	50.77	46.89	50.77	43.05	54.70	49.24
5.0 2,4-D + 1.0 2ip	39.23	35.24	39.23	43.05	50.77	41.50
5.0 2,4-D + 2.0 2ip	43.05	39.23	43.05	46.89	54.70	45.38
5.0 NAA + 2.0 Kn	46.89	43.05	46.89	39.23	50.77	45.37
5.0 IAA + 2.0 Kn	43.05	39.23	43.05	35.24	46.89	41.49
Mean	**44.39**	**39.50**	**40.11**	**38.05**	**48.31**	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	16.109	1.42^{NS}	Tree 1 0.958 ±0.249
Tree	4	184.78	16.29***	Tree 2 0.912 ±0.010
Treatment	10	110.76	9.77***	Tree 3 1.022 ±0.011
Tree × Treatment	40	8.38	0.74^{NS}	Tree 4 1.145 ±0.029
Error	108	11.34		Tree 5 0.974 ±0.099

*** Significant at 0.1% level of probability

NS Non-significant.

Table 32: Effect of auxins either singly or incombination with cytokinins on fresh weight of calli per culture from *in vitro* grown leaf explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with five culture tubes per treatment.

Growth regulators (mg/l)	Fresh weight of calli per culture (g)					Mean
	T_1	T_2	T_3	T_4	T_5	
5.0 2,4-D	0.32	0.26	0.21	0.31	0.37	0.29
5.0 NAA	0.26	0.21	0.16	0.24	0.33	0.24
5.0 IAA	0.21	0.16	0.12	0.17	0.27	0.18
5.0 2,4-D + 1.0 BA	0.38	0.36	0.36	0.39	0.43	0.38
5.0 2,4-D + 2.0 BA	0.42	0.41	0.40	0.43	0.46	0.42
5.0 2,4-D + 1.0 Kn	0.30	0.28	0.25	0.27	0.38	0.29
5.0 2,4-D + 2.0 Kn	0.33	0.32	0.30	0.29	0.42	0.33
5.0 2,4-D + 1.0 2ip	0.36	0.33	0.35	0.37	0.39	0.36
5.0 2,4-D + 2.0 2ip	0.41	0.38	0.38	0.41	0.44	0.40
5.0 NAA + 2.0 Kn	0.44	0.40	0.42	0.49	0.53	0.45
5.0 IAA + 2.0 Kn	0.33	0.36	0.37	0.39	0.42	0.37
Mean	**0.34**	**0.31**	**0.30**	**0.34**	**0.40**	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	0.005	0.83^{NS}	Tree 1 1.128 ±0.046
Tree	4	0.017	2.62*	Tree 2 1.000 ±0.015
Treatment	10	0.033	5.5***	Tree 3 0.782 ±0.180
Tree × Treatment	40	0.0003	-	Tree 4 0.843 ±0.013
Error	108	0.006		Tree 5 1.138 ±0.190

* Significant at 5% level of probability
*** Significant at 0.1% level of probability
NS Non-significant.

Table 33: Effect of auxins alone or incombination with cytokinins on percentage of callus formation from nucellar explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with five culture tubes per treatment.

Growth regulators (mg/l)	Percentage of callus forming explant					Mean
	T_1	T_2	T_3	T_4	T_5	
5.0 2,4-D	25.33	26.56	25.33	27.69	28.86	26.75
5.0 NAA	26.56	24.04	22.79	26.56	26.56	25.30
5.0 IAA	24.04	22.79	21.39	24.04	25.33	23.52
5.0 2,4-D + 1.0 BA	35.24	34.20	32.14	31.05	37.23	33.97
5.0 2,4-D + 2.0 BA	37.23	36.27	35.24	32.14	40.16	36.21
5.0 2,4-D + 1.0 Kn	32.14	31.05	28.86	26.56	34.20	30.56
5.0 2,4-D + 2.0 Kn	34.20	32.14	30.00	27.69	35.24	31.85
5.0 2,4-D + 1.0 2ip	34.20	32.14	31.05	28.86	34.20	32.09
5.0 2,4-D + 2.0 2ip	35.24	34.20	32.14	30.00	36.27	33.57
5.0 NAA + 2.0 Kn	38.23	37.23	33.21	32.14	41.14	36.39
5.0 IAA + 2.0 Kn	36.27	34.20	31.05	32.14	37.23	34.18
Mean	**32.61**	**31.35**	**29.38**	**28.99**	**34.22**	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	29.93	2.99^{NS}	Tree 1 0.859 ±0.007
Tree	4	52.98	5.29**	Tree 2 0.896 ±0.140
Treatment	10	94.63	9.45***	Tree 3 0.973 ±0.380
Tree × treatment	40	0.72	-	Tree 4 1.429 ±0.410
Error	108	10.01		Tree 5 0.827 ±0.140

** Significant at 1% level of probability
*** Significant at 0.1% level of probability
NS Non-significant.

Table 34: Effect of auxins alone or incombinations with cytokinins on fresh weight of calli per culture from nucellar explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with five culture tubes per treatment.

Growth regulators (mg/l)	Fresh weight of calli per culture (g)					Mean
	T_1	T_2	T_3	T_4	T_5	
5.0 2,4-D	0.46	0.38	0.37	0.43	0.48	0.42
5.0 NAA	0.40	0.34	0.30	0.39	0.44	0.37
5.0 IAA	0.36	0.29	0.26	0.32	0.39	0.32
5.0 2,4-D + 1.0 BA	0.61	0.58	0.49	0.53	0.69	0.58
5.0 2,4-D + 2.0 BA	0.68	0.65	0.56	0.60	0.74	0.64
5.0 2,4-D + 1.0 Kn	0.55	0.51	0.48	0.53	0.60	0.53
5.0 2,4-D + 2.0 Kn	0.59	0.57	0.55	0.49	0.68	0.57
5.0 2,4-D + 1.0 2ip	0.59	0.55	0.45	0.51	0.65	0.55
5.0 2,4-D + 2.0 2ip	0.63	0.61	0.50	0.56	0.69	0.59
5.0 NAA + 2.0 Kn	0.78	0.69	0.62	0.67	0.84	0.72
5.0 IAA + 2.0 Kn	0.62	0.61	0.53	0.59	0.68	0.60
Mean	**0.57**	**0.52**	**0.46**	**0.51**	**0.62**	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	0.017	2.99^{NS}	Tree 1 0.971 ±0.038
Tree	4	0.04	7.06***	Tree 2 0.897 ±0.250
Treatment	10	0.071	12.67***	Tree 3 1.047 ±0.109
Tree × Treatment	40	0.0003	-	Tree 4 1.158 ±0.009
Error	108	0.006		Tree 5 0.838 0.037

*** Significant at 0.1% level of probability

NS Non-significant.

3.3 PLANT REGENERATION THROUGH CALLUS CULTURE

Different experiments were conducted to investigate plant regeneration ability through callus culture to different explants of 5 trees. Data on percentage of organogenic callus and number of shoots per callus and after eight weeks of culture were collected. The results are discussed under separate heads.

3.3.1 Plant Regeneration from Embryo Derived Callus

Undifferentiated hard, green to greenish yellow calli composed of small compact cells obtained from embryo explants were cultured onto MS medium supplemented with various concentrations and combinations of cytokinins and auxins for inducing shoot regeneration. Morphogenic potentialities of cultured calli greatly varied with growth regulators supplements (tables 35 & 36). Calli cultured with 1.0 or 2.0 mgl^{-1} BA and 0.2 mgl^{-1} Kn alone or in combination with 0.1 or 0.2 mgl^{-1} NAA, 0.2 mgl^{-1} IAA and 0.5 mgl^{-1} GA_3 induced form shoot bud.

Among 10 different concentrations and combinations 1.0 mgl^{-1} BA + 0.5 mgl^{-1} GA_3 was the best combination in which 60.99% of calli developed shoot buds. Number of shoots per callus was also high in this formulation (plate 5C & 5D). Kn (2.0 mgl^{-1}) alone or in combination with 0.2 mgl^{-1} IAA and 0.5 mgl^{-1} GA_3 were found less effective in shoot bud differentiation and number of shoots per callus.

In the analysis of variance the main item treatment was highly significant against experimental error. However, there was no significant difference among trees, for frequency of organogenesis. The item tree was highly significant for number of shoots per culture. However, all the interaction items were non-significant in both parameters.

3.3.2 Plant Regeneration from Cotyledon Derived Callus

Undifferentiated calli derived from cotyledon explants were cultured on MS medium with various 10 (cytokinins alone or in combination with auxins or GA_3) growth regulator formulations (tables 37 & 38). Among 10 growth regulator formulations, the highest frequency of adventitious bud regenerating callus was 36.23% which was recorded at 1.0 mgl^{-1} BA + 0.5 mgl^{-1} GA_3. Maximum number of shoots per callus (17.50) was recorded at the same hormonal supplement (plate 5C). Analysis of variance indicates that the items trees and treatments were significant against the experimental error.

3.3.3 Plant Regeneration from Hypocotyl Derived Callus

Morphogenic differentiation of cultured callus was greatly influenced by concentrations and combinations of BA, Kn with NAA, IAA or GA_3 present in the culture media (table 39 & 40). Kinetin alone or in combination with NAA or IAA showed inhibitory effect on the frequency of organogenesis and the number of shoots per callus whereas, BA-GA_3 combination was found to be suitable than cytokinin alone or cytokinin-auxin combinations. The highest percentage of organogenic callus (39.77%) and number of shoots per callus (15.82) were recorded in media with 1.0 mgl^{-1} BA + 0.5 mgl^{-1} GA_3 (plate 6A & 6B).

Like frequency of organogenesis, variation concerning with number of shoot developing potentiality was significant for the item tree and for treatment.

3.3.4 Plant Regeneration from Leaf Derived Callus

Leaf explants derived callus of 5 different trees were cultured onto 10 different concentrations and combinations of BA, Kn, NAA, IAA and GA_3. Results concerning with shoot regeneration ability of 5 different trees over 10 different growth regulators are summarized in Tables 41 & 42. The shoot regeneration ability of the explants was found to vary with trees and with growth regulator

PLATE 5 (A-E)

Callus formation from cotyledon explant, subsequent differentiation of shoot.

Plate 5A: A callus proliferated from cotyledon explant in 5.0 mgl^{-1} 2,4-D after 3 weeks of culture.

Plate 5B: Calli proliferated from cotyledon in 5.0 mgl^{-1} 2,4-D + 2.0 mgl^{-1} Kn, after 8 weeks of culture.

Plate 5C: Bud primordias differentiated from cotyledon callus in 1.0 mgl^{-1} BA + 0.5 mgl^{-1} GA_3, after 3 weeks of culture.

Plate 5D: Multiple shoot developed from cotyledon callus in 1.0 mgl^{-1} BA + 0.5 mgl^{-1} GA_3 after 6 weeks of culture.

Plate 5E: Multiple shoot developed from cotyledon callus in 1.0 mgl^{-1} BA + 0.5 mgl^{-1} GA_3, after 8 weeks of culture.

PLATE 5 (A - D)

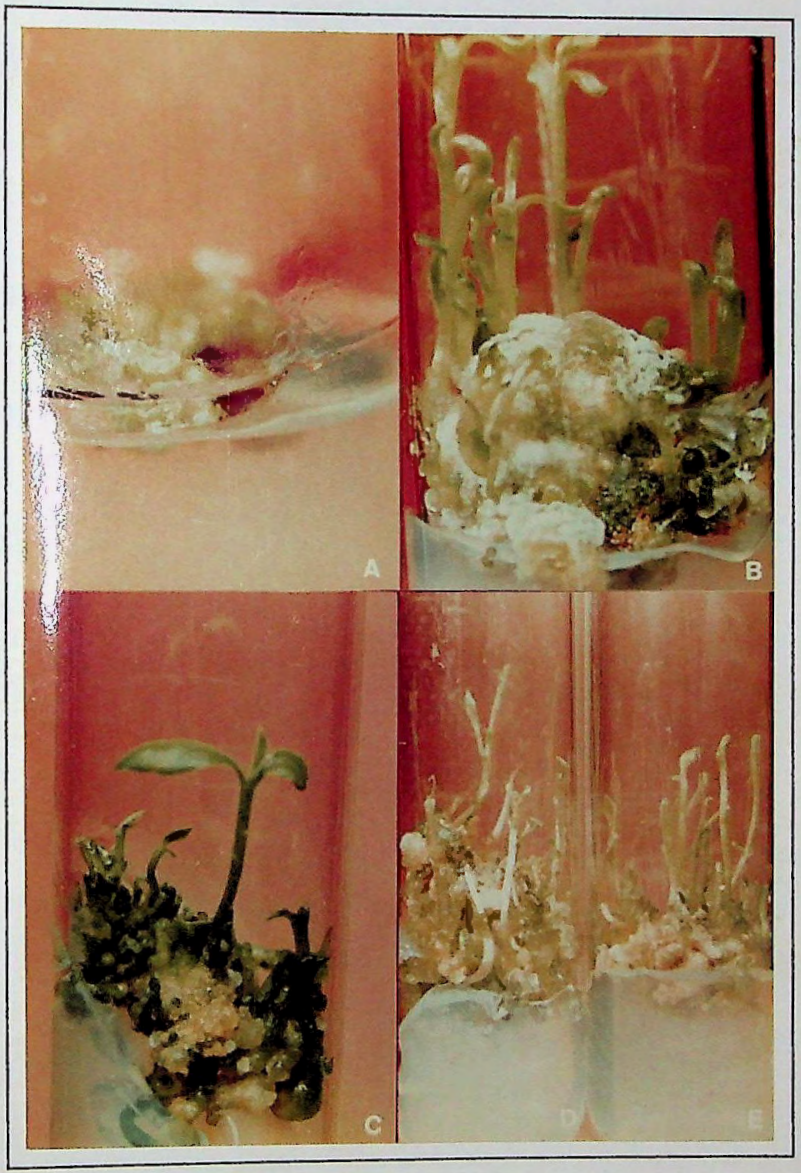


PLATE 6(A-E)

Plate regeneration from nucellar culture of *A. marmelos*.

Plate 6A: Bud primordias proliferated directly from nucellar tissue in 1.0 mgl^{-1} BA, after 6 weeks of culture.

Plate 6B: Shoot buds proliferated from nucellar surface in 1.0 mgl^{-1} BA + 0.1 mgl^{-1} NAA, after 6 weeks of culture.

Plate 6C: Multiple shoot differentiated from nucellar callus in 1.0 mgl^{-1} BA + 0.5 mgl^{-1} GA_3, after 8 weeks of culture.

Plate 6D: Multiple shoots developed from nucellar callus, after subculture in 1.0 mgl^{-1} BA + 0.5 mgl^{-1} GA_3 (5 weeks of subculture)

Plate 6E: Multiple shoots developed from nucellar callus, after subculture in 1.0 mgl^{-1} BA + 0.5 mgl^{-1} GA_3 (3 weeks after subculture)

PLATE 6 (A - E)

Table 35: Effect of cytokinins alone or incombination with auxins and GA_3 on percentage of organogenic green weight (g) of callus derived from embryo explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with ten culture tubes per treatment.

Growth regulators (mg/l)	Percentage of organogenic callus					Mean
	T_1	T_2	T_3	T_4	T_5	
BA 1.0	50.77	47.87	46.89	51.71	50.77	49.50
BA 2.0	51.71	52.71	47.06	52.71	53.73	51.58
Kn 2.0	33.21	32.14	31.05	30.00	34.20	32.12
BA 2.0 + NAA 0.1	56.79	52.71	53.73	54.70	55.73	54.73
BA 2.0 + NAA 0.2	58.89	57.80	55.73	57.80	56.79	57.40
BA 2.0 + IAA 0.2	50.77	53.73	51.71	53.73	52.71	52.53
BA 1.0 + GA_3 0.5	58.89	57.80	60.00	51.07	57.21	60.99
Kn 2.0 + NAA 0.2	43.05	42.13	39.23	38.23	44.03	41.33
Kn 2.0 + IAA 0.2	39.23	40.16	37.23	36.27	39.23	38.42
Kn 2.0 + GA_3 0.5	34.20	32.14	31.05	30.00	35.24	32.53
Mean	**47.75**	**46.92**	**45.37**	**46.62**	**48.96**	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	35.04	4.86*	Tree 1 0.945 ±0.063
Tree	4	17.89	2.48^{NS}	Tree 2 0.941 ±0.011
Treatment	9	532.55	73.86***	Tree 3 1.098 ±0.009
Tree × Treatment	36	3.22	-	Tree 4 0.986 ±0.99
Error	99	7.21		Tree 5 0.865 0.029

* Significant at 5% level of probability
*** Significant at 0.1% level of probability
NS Non-significant

Table 36: Effect of cytokinins alone or incombination with auxins and GA_3 on number of shoots per green weight (g) of callus derived from embryo explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with ten culture tubes per treatment.

Growth regulators (mg/l)	Number of shoots per culture					Mean
	T_1	T_2	T_3	T_4	T_5	
BA 1.0	41.6	31.6	29.4	24.6	43.5	34.14
BA 2.0	42.6	35.9	30.3	26.7	45.6	36.22
Kn 2.0	19.2	18.7	16.8	16.5	20.0	18.24
BA 2.0 + NAA 0.1	43.8	39.1	36.8	29.9	46.1	39.14
BA 2.0 + NAA 0.2	49.8	46.2	42.5	33.3	52.8	44.92
BA 2.0 + IAA 0.2	48.2	43.5	40.4	30.5	49.9	42.50
BA 1.0 + GA_3 0.5	51.3	49.8	45.6	44.4	57.6	49.74
Kn 2.0 + NAA 0.2	22.2	20.3	19.7	17.9	22.4	19.82
Kn 2.0 + IAA 0.2	20.5	19.7	18.6	17.9	22.4	19.82
Kn 2.0 + GA_3 0.5	23.5	21.5	20.0	19.3	24.6	21.78
Mean	**36.27**	**32.63**	**30.01**	**26.17**	**38.57**	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	107.97	4.68*	Tree 1 0.784 ±0.130
Tree	4	242.70	10.52***	Tree 2 0.876 ±0.240
Treatment	9	681.94	29.56***	Tree 3 0.958 ±0.019
Tree × Treatment	36	9.41	-	Tree 4 1.150 ±0.003
Error	99	23.07		Tree 5 0.726 ±0.029

* Significant at 5% level of probability
*** Significant at 0.1% level of probability

Table 37: Effect of cytokinins alone or incombination with auxins and GA_3 on percentage of organogenic green weight (g) of callus derived from cotyledon explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with ten culture tubes per treatment.

Growth regulators (mg/l)	Percentage of organogenic callus					Mean
	T_1	T_2	T_3	T_4	T_5	
BA 1.0	31.05	30.00	27.69	26.56	32.14	29.49
BA 2.0	32.14	32.14	31.05	28.86	34.20	31.68
Kn 2.0	21.39	19.91	18.44	16.74	22.79	19.85
BA 2.0 + NAA 0.1	34.20	33.21	32.14	30.00	35.24	32.96
BA 2.0 + NAA 0.2	35.24	34.20	33.21	31.05	36.27	33.99
BA 2.0 + IAA 0.2	33.21	32.14	31.05	28.86	33.21	31.69
BA 1.0 + GA_3 0.5	37.23	36.27	35.24	34.20	38.23	36.23
Kn 2.0 + NAA 0.2	26.56	25.33	24.04	22.79	27.69	25.28
Kn 2.0 + IAA 0.2	25.33	24.04	22.79	21.39	26.56	24.02
Kn 2.0 + GA_3 0.5	30.00	28.86	27.69	25.33	31.05	28.59
Mean	**30.64**	**29.61**	**28.33**	**26.58**	**31.74**	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	23.39	3.05^{NS}	Tree 1 0.922 ±0.009
Tree	4	40.34	5.26**	Tree 2 0.887 ±0.039
Treatment	9	33.74	4.4*	Tree 3 0.862 0.220
Tree × Treatment	36	31.28	4.09***	Tree 4 0.877 ±0.055
Error	99	7.67		Tree 5 1.458 ±0.037

* Significant at 5% level of probability
** Significant at 1% level of probability
*** Significant at 0.1% level of probability
NS Non-significant

Table 38: Effect of cytokinins alone or incombination with auxins and GA_3 on number of shoots per green weight (g) of callus derived from cotyledon explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with ten culture tubes per treatment.

Growth regulators (mg/l)	Number of shoots per culture					Mean
	T_1	T_2	T_3	T_4	T_5	
BA 1.0	9.2	8.2	7.5	7.3	9.8	8.40
BA 2.0	11.8	8.9	9.2	8.5	12.5	10.18
Kn 2.0	6.2	6.0	5.8	5.3	7.0	6.06
BA 2.0 + NAA 0.1	16.4	15.2	14.5	13.6	18.2	15.58
BA 2.0 + NAA 0.2	18.5	17.8	15.0	14.5	20.6	17.28
BA 2.0 + IAA 0.2	15.8	15.6	13.2	11.4	15.3	14.26
BA 1.0 + GA_3 0.5	19.3	17.9	15.6	15.2	19.5	17.50
Kn 2.0 + NAA 0.2	10.5	10.0	9.4	8.6	11.8	10.06
Kn 2.0 + IAA 0.2	9.8	8.7	8.2	7.9	10.0	8.92
Kn 2.0 + GA_3 0.5	20.0	18.2	16.5	15.0	21.2	18.18
Mean	**13.75**	**12.65**	**11.49**	**10.73**	**14.59**	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	1.34	2.98^{NS}	Tree 1 0.816 ±0.012
Tree	4	25.01	55.58***	Tree 2 0.833 ±0.003
Treatment	9	97.18	215.96***	Tree 3 1.022 ±0.220
Tree × Treatment	36	0.66	1.47*	Tree 4 1.082 ±0.018
Error	99	0.45		Tree 5 1.045 ±0.055

* Significant at 5% level of probability
*** Significant at 0.1% level of probability
NS Non-significant

Table 39: Effect of cytokinins alone or incombinaton with auxins and GA_3 on percentage of organogenic green weight (g) of callus derived from hypocotyl explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with ten culture tubes per treatment.

Growth regulators (mg/l)	Percentage of organogenic callus					Mean
	T_1	T_2	T_3	T_4	T_5	
BA 1.0	33.21	32.14	27.69	25.33	36.27	30.93
BA 2.0	36.27	33.21	31.05	28.86	37.23	33.32
Kn 2.0	30.00	28.86	27.69	28.86	32.14	29.51
BA 2.0 + NAA 0.1	37.23	34.20	32.14	30.00	36.27	33.97
BA 2.0 + NAA 0.2	39.23	38.23	35.24	32.14	40.16	37.00
BA 2.0 + IAA 0.2	38.23	36.27	37.23	34.20	39.23	37.03
BA 1.0 + GA_3 0.5	41.15	38.23	40.16	36.27	43.05	39.77
Kn 2.0 + NAA 0.2	32.14	31.05	30.00	30.00	33.21	31.28
Kn 2.0 + IAA 0.2	31.05	30.00	31.05	28.86	32.14	30.62
Kn 2.0 + GA_3 0.5	37.23	38.23	37.23	35.24	39.23	37.43
Mean	**35.37**	**34.04**	**32.95**	**30.98**	**36.89**	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	44.37	4.45*	Tree 1 0.806 ±0.044
Tree	4	52.66	5.28**	Tree 2 0.863 ±0.200
Treatment	9	62.27	6.25***	Tree 3 1.040 ±0.029
Tree × Treatment	36	1.95	-	Tree 4 1.254 ±0.011
Error	99	9.97		Tree 5 0.828 ±0.037

* Significant at 5% level of probability
** Significant at 1% level of probability
*** Significant at 0.1% level of probability

Table 40: Effect of cytokinins alone or incombination with auxins and GA_3 on number of shoots per green weight (g) of callus derived from hypocotyl explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with ten culture tubes per treatment.

Growth regulators (mg/l)	Number of shoots per culture					Mean
	T_1	T_2	T_3	T_4	T_5	
BA 1.0	7.3	5.9	6.6	5.4	8.8	6.8
BA 2.0	9.1	6.8	7.4	6.6	10.2	8.02
Kn 2.0	5.3	4.4	3.6	2.9	6.5	4.54
BA 2.0 + NAA 0.1	13.6	12.4	12.1	10.8	14.8	12.74
BA 2.0 + NAA 0.2	15.4	14.8	13.7	12.2	16.7	14.56
BA 2.0 + IAA 0.2	14.2	13.8	13.4	11.1	15.6	13.62
BA 1.0 + GA_3 0.5	16.4	15.3	14.8	14.2	18.4	15.82
Kn 2.0 + NAA 0.2	10.4	8.6	7.8	5.8	12.7	9.06
Kn 2.0 + IAA 0.2	9.1	7.8	6.3	4.6	10.2	7.6
Kn 2.0 + GA_3 0.5	13.4	13.1	12.6	11.3	15.8	13.24
Mean	**11.42**	**10.29**	**9.83**	**8.49**	**12.97**	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	23.72	4.45*	Tree 1 1.075 ±0.150
Tree	4	28.57	5.36***	Tree 2 0.855 ±0.005
Treatment	9	73.77	13.84***	Tree 3 0.882 ±0.039
Tree × Treatment	36	0.31	-	Tree 4 0.886 ±0.290
Error	99	5.33		Tree 5 1.122 ±0.009

* Significant at 5% level of probability
*** Significant at 0.1% level of probability

Table 41: Effect of cytokinins alone or incombination with auxins and GA_3 on percentage of organogenic green weight (g) of callus derived from *in vitro* grown leaf explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with ten culture tubes per treatment.

Growth regulators (mg/l)	Percentage of organogenic callus					Mean
	T_1	T_2	T_3	T_4	T_5	
BA 1.0	25.33	26.56	24.04	22.79	26.56	25.06
BA 2.0	27.69	26.56	25.33	24.04	28.86	26.50
Kn 2.0	22.79	21.39	19.91	18.44	24.04	21.31
BA 2.0 + NAA 0.1	30.00	27.69	26.56	25.33	31.05	28.13
BA 2.0 + NAA 0.2	31.05	30.00	28.86	27.69	32.14	29.95
BA 2.0 + IAA 0.2	28.86	27.69	26.56	25.33	30.00	27.69
BA 1.0 + GA_3 0.5	32.14	31.05	30.00	28.86	33.21	31.05
Kn 2.0 + NAA 0.2	24.04	22.79	21.39	22.79	25.33	23.27
Kn 2.0 + IAA 0.2	25.33	24.04	26.56	22.79	27.69	25.28
Kn 2.0 + GA_3 0.5	30.00	28.86	27.69	27.69	31.05	29.06
Mean	**27.72**	**26.66**	**25.69**	**24.57**	**28.99**	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	24.54	3.06^{NS}	Tree 1 0.848 ±0.001
Tree	4	29.59	3.69*	Tree 2 0.861 ±0.260
Treatment	9	47.77	5.96***	Tree 3 0.836 ±0.006
Tree × Treatment	36	0.21	-	Tree 4 1.174 ±0.259
Error	99	8.02		Tree 5 0.897 ±0.240

* Significant at 5% level of probability
*** Significant at 0.1% level of probability
NS Non-significant

Table 42: Effect of cytokinins or incombination with auxins and GA_3 on number of shoots per green weight (g) of callus derived from *in vitro* grown leaf explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with ten culture tubes per treatment.

Growth regulators (mg/l)	Number of shoots per culture					Mean
	T_1	T_2	T_3	T_4	T_5	
BA 1.0	5.1	5.2	4.4	3.7	8.2	5.2
BA 2.0	6.2	6.7	5.2	4.6	7.6	6.1
Kn 2.0	4.0	3.5	3.9	2.7	3.8	3.6
BA 2.0 + NAA 0.1	7.3	6.3	7.2	5.1	10.3	8.1
BA 2.0 + NAA 0.2	8.9	7.7	8.4	9.2	12.2	10.7
BA 2.0 + IAA 0.2	10.7	9.9	11.7	9.2	12.2	10.7
BA 1.0 + GA_3 0.5	8.6	7.5	9.4	7.8	10.5	8.8
Kn 2.0 + NAA 0.2	4.8	3.9	4.2	3.6	4.5	4.2
Kn 2.0 + IAA 0.2	5.6	4.9	5.7	4.7	7.2	5.6
Kn 2.0 + GA_3 0.5	6.9	5.7	6.5	4.5	7.4	6.2
Mean	**6.81**	**6.13**	**6.66**	**5.12**	**8.06**	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	4.71	2.06^{NS}	Tree 1 0.916 ±0.026
Tree	4	11.45	4.98**	Tree 2 0.986 ±0.009
Treatment	9	22.98	10.03***	Tree 3 0.753 ±0.249
Tree × Treatment	36	0.55	-	Tree 4 0.942 ±0.099
Error	99	2.29		Tree 5 0.715 ±0.029

** Significant at 1% level of probability
*** Significant at 0.1% level of probability
NS Non-significant

Table 43: Effect of cytokinins alone or incombination with auxins and GA_3 on percentage of organogenic green weight (g) of callus derived from nucellar explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with ten culture tubes per treatment.

Growth regulators (mg/l)	Percentage of organogenic callus					Mean
	T_1	T_2	T_3	T_4	T_5	
BA 1.0	34.20	30.0	27.69	26.56	34.20	30.53
BA 2.0	36.27	31.05	32.14	27.69	36.27	32.68
Kn 2.0	26.56	25.33	24.04	22.79	27.69	25.28
BA 2.0 + NAA 0.1	37.23	32.14	31.05	28.86	37.23	33.30
BA 2.0 + NAA 0.2	38.23	33.21	32.14	30.00	39.23	34.56
BA 2.0 + IAA 0.2	36.27	35.24	34.20	31.05	38.23	34.99
BA 1.0 + GA_3 0.5	38.23	39.23	36.27	34.20	39.23	37.43
Kn 2.0 + NAA 0.2	27.69	26.56	25.33	24.04	28.86	26.50
Kn 2.0 + IAA 0.2	28.86	30.00	27.69	26.56	31.05	28.83
Kn 2.0 + GA_3 0.5	32.14	31.05	32.14	30.00	34.20	31.90
Mean	**33.57**	**31.38**	**30.27**	**28.17**	**34.62**	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	41.96	6.89**	Tree 1 0.928 ±0.009
Tree	4	66.34	10.89***	Tree 2 0.836 ±0.029
Treatment	9	74.04	12.16***	Tree 3 0.856 ±0.022
Tree × Treatment	36	1.65	-	Tree 4 1.147 ±0.055
Error	99	6.09		Tree 5 0.804 ±0.037

** Significant at 1% level of probability
*** Significant at 0.1% level of probability

Table 44: Effect of cytokinins alone or incombination with auxins and GA_3 on number of shoots per green weight (g) of callus derived from nucellar explants of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is an average of three replications with ten culture tubes per treatment.

Growth regulators (mg/l)	Number of shoots per culture					Mean
	T_1	T_2	T_3	T_4	T_5	
BA 1.0	5.7	5.9	5.2	6.9	7.2	6.18
BA 2.0	7.2	6.0	7.6	8.9	9.0	7.74
Kn 2.0	2.9	3.1	2.2	2.1	4.5	2.96
BA 2.0 + NAA 0.1	16.2	15.8	16.1	14.2	17.2	15.9
BA 2.0 + NAA 0.2	16.3	16.4	14.9	15.0	16.7	15.86
BA 2.0 + IAA 0.2	17.4	16.3	16.5	15.8	17.5	16.7
BA 1.0 + GA_3 0.5	24.3	23.4	22.6	20.2	25.3	23.16
Kn 2.0 + NAA 0.2	13.2	13.8	13.9	12.8	13.8	13.5
Kn 2.0 + IAA 0.2	14.0	14.6	15.5	14.6	15.2	14.78
Kn 2.0 + GA_3 0.5	16.2	15.4	15.5	14.9	16.8	15.76
Mean	**13.34**	**13.07**	**13.0**	**12.54**	**14.32**	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response b $\pm S_b$
Replication	2	56.15	5.0**	Tree 1 0.836 ±0.029
Tree	4	49.41	4.4*	Tree 2 0.862 ±0.249
Treatment	9	177.15	15.77***	Tree 3 0.871 ±0.099
Tree × Treatment	36	0.68	-	Tree 4 1.004 ±0.009
Error	99	11.23		Tree 5 0.885 ±0.026

* Significant at 5% level of probability
** Significant at 1% level of probability
*** Significant at 0.1% level of probability

tree 5 performed better in respect of shoot organogenesis. The highest percentage of organogenesis (34.62%) and number of shoots per callus (14.32) were recorded in tree 5 (plate 6D & 6E). The lowest percentage of organogenesis (28.17%) and number of shoots per callus were recorded in tree 4 at the end of 8 weeks of culture.

The analysis of variance reveals that the items tree and treatment were significant. Both interaction items T×E concerning with the percentage of organogenesis and number of shoots per culture was non-significant.

3.4 INDUCTION OF ADVENTITIOUS ROOTS ON MICROCUTTINGS

Proliferating shoots from different types of explants of 5 different trees took 8 weeks from the time of establishment of explant to attain suitable size for rooting (2 cm. or more). Different experiments were conducted for rooting. Results of these experiments are discussed below on the basis of types and objectives of the experiments.

3.4.1 Effect of IBA on Adventitious Root Induction

An experiment on *in vitro* induction of adventitious roots was conducted using microcuttings of 5 trees of *A. marmelos*. Individual shoots from *in vitro* grown shoot clumps were excised and after trimming of basal leaves they were transferred to rooting media. The rooting media composed of MS basal salt having 0.6 agar, 3% sucrose and fortified with different concentrations of IBA (0.5 - 40.00 mgl^{-1}). Quantitative data on the percentage of root forming microcuttings, number of roots per culture and length of the longest root in cm. are presented in Tables 45, 46 & 47. In most of the cases root initiation started within 2-3 weeks of culture and continued to grow upto the end of 8 weeks. Out of 8 IBA concentrations tested, 30 mgl^{-1} IBA produced roots rapidly than other IBA concentrations. In high concentration

PLATE 7 (A-L)

Development of plantlets from *in vitro* regeneration shoots.

Plate 7 (A&B): A rooted shoot showing vigorous callus at its base in 40 mgl^{-1} BA, after 8 weeks of culture.

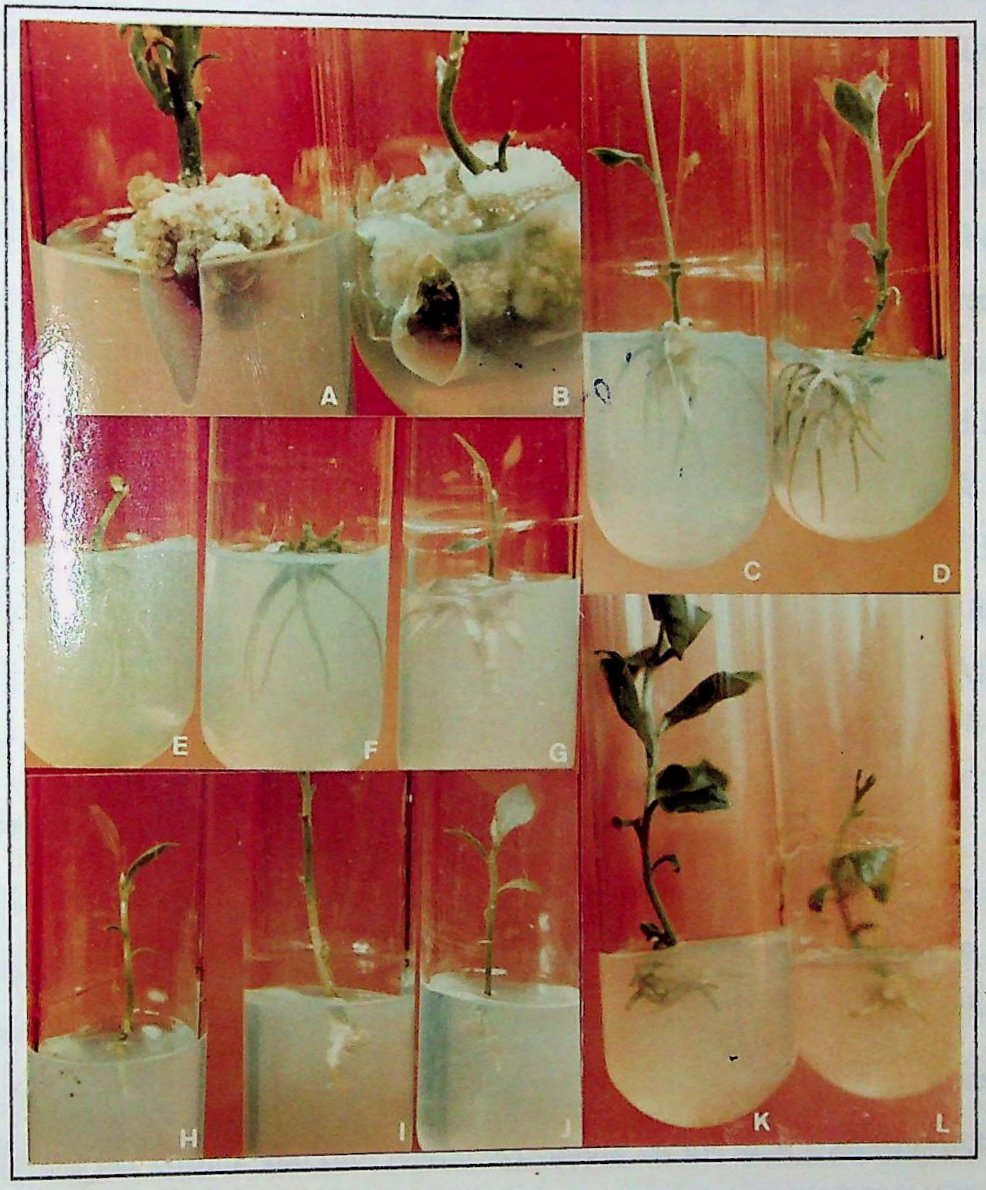
Plate 7(C&D): A rooted shoot showing multiple roots at its base in 30 mgl^{-1} IBA, after 8 weeks of culture.

Plate 7(E,F&G): A rooted shoot showing fewer but healthier roots formed directly at the base in 30 mgl^{-1} IBA, after 8 weeks of culture.

Plate (H,I&J): A rooted shoot showing thin, short and slow growing roots in low concentration (below 30 mgl^{-1} IBA) of IBA, after 8 weeks of culture.

Plate 7(K&L): A rooted shoot showing callus at its base with short roots in 40 mgl^{-1} IBA, after 8 weeks of culture

PLATE 7 (A - L)



(40 mgl^{-1}) of IBA, the shoots which failed to form roots within this period were unable to develop roots even after 8 weeks of culture but induced callus development at the base of the microcuttings (plate 7A & 7B). The percentage of microcuttings formed roots (87%), number of roots per culture (6.57) and root length (3.50 cm.) was maximum in 30 mgl^{-1} IBA (plate 7C & 7D). Some of the cultured microcuttings in all IBA concentrations induced to develop trace of callus at the cut surface of the microcutting (plate 7E & 7F). At 30 mgl^{-1} IBA, fewer but healthier and more vigorously growing roots formed directly at the base of the shoots with no callus formation (plate 7E, 7F & 7G). In low concentrations of IBA (below 30 mgl^{-1}), roots were thin, short and slow growing (plate 7H, 7I & 7J). However, 30 mgl^{-1} IBA improved the rooting percentage, root quality and the number of roots per cutting. When IBA concentration was increased to 40 mgl^{-1}, the roots induced from elongated shoots failed to elongate and sometimes were malformed (plate 7K & 7L). Generally, the roots were unbranched but in some cultures containing 30 mgl^{-1} IBA, secondary roots were found to develop from the primary roots (plate 8A, 8B, 8C & 8D). The results of analysis of variance are shown in Tables 46, 47 and 54. All the main items in the analysis of variance were significant.

3.4.2 Effect of Agar on Adventitious Root Development

Microcuttings of 5 different trees were cultured on MS medium employed with different concentrations of agar, viz., 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 and 10.0 gl^{-1} fortified with fixed hormonal concentration, like 30 mgl^{-1} IBA which are presented in Tables 48 & 49. Microcuttings showed variable response to number of roots per cutting and root length depending on the agar concentrations. It was observed that number of roots per cutting and root length was high in cultured microcuttings receiving 7.5 gl^{-1} agar concentration and was low in 4.0 gl^{-1}. The rooting performance decreased with increasing agar concentration. Agar concentration 7.5 gml^{-1} was optimum for root induction in *A. marmelos*

The highest 8.89 number of roots per cutting (plate 8E) and 3.86 cm. root length were found in 7.5 gl^{-1} agar with 30 mgl^{-1} IBA while the lowest 1.48 number of roots per cutting and 1.08 cm. root length was in 4.0 gml^{-1} agar with 30 mgl^{-1} IBA.

Analysis of variance showed that the items tree and treatment in number of roots per cutting and root length were significant at 5%, 1% and 0.1% level of probability. Variable effect of different agar concentration on number of roots per cutting and root length were highly significant. Nevertheless, agar and tree interaction item concerning with number of roots per cutting was also highly significant but the interaction item concerning with root length was non-significant.

3.4.3 Effect of Sucrose on Adventitious Root Development

Sucrose being an important source of carbon and energy in culture media has considerable influence on growth and morphogenesis. Thus the root induction medium was supplemented with some particular concentrations of sucrose, viz., 10-80 gl^{-1} fortified with 30 mgl^{-1} IBA and 7.5 gl^{-1} agar. The results are presented in Tables 50 & 51. Microcuttings of 5 different trees showed variable response to root initiation depending on the sucrose concentrations. Sucrose 30gl^{-1} gave significantly more roots, higher rooting frequency, higher root length and maximum shoot length increment as compared with lowest (10-25 gl^{-1}) and higher (35-80 gl^{-1}) concentrations. The results indicate that the adventitious root formation is an energy requiring process and presence of certain level of sucrose is essential for optimum rooting. The microcuttings cultured with 30 gl^{-1} sucrose showed optimum number of roots (plate 8F & 8G) per cutting (8.43) and root length (3.38 cm.). Number of roots per cutting and root length was found lower at low concentration (10 gl^{-1}) and high

PLATE 8(A-G)

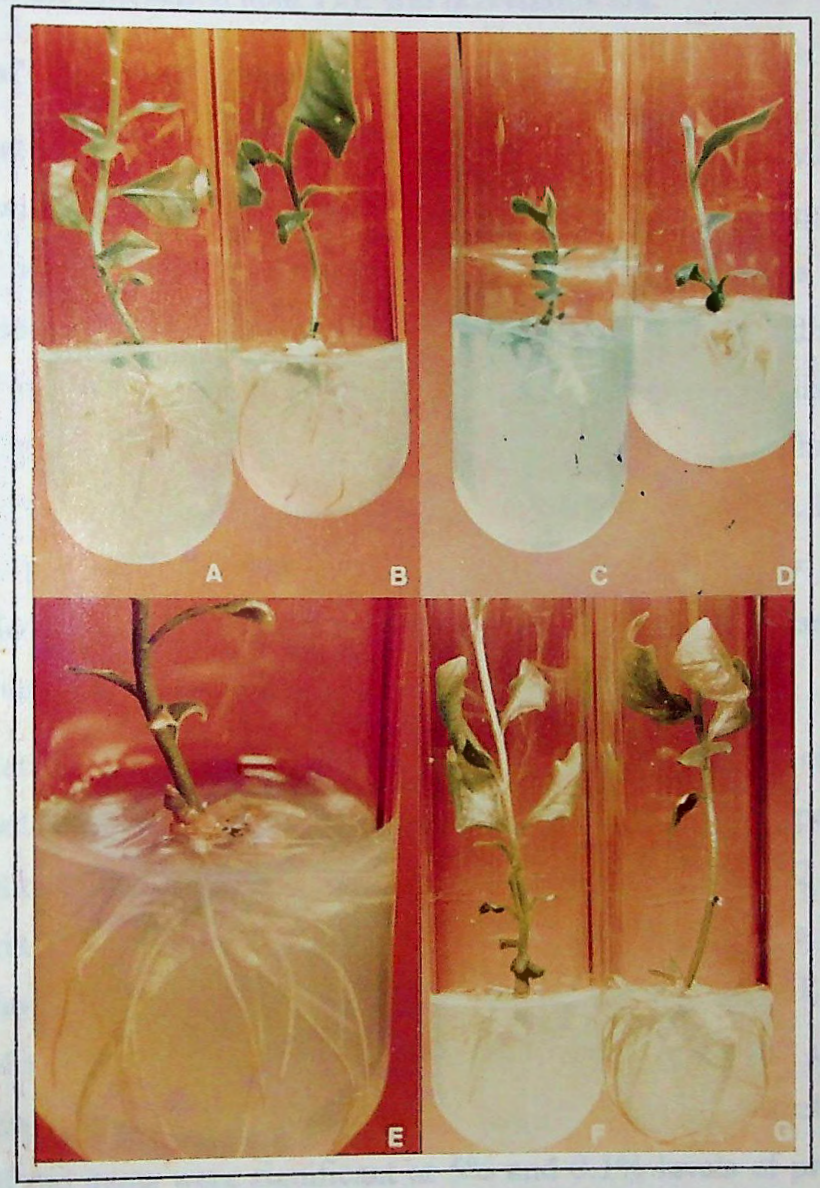
Development of plantlets from *in vitro* regeneration shoots.

Plate 8(A,B,C&D): A rooted shoot showing secondary roots were found to develop from the primary roots in 30 mgl^{-1} IBA, after 8 weeks of culture.

Plate 8(E): A rooted shoot showing multiple roots in 30 mgl^{-1} IBA with 7.5 gl^{-1} agar, after 8 weeks of culture.

Plate 8(F&G): A rooted shoot showing multiple roots in 30 mgl^{-1} IBA with 30 gl^{-1} sucrose, after 8 weeks of culture.

PLATE 8 (A - G)



concentration (80 gl^{-1}) sucrose. Medium containing 80 gl^{-1} sucrose level showed very poor response.

The results of the analysis of variance are shown in Tables 49 & 55. In the analysis of variance, all the main items were highly significant against experimental error except that the interaction item T×E was non-significant.

3.4.4 Effect of pH on Adventitious Root Development

Microcuttings of 5 different trees were cultured onto MS medium supplemented with nine different pH level (5.0 - 8.0) fortified with 30 mgl^{-1} IBA, 7.5 gl^{-1} agar and 30 gl^{-1} sucrose. The results are presented in Tables 52 & 53. Microcuttings showed variable response to root initiation and root length depending on pH level. It was observed that the microcuttings cultured onto media having all pH level developed moderate callus at the cut end (plate 9A & 9B). The highest of 6.24 number of roots (plate 9C & 9D) per cutting and 2.59 cm. root length were recorded in 7.0 pH level while the lowest of 1.34 number of roots per cutting and 0.86 cm. root length in 4.0 pH level. Higher and lower pH level (compared with 7.0 pH level) inhibited root development in *A. marmelos*.

Quantitative data for different rooting parameters (number of roots per cutting and root length) was collected 8 weeks after culture. Mean performances over 3 replications under different pH levels are shown in Tables 52 & 53 with calculated variance at 5%, 1% and 0.1% level of probability. The analysis of variance as shown in Table 52 reveals that the items tree and T×E were non-significant and the item treatment was highly significant in number of roots per cutting indicating a real difference existed among the different pH levels. Table 53 reveals that all the items were significant at 5% and 0.1% levels of probability in root length indicating a real difference existed among the trees and treatments. The interaction item was also significant in root length at 1% level of probability.

3.4.5 Effect of Media Composition on Adventitious Root Development

Microcuttings of 5 different trees were cultured onto different media composition, viz., LS, MS, N_6, B_5 and Whites media fortified with 30 mgl^{-1} IBA, 7.5 gl^{-1} agar and 30 gl^{-1} sucrose. The results are presented in Tables 54 & 55. Microcuttings showed variable response to root initiation and root length depending on different media composition. MS medium gave more roots and higher root length (plate 9E & 9F). Among the 5 media composition, White's medium, showed the lowest performances in number of roots per cutting and root length.

The results of the analysis of variance shown in Tables 54 & 55 indicates the all the main items were highly significant.

3.4.6 Effect of Different Sources of Explants on Adventitious Root Development

Microcuttings from different sources of explant of 5 different trees were cultured onto MS medium supplemented with fixed hormone, agar and sucrose concentrations, like 30 mgl^{-1} IBA, 7.5 gl^{-1} agar and 30 gl^{-1} sucrose. Data on the percentage of root forming microcuttings, number of roots per cutting and root length are presented in Tables 56, 57 & 58. Rooting potentiality of microcuttings markedly varied with sources of explants from which they were originated. In general, rooting frequency of microcutting derived from immature embryo germinated embryo and nucellus were higher than the microcuttings derived from mature embryo, cotyledon, hypocotyl, leaf and callus. Microcuttings derived from germinated embryo yielded 84% rooting. Higher frequency of rooting was also recorded for immature embryo (77.39%) and nucellus (74.39%) initiated culture. Rooting frequency was the lowest (48.84%) for microcuttings derived from leaf explant culture.

Microcuttings derived from immature tissues (embryo, germinated embryo & nucellus) produced higher number of roots per cutting. Number of roots per cutting

PLATE 9(A-L)

Development of plantlets from *in vitro* regeneration shoots.

Plate 9(A,B,C&D): A rooted shoot showing multiple roots in 30 mgl^{-1} IBA depending on 7.0 pH level, after 8 weeks of culture.

Plate 9(E&F): A rooted shoot showing multiple roots and maximum root length in 30 mgl^{-1} IBA depending on MS medium, after 8 weeks of culture.

Plate 9(G,H,I,J,K&L): A rooted shoot showing fewer roots in low concentration (below 30 mgl^{-1} IBA) of IBA, after 8 weeks of culture

PLATE 9 (A - L)

Table 45: Effect of IBA on percentage of root regenerated from microcuttings of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is a mean of three replications with five culture tubes per replicate.

Concentrations of IBA (mg/l)	Percentage of root forming microcuttings					Mean
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	
0.5	21.39	14.89	21.39	14.89	26.56	19.82
1.0	26.56	21.39	26.56	21.39	35.24	26.23
2.0	31.05	35.24	31.05	26.56	39.23	32.63
5.0	39.23	39.23	35.24	31.05	43.05	37.56
10.0	46.89	43.05	39.23	35.24	54.70	43.82
20.0	63.44	58.89	54.70	50.77	75.00	60.56
30.0	90.0	90.0	90.0	75.0	90.0	87.0
40.0	31.05	39.23	35.24	26.56	43.05	35.02
Mean	**43.70**	**42.74**	**41.67**	**35.18**	**50.85**	

ANALYSIS OF VARIANCE

Item	df	MS	F	**Response b $\pm S_b$**
Replication	2	133.52	2.96^{NS}	Tree 1 0.821 ±0.010
Tree	4	249.93	5.54***	Tree 2 0.803 ±0.063
Treatment	7	2331.45	51.68***	Tree 3 0.856 ±0.032
Tree × Treatment	28	4.17	-	Tree 4 0.980 ±0.011
Error	78	45.11		Tree 5 0.869 0.029

*** Significant at 0.1% level of probability.
NS Non-significant.

Table 46: Effect of IBA on number of roots per culture from microcuttings of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is a mean of three replications with five culture tubes per replicate.

Concentrations of IBA (mg/l)	Number of roots per culture					Mean
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	
0.5	1.42	1.32	1.01	0.57	1.57	1.14
1.0	1.74	1.64	0.54	1.0	1.83	1.35
2.0	1.04	1.36	1.05	1.0	2.0	1.29
5.0	2.66	2.57	1.38	1.58	2.36	2.11
10.0	3.17	3.48	3.27	2.0	3.62	3.11
20.0	4.58	5.29	3.66	2.27	5.85	4.33
30.0	7.81	6.75	5.75	3.89	8.63	6.57
40.0	2.89	3.42	2.21	1.32	2.34	2.44
Mean	**3.16**	**3.23**	**2.36**	**1.70**	**3.53**	

ANALYSIS OF VARIANCE

Item	df	MS	F	**Response b $\pm S_b$**
Replication	2	2.96	2.56^{NS}	Tree 1 0.739 ±0.249
Tree	4	4.48	3.86**	Tree 2 0.821 ±0.063
Treatment	7	17.25	14.87***	Tree 3 0.907 ±0.009
Tree × Treatment	28	0.44	-	Tree 4 1.537 ±0.029
Error	78	1.16		Tree 5 0.646 ±0.010

** Significant at 1% level of probability.
*** Significant at 0.1% level of probability.
NS Non-significant.

Table 47: Effect of IBA on length of root (cm) from microcuttings of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is a mean of three replications with five culture tubes per replicate.

Concentrations of IBA (mg/l)	Length of root in cm					Mean
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	
0.5	0.5	0.7	0.6	0.3	0.5	0.52
1.0	0.8	0.8	0.9	0.6	0.7	0.76
2.0	1.2	0.9	1.2	0.9	1.2	1.14
5.0	1.4	1.1	1.6	1.2	1.5	1.36
10.0	1.9	1.4	1.9	1.5	1.8	1.70
20.0	2.4	1.9	2.0	1.8	2.1	2.04
30.0	2.9	2.2	2.8	2.3	4.2	3.50
40.0	3.8	2.9	3.0	3.6	4.2	3.50
Mean	**1.86**	**1.49**	**1.75**	**1.53**	**1.90**	

ANALYSIS OF VARIANCE

Item	df	MS	F	**Response b $\pm S_b$**
Replication	2	0.19	3.07^{NS}	Tree 1 0.781 ±0.011
Tree	4	0.29	4.62**	Tree 2 1.110 ±0.030
Treatment	7	5.16	83.23***	Tree 3 1.005 ±0.025
Tree × Treatment	28	0.03	-	Tree 4 0.823 ±0.019
Error	78	0.06		Tree 5 0.694 ±0.009

** Significant at 1% level of probability.
*** Significant at 0.1% level of probability.
NS Non-significant.

Table 48: Effect of agar on number of roots per culture from microcuttings of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is a mean of three replications with three culture tubes per replicate (all cultured onto 30 mg/l IBA)

Agar concentrations (mg/l)	Number of roots per culture					Mean
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	
4.0	1.33	2.05	1.35	1.09	1.56	1.48
4.5	1.54	1.78	1.66	1.26	1.73	1.59
5.0	2.01	2.76	2.0	1.82	2.0	2.12
5.5	2.67	3.25	2.23	2.33	3.55	2.81
6.0	4.58	3.76	3.24	2.61	3.80	3.60
6.5	5.02	5.88	5.53	4.23	6.79	5.49
7.0	7.30	7.90	6.74	6.54	9.87	7.69
7.5	8.57	9.58	8.39	7.85	10.55	8.98
8.0	5.34	6.17	5.68	4.67	6.42	5.66
8.5	3.28	4.34	2.35	2.74	3.51	3.24
9.0	3.11	2.07	2.34	2.17	2.21	2.38
10.0	1.12	1.55	2.00	1.68	1.80	1.63
Mean	**3.82**	**4.26**	**3.63**	**3.25**	**4.48**	

ANALYSIS OF VARIANCE

Item	df	MS	F	**Response b $\pm S_b$**
Replication	2	1.60	1.47^{NS}	Tree 1 0.945 ±0.017
Tree	4	2.91	2.65*	Tree 2 0.876 ±0.002
Treatment	11	31.40	28.80***	Tree 3 0.974 ±0.111
Tree × Treatment	44	5.64	5.17***	Tree 4 1.065 ±0.012
Error	188	1.09		Tree 5 0.858 ±0.025

* Significant at 5% level of probability.
*** Significant at 0.1% level of probability.
NS Non-significant.

Table 49: Effect of agar on length of root (cm) from microcuttings of five different trees of *Aegle marmelos*. Scroing was done after eight weeks of culture. Each value is a mean of three replications with three culture tubes per replicate (all cultured onto 30 mg/l IBA).

Agar concentrations (gm/l)	Length of root in cm.					Mean
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	
4.0	1.4	1.0	1.09	0.7	1.3	1.08
4.5	1.6	2.0	1.2	1.2	1.8	1.56
5.0	2.2	2.3	1.8	1.5	1.5	1.86
5.5	2.8	2.5	2.0	1.9	2.1	2.26
6.0	3.2	3.0	2.4	2.0	2.5	2.74
6.5	3.3	3.4	2.6	1.9	2.5	2.74
7.0	3.4	3.5	2.9	2.4	3.8	3.20
7.5	3.7	3.9	3.8	3.9	4.0	3.86
8.0	4.0	3.4	3.1	3.0	3.3	3.36
8.5	3.1	3.7	3.3	3.5	2.9	3.30
9.0	2.8	2.8	2.5	2.7	2.8	2.72
10.0	1.9	2.1	2.9	2.6	2.9	2.48
Mean	**2.78**	**2.80**	**2.46**	**2.28**	**2.67**	

ANALYSIS OF VARIANCE

Item	df	MS	F	**Response b $\pm S_b$**
Replication	2	0.23	1.46^{NS}	Tree 1 0.798 ±0.017
Tree	4	0.68	2.72*	Tree 2 0.818 ±0.007
Treatment	11	3.24	12.96***	Tree 3 0.854 ±0.180
Tree × Treatment	44	0.12	-	Tree 4 0.735 ±0.108
Error	188	0.25		Tree 5 0.799 ±0.009

* Significant at 5% level of probability.
*** Significant at 0.1% level of probability.
NS Non-significant.

Table 50: Effect of sucrose on number of roots per culture from microcuttings of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is a mean of three replications with three culture tubes per replicate (all cultured onto 30 mg/l IBA & 7.5 mg/l agar).

Sucrose concentrations (gm/l)	Number of roots per culture					Mean
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	
10.0	2.67	1.0	1.56	1.09	2.35	1.73
15.0	2.84	2.37	2.13	2.08	2.42	2.37
20.0	3.15	2.17	3.08	2.34	3.23	2.79
25.0	5.31	4.56	4.80	4.65	5.54	4.97
30.0	9.24	7.94	8.62	6.78	9.59	8.43
35.0	6.56	5.81	6.34	4.76	7.25	6.14
40.0	5.0	4.43	4.89	3.56	5.16	4.61
45.0	3.05	2.05	2.14	2.38	3.24	2.57
50.0	2.48	1.86	1.36	2.56	3.62	2.38
55.0	1.69	1.38	1.24	1.14	2.56	1.60
60.0	1.0	1.0	1.18	1.0	2.35	1.31
80.0	0.78	1.28	1.05	0.93	1.24	1.06
Mean	**3.65**	**2.99**	**3.20**	**2.77**	**4.04**	

ANALYSIS OF VARIANCE

Item	df	MS	F	**Response b $\pm S_b$**
Replication	2	1.06	4.25*	Tree 1 0.829 ±0.106
Tree	4	3.18	12.93***	Tree 2 0.928 ±0.018
Treatment	11	25.36	101.44***	Tree 3 0.837 ±0.200
Tree × Treatment	44	0.20	-	Tree 4 1.108 ±0.036
Error	118	0.25		Tree 5 0.843 ±0.011

* Significant at 5% level of probability.
*** Significant at 0.1% level of probability.
NS Non-significant.

Table 51: Effect of sucrose on length of root (cm) from microcuttings of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is a mean of three replications with three culture tubes per replicate (all cultured onto 30 mg/l IBA and 7.5 gm/l agar).

Sucrose concentrations (gm/l)	Length of the longest root in cm.					Mean
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	
10.0	1.2	0.7	1.0	1.5	1.0	1.08
15.0	0.9	1.2	0.7	1.5	1.8	1.22
20.0	1.4	1.6	1.3	2.6	2.3	1.84
25.0	2.8	2.6	2.8	2.8	3.2	2.84
30.0	3.7	2.9	3.5	3.0	3.8	3.38
35.0	3.1	2.7	3.1	2.5	3.6	3.0
40.0	2.7	1.8	2.4	2.4	3.5	2.56
45.0	1.2	1.5	1.9	2.2	3.2	2.0
50.0	1.4	1.0	1.6	1.4	1.9	1.46
55.0	0.9	0.9	1.5	1.4	1.7	1.28
60.0	0.7	0.7	1.1	1.0	1.4	0.98
80.0	0.6	0.6	1.0	1.2	1.0	0.88
Mean	**1.72**	**1.52**	**1.83**	**1.96**	**2.37**	

ANALYSIS OF VARIANCE

Item	df	MS	F	**Response b $\pm S_b$**
Replication	2	0.105	3.25*	Tree 1 0.950 ±0.249
Tree	4	1.215	37.65***	Tree 2 1.080 ±0.029
Treatment	11	3.785	117.35***	Tree 3 0.834 ±0.099
Tree × Treatment	44	0.107	3.31***	Tree 4 1.064 ±0.009
Error	118	0.0323	-	Tree 5 0.885 ±0.026

* Significant at 5% level of probability.
*** Significant at 0.1% level of probability.

Table 52: Effect of p^H on number of roots per culture from microcuttings of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value of a mean of three replications with three culture tubes per replicate (all cultured onto 30 mg/l IBA, 7.5 gm/l agar and 30 gm/l sucrose).

pH level	Number of roots per culture					Mean
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	
4.0	1.33	1.83	1.05	1.23	1.26	1.34
4.5	1.89	2.97	1.51	1.35	1.44	1.83
5.0	2.48	3.12	2.53	1.92	2.19	2.45
5.5	4.75	5.36	6.85	4.86	5.28	5.42
6.0	9.34	7.69	9.89	10.53	11.61	9.81
6.5	8.16	9.45	10.67	11.21	11.96	10.29
7.0	9.89	12.40	11.38	11.84	12.51	11.60
7.5	5.26	7.31	6.82	6.38	7.54	6.66
8.0	3.24	2.52	2.0	1.86	2.33	2.39
Mean	**5.15**	**5.85**	**5.86**	**5.69**	**6.24**	

ANALYSIS OF VARIANCE

Item	df	MS	F	**Response b $\pm S_b$**
Replication	2	2.51	3.30*	Tree 1 1.079 ±0.063
Tree	4	2.17	2.85*	Tree 2 0.959 ±0.011
Treatment	8	80.82	106.34***	Tree 3 0.847 ±0.029
Tree × Treatment	32	0.83	1.09^{NS}	Tree 4 0.961 ±0.099
Error	88	0.76		Tree 5 0.744 ±0.037

* Significant at 5% level of probability.
*** Significant at 0.1% level of probability.
NS Non-significant.

Table 53: Effect of pH on length of root from microcuttings of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is a mean of three replications with three culture tubes per replicate (all cultured onto 30 mg/l IBA, 7.5 gm/l agar and 30 gm/l sucrose).

pH level	Length of the longest root in cm.					Mean
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	
4.0	0.9	0.8	0.7	0.6	1.3	0.86
4.5	1.7	1.1	1.5	1.2	1.4	1.38
5.0	2.2	1.4	1.8	1.8	2.7	1.98
5.5	2.0	1.8	2.3	1.7	3.0	2.16
6.0	2.3	2.1	2.4	2.3	2.8	2.38
6.5	2.9	2.6	2.8	2.9	3.2	2.88
7.0	3.6	2.9	3.5	3.4	3.7	3.42
7.5	3.2	2.4	3.0	3.0	3.4	3.0
8.0	1.6	1.4	1.4	1.5	1.8	1.54
Mean	**2.27**	**1.83**	**2.16**	**2.04**	**2.59**	

ANALYSIS OF VARIANCE

Item	df	MS	F	**Response b $\pm S_b$**
Replication	2	0.97	3.36*	Tree 1 1.013 ±0.038
Tree	4	0.71	2.60*	Tree 2 1.018 ±0.160
Treatment	8	1.28	4.74***	Tree 3 0.832 ±0.009
Tree × Treatment	32	0.59	2.16**	Tree 4 0.795 ±0.154
Error	88	0.27	-	Tree 5 0.952 ±0.009

* Significant at 5% level of probability.
** Significant at 1% level of probability
*** Significant at 0.1% level of probability.

Table 54: Effect of media composition on number of roots per culture from microcuttings of five trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is a mean of three replications with three culture tubes per replicate (all cultured onto 30 mg/l IBA, 7.5 gm/l agar, 30 gm/l sucrose and 7.0 pH level.)

Media composition	Number of roots per culture					Mean
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	
LS	9.65	7.53	10.16	9.17	12.69	9.84
MS	10.22	6.88	11.43	10.72	13.24	10.50
N_6	7.96	4.47	6.60	8.33	10.27	7.53
B_5	7.21	5.56	7.87	7.61	9.88	7.63
White's	6.98	6.23	6.54	7.45	8.56	7.15
Mean	**8.40**	**6.13**	**8.52**	**8.66**	**10.93**	

ANALYSIS OF VARIANCE

Item	df	MS	F	**Response b $\pm S_b$**
Replication	2	3.99	3.65*	Tree 1 0.818 ±0.115
Tree	4	14.40	12.86***	Tree 2 1.189 ±0.026
Medium	4	11.64	10.40***	Tree 3 0.797 ±0.035
Tree × Treatment	16	0.59	-	Tree 4 0.855 ±0.001
Error	48	1.12		Tree 5 0.939 ±0.025

* Significant at 5% level of probability.
*** Significant at 0.1% level of probability.

Table 55: Effect of media composition on length of root (cm) from microcuttings of five different trees of *Aegle marmelos*. Scoring was done after eight weeks of culture. Each value is a mean of three replications with three culture tubes per replicate (all cultured onto 30 mg/l IBA, 7.5 gm/l agar, 30 gm/l sucrose and 7.0 pH level).

Media composition	Length of the longest root in cm					Mean
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	
LS	2.9	3.1	2.7	3.4	3.7	3.16
MS	3.4	3.6	2.9	2.2	4.0	3.22
N_6	2.1	2.6	2.4	1.9	3.2	2.44
B_5	2.5	1.8	2.7	2.2	3.0	2.44
White's	1.9	2.2	1.8	1.6	2.9	2.08
Mean	**2.56**	**2.66**	**2.50**	**2.26**	**3.36**	

ANALYSIS OF VARIANCE

Item	df	MS	F	Response (b)
Replication	2	0.68	4.52*	Tree 1 1.050 ±0.120
Tree	4	0.86	5.69**	Tree 2 0.870 ±0.049
Medium	4	1.25	8.27***	Tree 3 0.870 ±0.008
Tree × Treatment	16	0.12	-	Tree 4 1.150 ±0.025
Error	48	0.15		Tree 5 0.980 ±0.012

* Significant at 5% level of probability.
** Significant at 1% level of probability
*** Significant at 0.1% level of probability.

Table 56: Microcuttings from different sources of explants of five different trees of *Aegle marmelos* influenced on percentage of root induction. Scoring was done after eight weeks of culture. Each value is a mean of three replications with five cultrue tubes per replicate (shoots were cultured onto 30 mg/l IBA, 7.5 gm/l agar, 30 gm/l sucrose and 7.0 pH level).

Sources of microcuttings	Percentage of root forming microcuttings					Mean
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	
Immature embryo	90.0	63.44	75.00	68.53	90.0	77.39
Mature embryo	61.07	54.70	50.77	48.79	63.44	55.75
Cotyledon	54.70	50.77	48.79	45.0	61.07	52.07
Hypocotyl	68.53	63.44	54.70	50.77	75.0	62.49
Leaf	50.77	46.89	48.79	43.05	54.70	48.84
Nucellus	75.0	68.53	63.44	75.0	90.0	74.39
Seedling	90.0	75.0	90.0	75.0	90.0	84.0
Callus	45.0	39.23	37.23	39.23	48.79	48.89
Mean	**66.88**	**57.75**	**58.59**	**55.67**	**71.63**	

ANALYSIS OF VARIANCE

Item	df	MS	F	**Response b $\pm S_b$**
Replication	2	21.48	1.32^{NS}	Tree 1 0.755 ±0.010
Tree	4	372.36	22.89***	Tree 2 1.059 ±0.249
Explant	28	1135.57	69.79***	Tree 3 0.872 ±0.008
Tree × Explant	28	23.17	1.42*	Tree 4 1.034 ±0.159
Error	78	16.27		Tree 5 0.937 ±0.022

* Significant at 5% level of probability.
*** Significant at 0.1% level of probability.
NS Non-significant

Table 57: Microcuttings from different sources of explants of five different trees of *Aegle marmelos* influenced on number of roots induction per cutting. Scoring was done after eight weeks of culture. Each value is a mean of three replications with five culture tubes per replicate (cuttings were cultured onto 30 mg/l IBA, 7.5 gm/l agar, 30 gm/l sucrose and 7.0 pH level).

Sources of microcuttings	Number of roots per cutting					Mean
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	
Immature embryo	10.40	9.50	10.97	8.31	12.60	10.36
Mature embryo	8.25	7.64	8.32	5.42	9.33	7.79
Cotyledon	6.78	7.34	6.76	4.25	8.78	6.78
Hypocotyl	8.64	6.70	9.65	4.63	10.29	7.98
Leaf	5.98	4.69	5.81	3.56	7.50	5.51
Nucellus	9.63	8.67	6.93	6.70	10.67	8.52
Germinateing embryo	11.19	10.23	8.82	9.47	13.53	10.65
Callus	2.24	1.41	1.97	2.28	3.42	2.26
Mean	**7.89**	**7.02**	**7.40**	**5.58**	**9.52**	

ANALYSIS OF VARIANCE

Item	df	MS	F	**Response b $\pm S_b$**
Replication	2	8.31	2.98^{NS}	Tree 1 0.970 ±0.029
Tree	4	16.29	5.82***	Tree 2 0.960 0.249
Explant	7	36.66	13.14***	Tree 3 0.940 0.099
Tree × Explant	28	0.78	-	Tree 4 0.900 0.009
Error	78	2.79		Tree 5 0.990 ±0.026

*** Significant at 0.1% level of probability.
NS Non-significant

Table 58: Microcuttings from different sources of explants of five different trees of *Aegle marmelos* influenced on length (cm) of root. Scoring was done after eight weeks of culture. Each value is a mean of three replications with five culture tubes per replicate (cuttings were cultured onto 30 mg/l IBA, 7.5 gm/l agar, 30 gm/l sucrose and 7.0 pH level).

Sources of microcuttings	Length of the longest root in cm.					Mean
	Tree 1	Tree 2	Tree 3	Tree 4	Tree 5	
Immature embryo	2.6	2.5	1.9	2.6	2.7	2.46
Mature embryo	1.9	2.3	1.8	2.6	2.5	2.22
Cotyledon	2.9	2.4	2.8	3.2	2.9	2.84
Hypocotyl	2.7	3.2	2.3	3.7	1.9	2.76
Leaf	2.6	3.1	3.0	3.3	2.1	2.82
Nucellus	2.2	2.3	2.9	3.0	3.6	2.80
Seedling	3.1	3.7	3.9	3.6	2.9	3.44
Callus	3.0	3.4	3.2	2.2	2.9	2.94
Mean	**2.63**	**2.86**	**2.73**	**3.03**	**2.69**	

ANALYSIS OF VARIANCE

Item	df	MS	F	**Response b $\pm S_b$**
Replication	2	0.23	2.58^{NS}	Tree 1 0.82 ±0.063
Tree	4	0.23	2.55*	Tree 2 1.20 ±0.011
Explant	7	0.63	7.04***	Tree 3 0.92 ±0.029
Tree × Explant	28	0.22	2.46***	Tree 4 1.06 ±0.099
Error	78	0.09		Tree 5 0.94 ±0.009

*** Significant at 0.1% level of probability.
NS Non-significant

derived from callus (type of explant) was also low (2.26) but healthy and grew faster. Maximum root length was recorded in microcuttings derived from germinated embryo (3.44 cm) and was lowest in microcuttings derived from mature embryo (2.22 cm.).

The regression values (b) are in effect measures of responses to increments in an improving environments (treatments). Since these environments are measured by the mean of all populations under consideration the trees must have a mean regression value of 1.0. Considering the five trees individually, a linear regression (b) of each tree against treatment (E) means was calculated and the values are shown in Table (1-58). Regression value <1.0 and >1.0 indicated below and above average response respectively by a individual tree for any set of populations under consideration. The distribution of five b values were heterogeneous as revealed by regression analysis and hence all these trees had different response to different treatment (E).

The regression lines are illustrated in Figs. 1-11. In all the cases crossing of regression lines was marked and in most cases it turned out that significant different could still be detected even in the poor as well as in good treatment (E).

3.5 PERFORMANCE OF REGENERATED PLANTLETS UNDER *EX VITRO* CONDITION

3.5.1 Initial Establishment

Rooted microcuttings were initially planted in polybag containing garden soil and compost (1:1) or sand and compost (1:1). After a few days indoor acclimatization, the plantlets from polybag were transplanted directly to the field or to the larger pots. Plants were initially established in the polybags with a view to easy handling during transplantation to the field. It was found that more than 75% of

the planted cuttings survived during initial establishment. Survival % of the plants was also higher in the field under natural environment (92%). Transplantation was done throughout the year. It was found that prevailing atmospheric condition had no effect on the initial survival of the transplanted plants. However, plants transplanted during warm humidity condition showed necrotic lesion on their leaf and shoot tips. This type of necrosis generally did not affect the survival of the plantlets. Nevertheless, there was no difference in survivability of the transplanted plants among their sources of origin. However, survival of the plantles on soil varied with duration of proliferation period. Plantlets originated from first harvest of microcuttings showed the highest survivability (96%) on soil. Whereas, survivability of the plantlets decreased when they were harvested from 12th or on ward subcultures. In general, plantlets with the active growth of primary roots (1-2 cm) showed greater survival and faster initial growth as compared to the plantlets having longer and branched root system (3-5 cm) at the time of transplantation.

3.5.2 Field Performance

Most of the transplanted plantlets (75%) showed orthotopic mode of development upto 6-8 months. Transplants started primary branching only after the period. In some of the cases the transplants started primary branching within 2-3 months after transplantation. Somaclonal variation among the transplants was not studied.

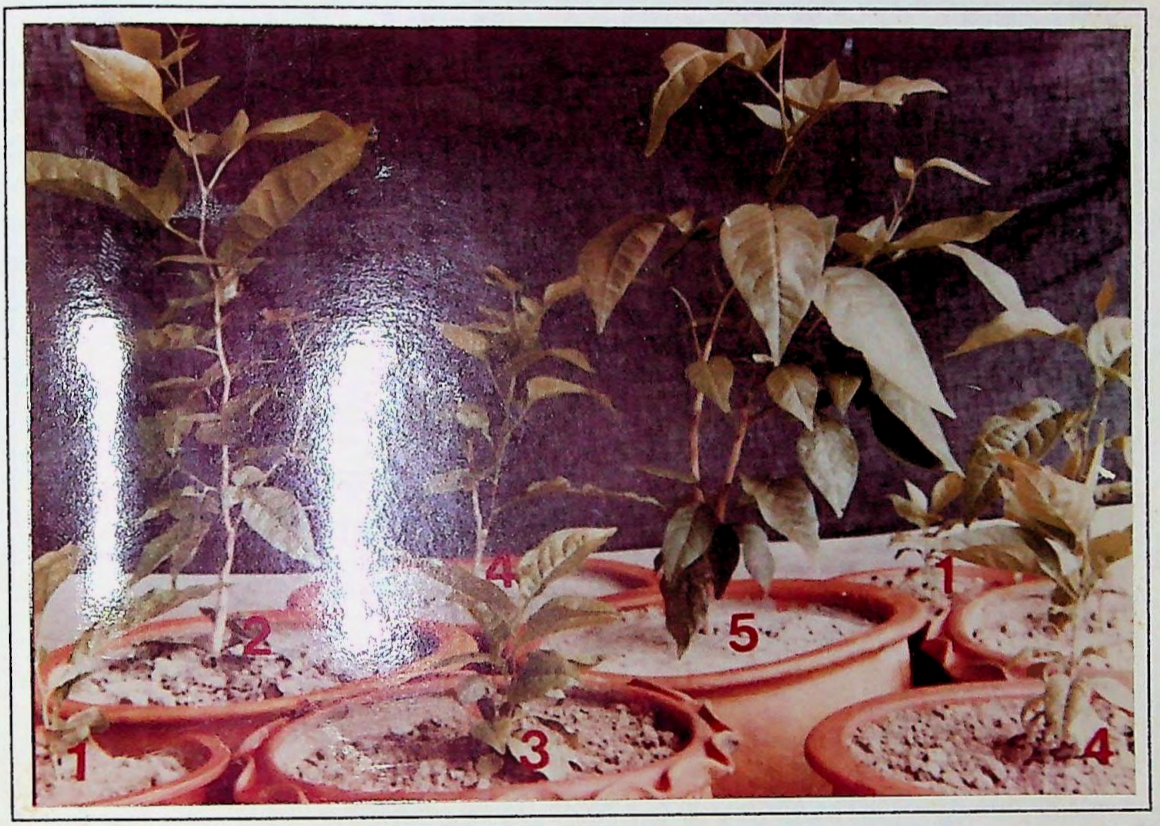
PLATE 10

Regenerated plantlets establishment in soil.

Plate 10: Potted plants derived from different trees:

1. A potted plant (tree 1) 2 months after transplantation.
2 A potted plant (tree 3) 2 months after transplantation.
3. A potted plant (tree 4) 2 months after transplantation.
4. A potted plant (tree 4) 3 months after transplantation.
5. A potted plant (tree 5) 3 months after transplantation.
6. A potted plant (tree 2) 3 months after transplantation.
7. A potted plant (tree 1) 2 months after transplantation.

PLATE 10



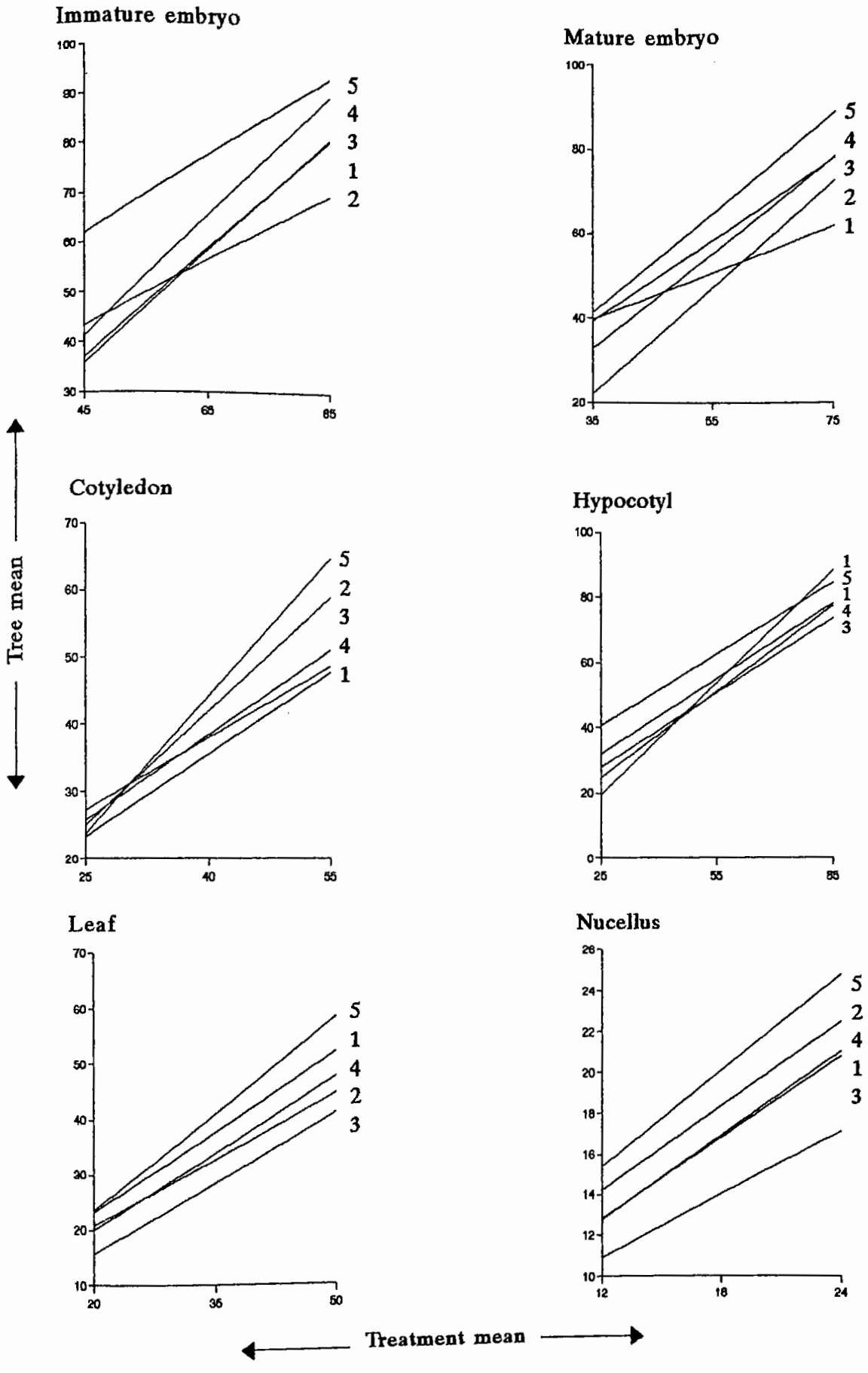


Fig. 1 Regression of individual tree mean on treatment (environment) mean for 5 trees in percentage of shoot forming explants.

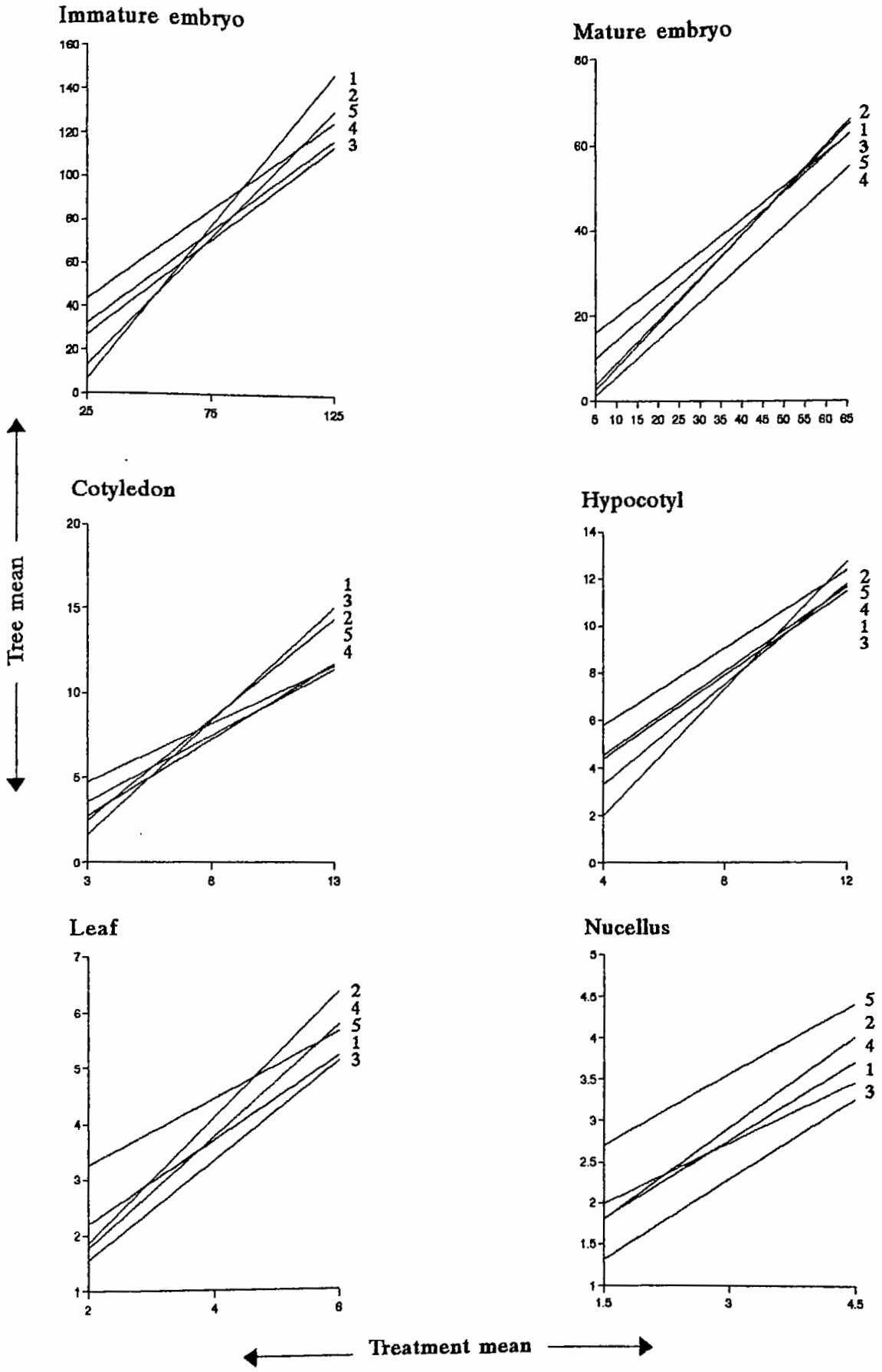


Fig. 2 Regression of individual tree mean on treatment (environment) mean for 5 trees in number of shoots per culture.

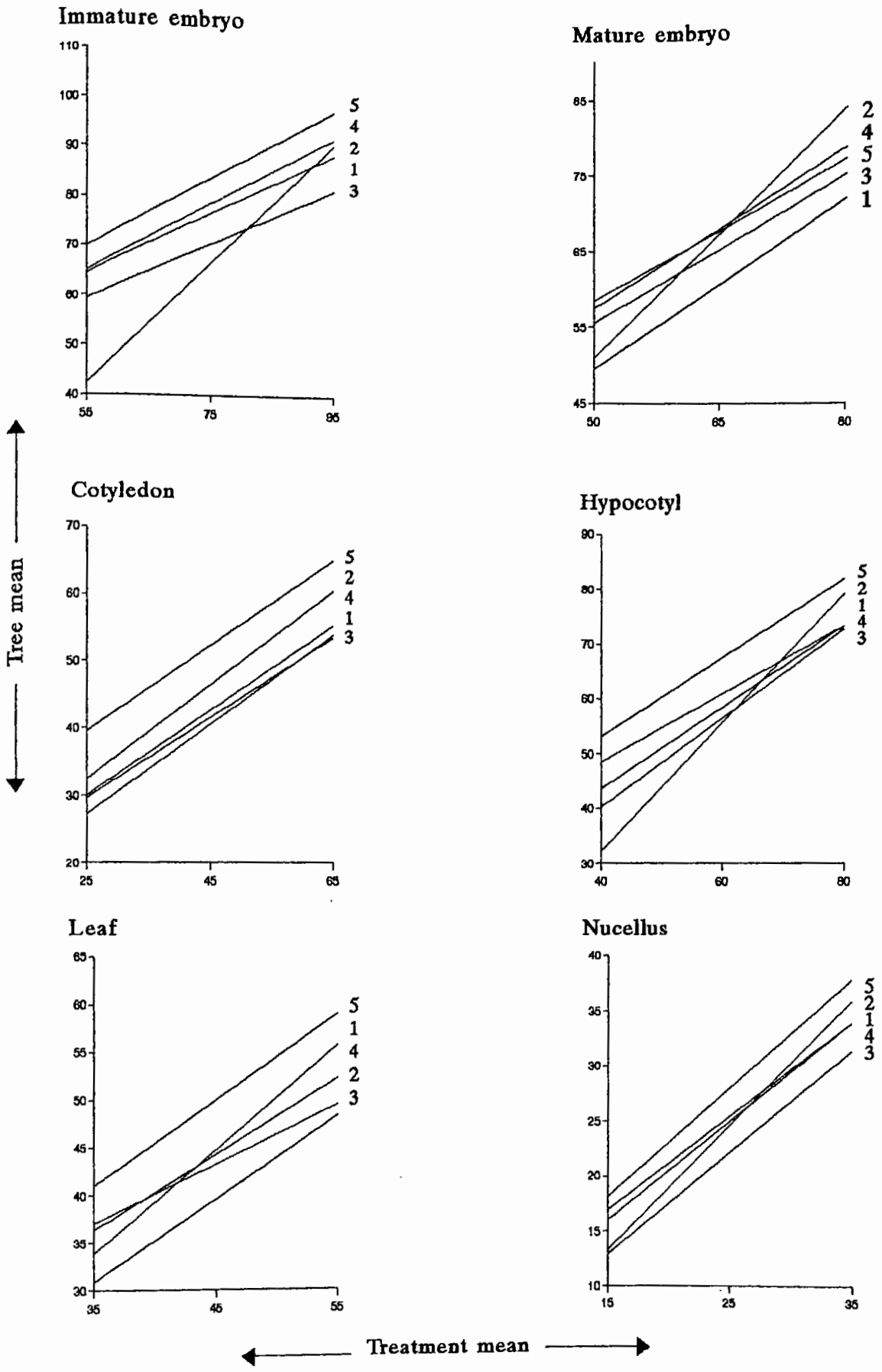


Fig. 3 **Regression of individual tree mean on treatment (environment) mean for 5 trees in percentage of shoot forming explants.**

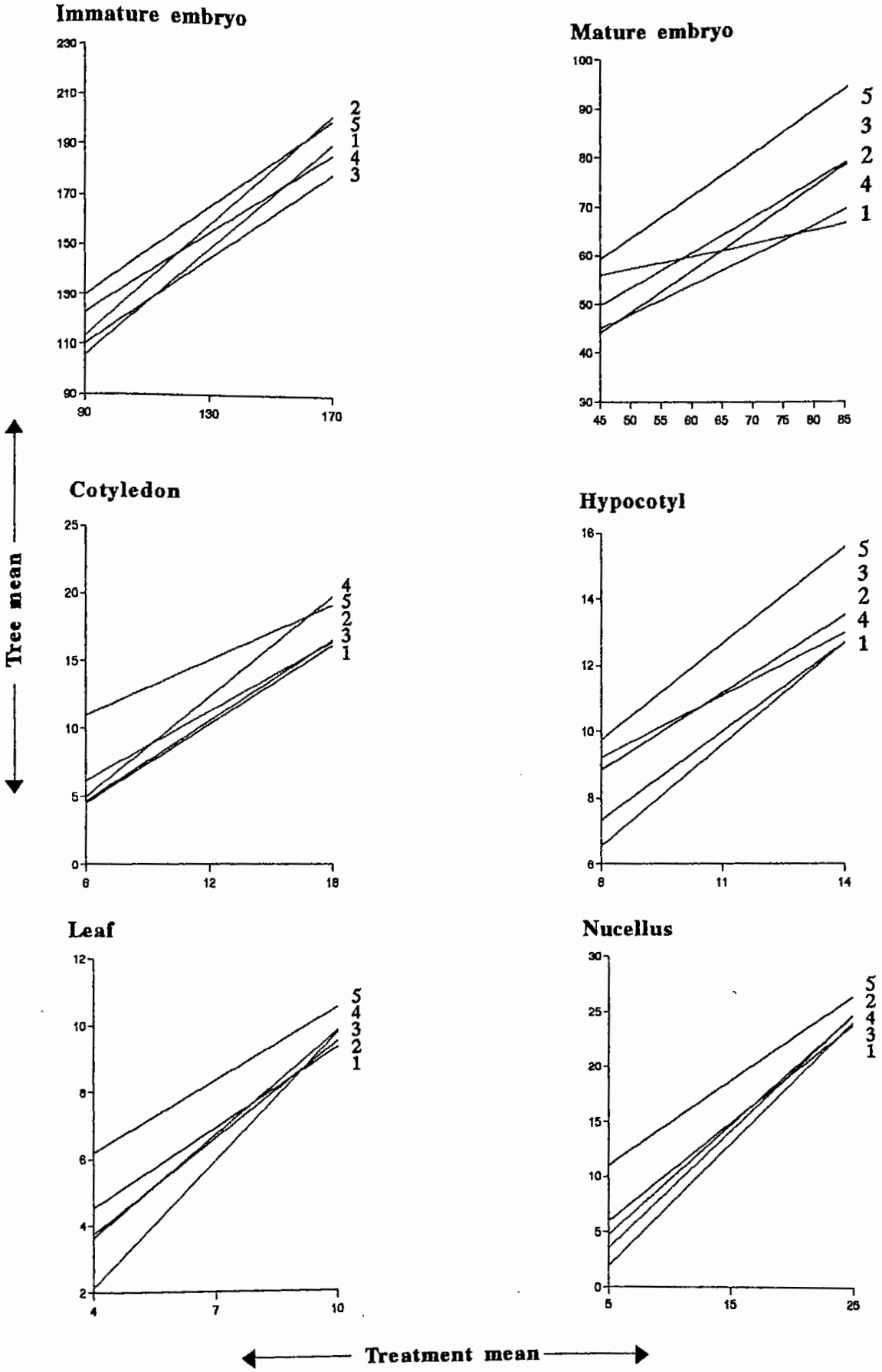


Fig. 4 **Regression of individual tree mean on treatment (environment) mean for 5 trees in number of shoots per culture.**

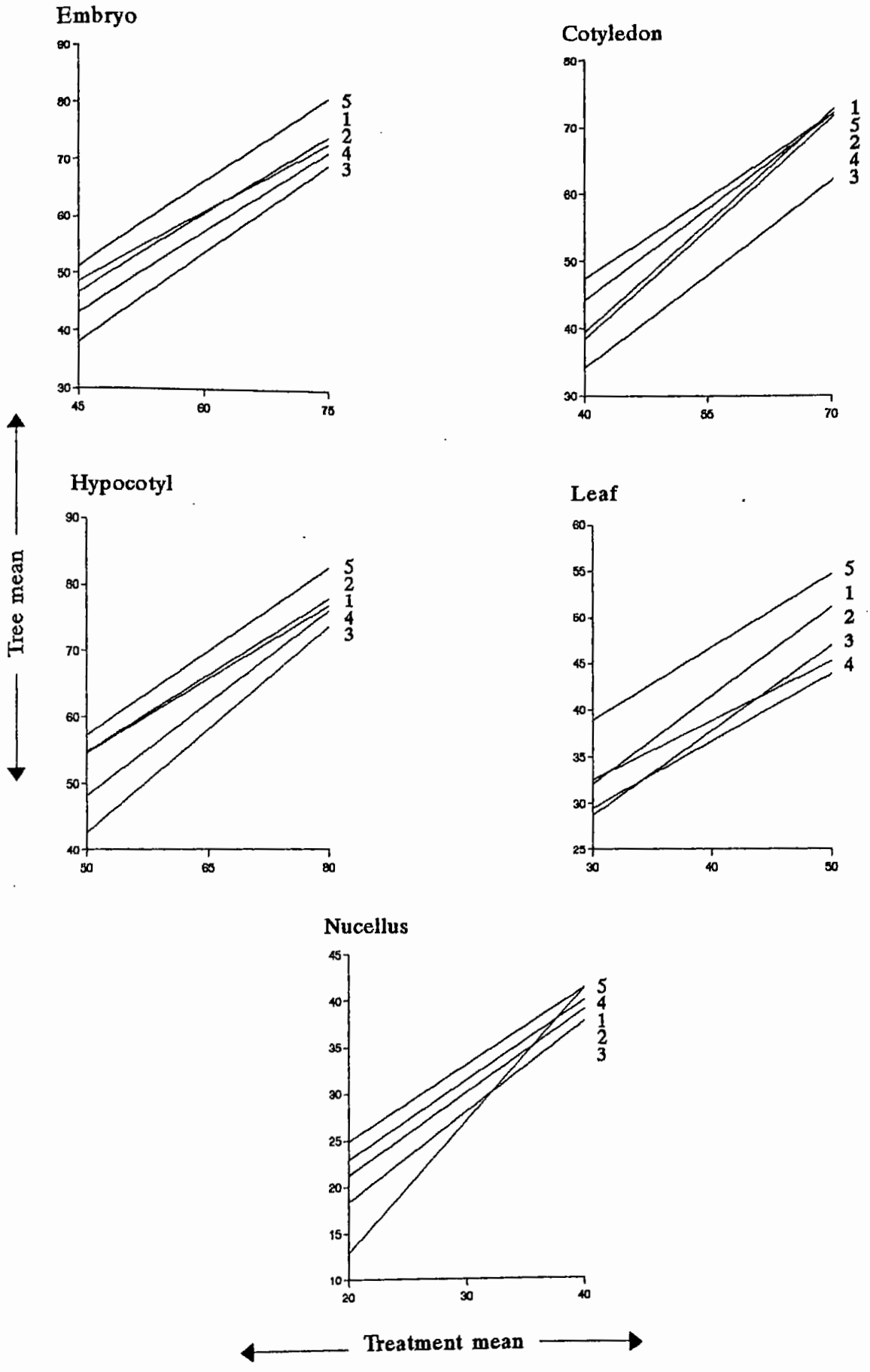


Fig. 5 Regression of individual tree mean on treatment (environment) mean for 5 trees in percentage of callus forming explant.

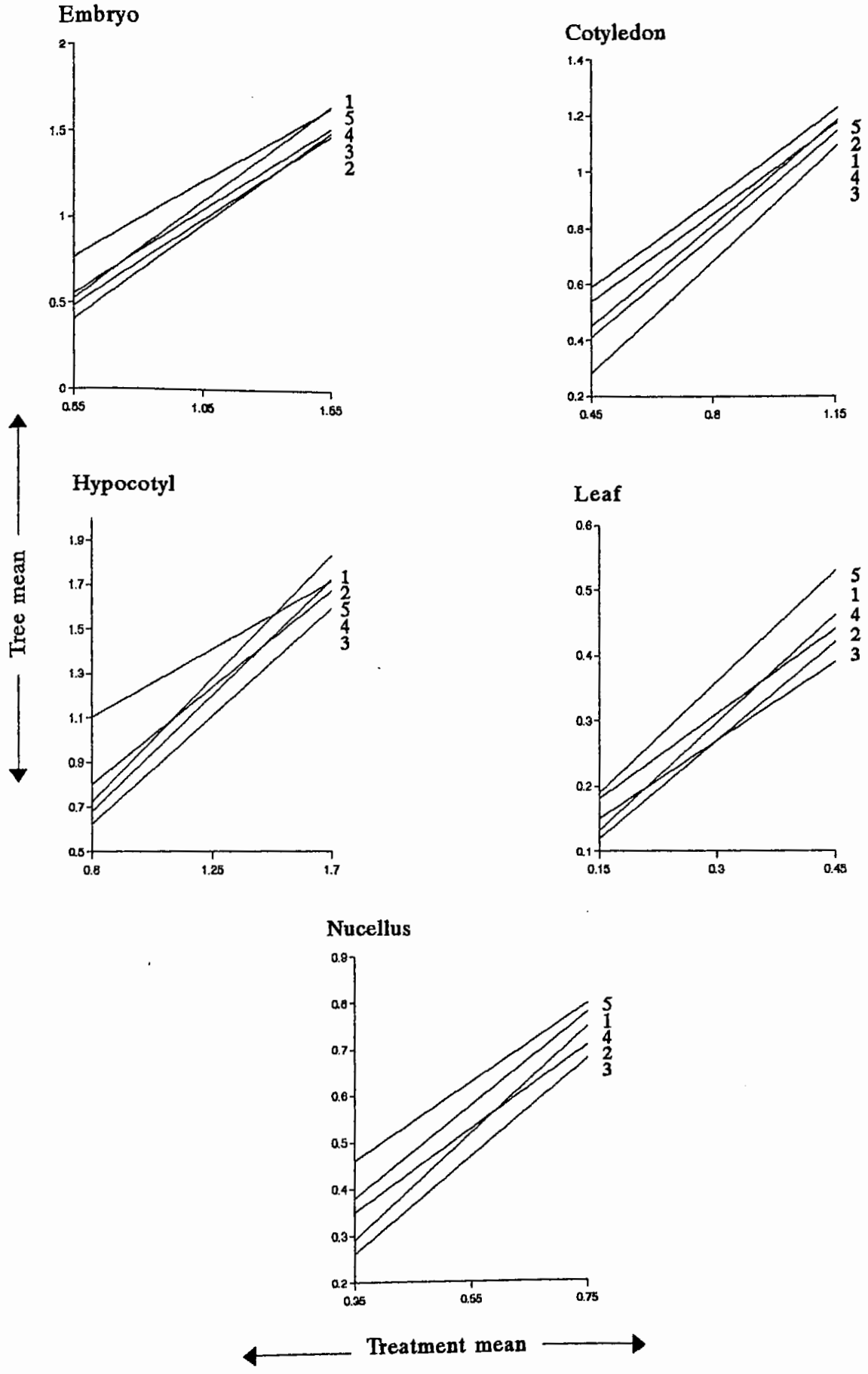


Fig. 6 **Regression of individual tree mean on treatment (environment) mean for 5 trees in fresh weight of calli per culture.**

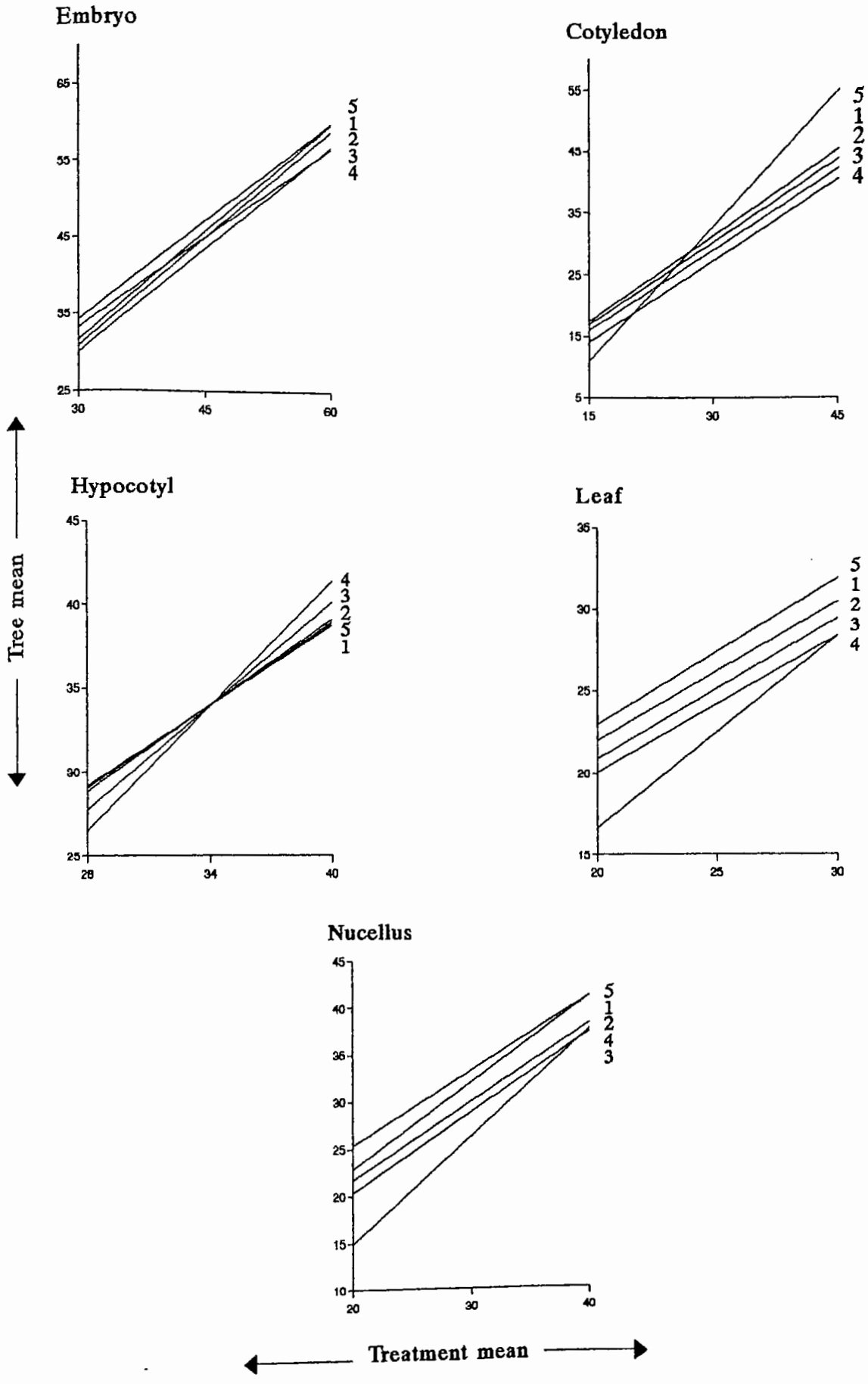


Fig. 7 **Regression of individual tree mean on treatment (environment) mean for 5 trees in percentage of organogenic callus.**

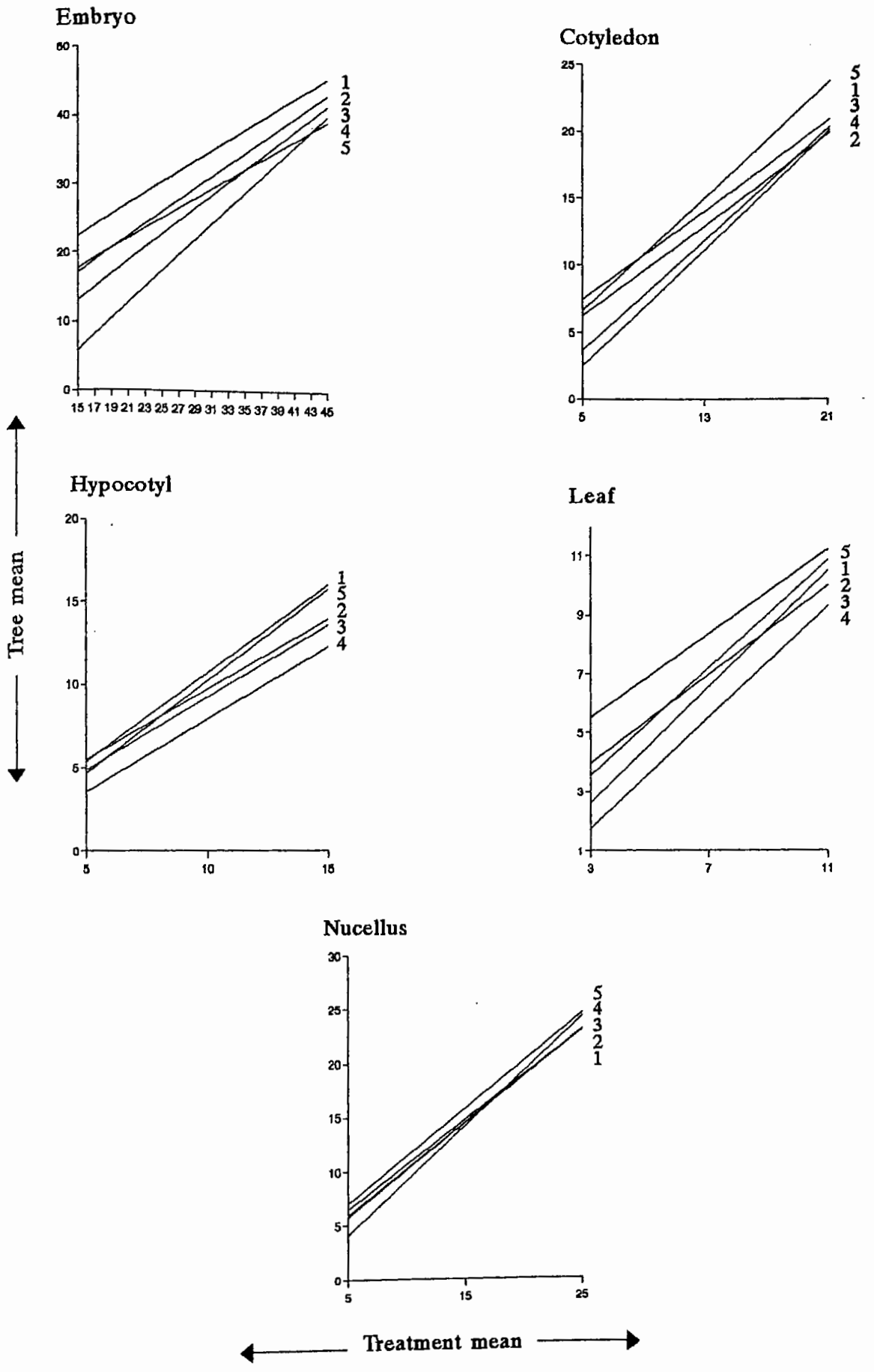


Fig. 8 Regression of individual tree mean on treatment (environment) mean for 5 trees in number of shoots per callus.

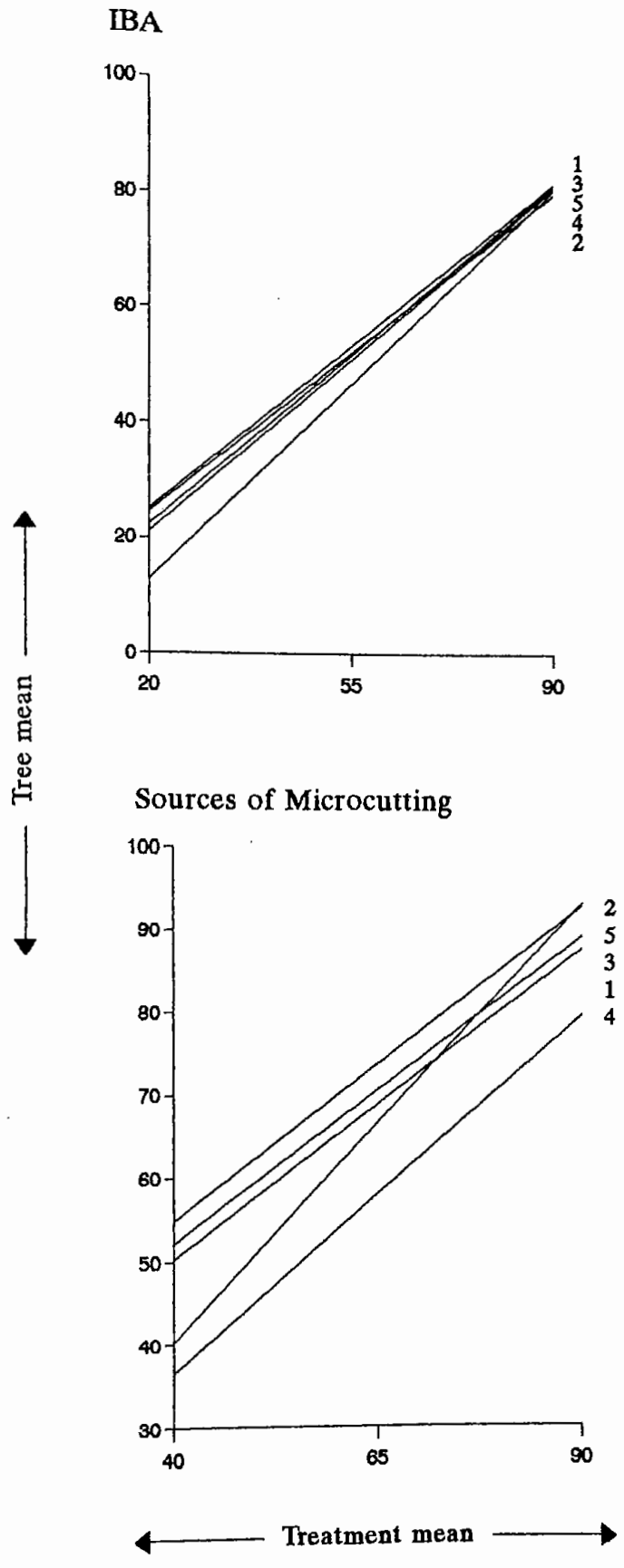


Fig. 9 Regression of individual tree mean on treatment (environment) mean for 5 trees in percentage of root forming microcuttings.

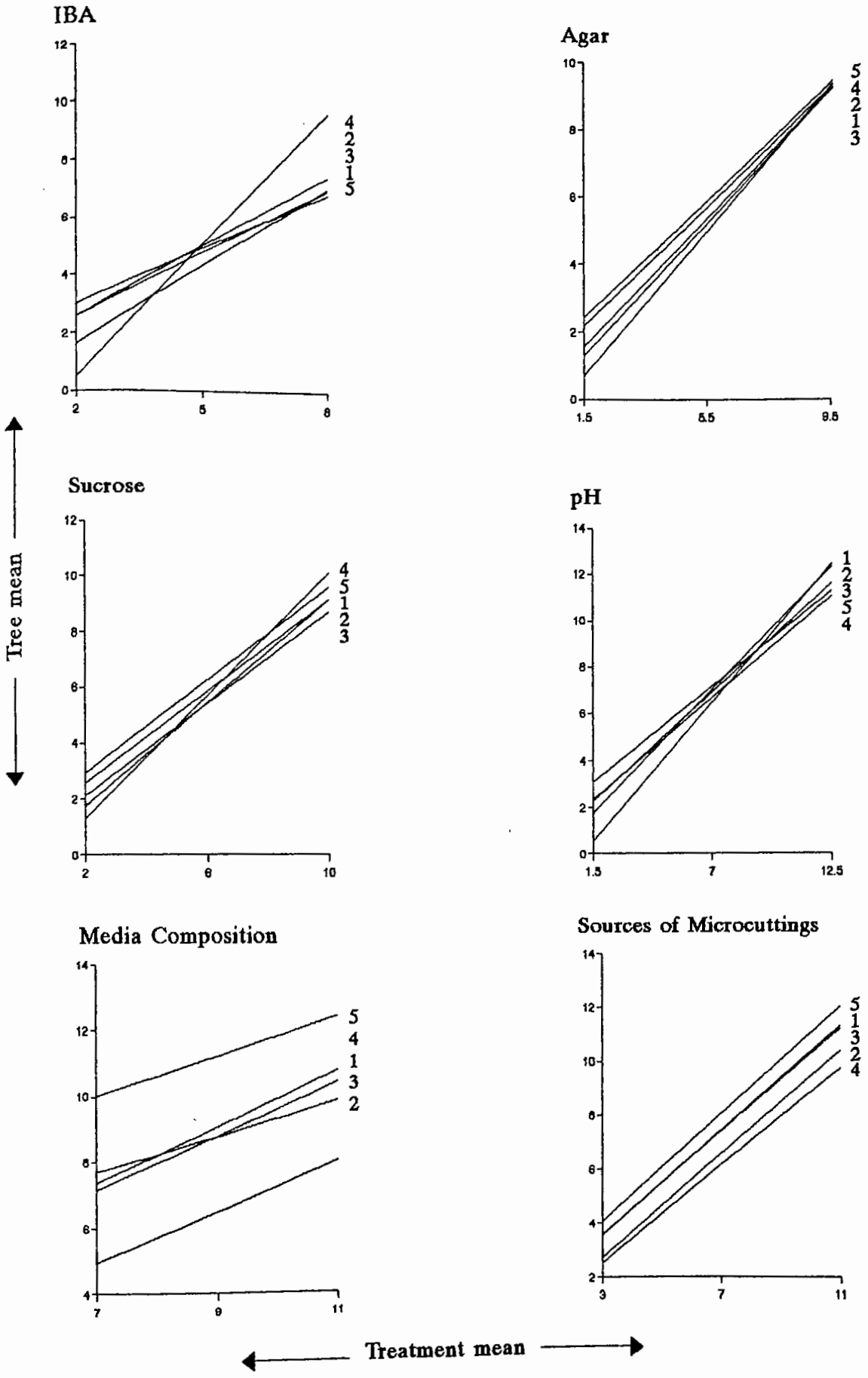


Fig. 10 Regression of individual tree mean on treatment (environment) mean for 5 trees in number of roots per cuttings.

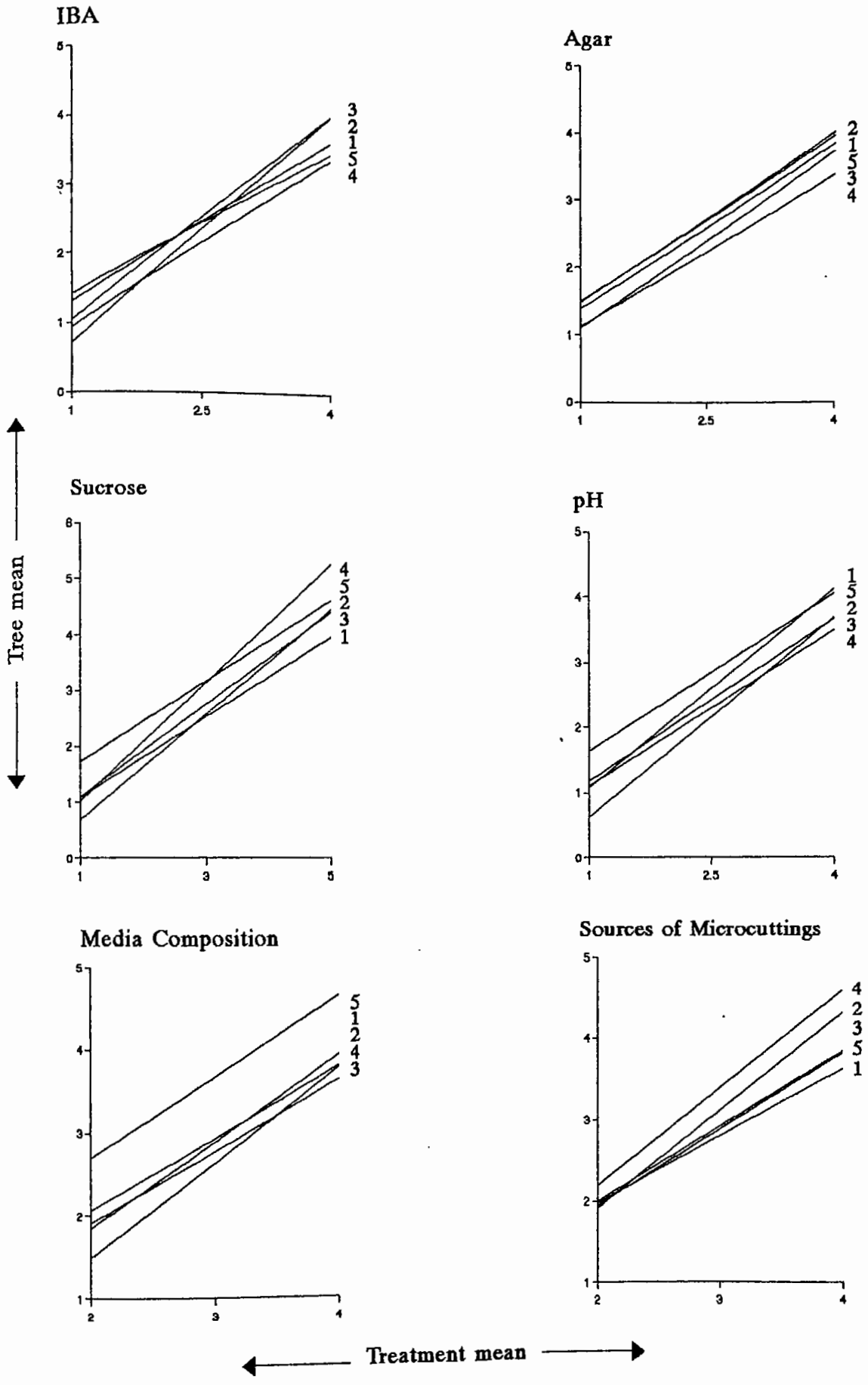


Fig. 11 **Regression of individual tree mean on treatment (environment) mean for 5 trees in length of the longest root.**

Chapter IV

Discussion

DISCUSSION

Woody species in general have been considered to be difficult in terms of ease of manipulation in culture, yet persistent research by a number of group have been that regeneration of plantlets *in vitro* could be achieved. Fruit species such as apple (Liu *et al*, 1983), jackfruit (Rahman and Black, 1988; Roy *et al.*, 1990), carambola (Litz and Conover, 1980; Amin and Jaiswal, 1987; Litz and Griffis, 1989; Amin and Razzaque, 1993) and elephant apple (Hossain *et al.*, 1994) have been successfully grown *in vitro*. In any preliminary study on the tissue culture of any species, it has been customary to use immature tissue rather than those from mature trees (Bonga, 1987). It has been reported that immature tissues such as embryo, cotyledon, nucellus and different parts of seedlings are good sources of starting materials specially important for callus through organogenesis or embryogenesis (Arnold and Erikson, 1978; Hammerschlag *et al.*, 1985; Espinasse *et al.*, 1989; Miller and Chandler, 1990; Karim, 1991; Dong and Jia, 1991; Islam *et al.*, 1993; Hossain *et al.*, 1994). Nevertheless, mature tissues are also less responsive when compared with immature tissue (Sommer and Caldras, 1981; Lazzeri *et al.*, 1985. Hence, in the present study on *Aegle marmelos.*, juveniles tissues such as, embryo, cotyledon, hypocotyl, leaf and nucellus of 5 different trees that were distinctly different in fruit morphology, were used and present investigation was designed as and result of which were discussed in the for going chapters.

The immature and mature embryo explants of 5 trees were cultured onto MS medium employed with different concentrations and combinations of cytokinins alone or BA-auxins, BA-GA_3 and BA-NAA-GA_3. Morphogenesis of the cultured embryo varied with growth regulators and nature of the explants. Overall

results of immature and mature embryo culture indicate that immature embryo had more potentiality to adventitious shoot regeneration than that of mature embryo. Most of the cultured embryo differentiated into normal plantlets in present of low (0.5 mgl^{-1} BA, 1.0 mgl^{-1} Kn and 2ip) and high (5.0 mgl^{-1} Kn and 2ip) concentrations of cytokinins. However, in some cases few cultures were found to differentiate into normal plantlets at all concentrations and types of cytokinins tested. Besides, callus proliferation was observed in a number of treatments. On the other hand at least certain percentage of the embryo underwent morphological changes which led them to differentiate multiple shoots. In the present study it was noticed that morphological changes of multiple shoot forming embryos during culture were different than those of callus forming embryos or those embryos developed from normal plantlets. These embryos became swell up considerably and buds emerged out in the form of tube like protuberances which grew rapidly into buds and then to shoot. These results are in consistent with the reports of Ohyama and Oka (1980), Wehner and Locy (1981) for embryonic tissue of *Cucumis sativus*, Konar and Oberoi (1965); Hu and Sussex (1971) for embryo of some plants.

The preceding observations indicate that application of cytokinins was every effective in inducing adventitious buds from embryos of *A. marmelos*. Among the three cytokinins tested BA was the best in inducing adventitious bud than Kn or 2ip. Superior effect of BA on adventitious bud regeneration has been described by Ohyama and Oka (1980) for hypocotyl of *Broussonetia* Kazinol; Ishikana (1975) in *Cryptomeria japonica*. BA at 1.0 mgl^{-1} was found to be best which induced maximum percentage of immature embryo to develop buds with the highest number of shoots per culture. However, mature embryo showed optimum bud regeneration at 2.0 mgl^{-1} BA. It indicated that endogenous hormones of the mature and immature embryos interacted differentially with the growth regulator level

present in the culture media. Similar response of apple embryo and cotyledon explants was reported by Kouider *et al.* (1985).

Present study showed that addition of auxins (NAA and IAA) and GA_3 with BA in the culture medium dramatically increased adventitious bud regeneration from embryo explant. However, simultaneously increased callusing. Degree of callusing was directly proportional to the concentration of auxin present in the media. Auxins and GA_3 at high concentration tested suppressed adventitious bud regeneration. Bud regeneration was the lowest when the culture media contained higher concentration of auxins and GA_3. The morphogenic differentiation of the cultured explant could be controlled by the auxin-cytokinin ratios present in the culture media (Skoog and Miller, 1957). Similar results also demonstrated by Ohyama and Oka, 1980; Hammersehlag *et al.*, 1985, Niedz *et al.*, 1989; Verhagen Wann, 1989; Roy and De, 1990.

Among the different combinations of BA-NAA, BA-IAA, BA-GA_3 and BA-NAN-GA_3, BA-NAA was found the best combination for frequency of adventitious bud regeneration for both mature and immature embryo and BA-GA_3 was found to be the best for degree of shoot regeneration per culture for immature embryo. Addition of NAA with BA-GA_3 was the best for mature embryo in respect of number of shoots per culture.

Adventitious bud regeneration ability of the embryo explants of 5 trees of *A. marmelos* differed significantly. The degree of shoot regeneration per culture was the highest for tree 5 and the lowest for tree 1 (tables 7 and 19) and tree 4 (tables 8 and 20). The highest frequency (%) of explants showing adventitious bud regeneration was observed in tree 5 and the lowest in tree 2 for both immature and mature embryo explants. Tree to tree variation in morphogenic competency was also observed by Lutova and Zabelina (1988); Kim *et al.* (1988) and Hammat *et al.* (1989) in different crop plants.

In the analysis of variance, all the main items (tree and treatment) were significant against experimental error. The item tree was significant in the characters indicating that a real difference existed among trees. A real effect of different treatment (E) was also noted, as the item treatment was significant in all cases. It can be concluded that factors in addition to genes controlling the regeneration ability influenced tree × culture protocol (Kris and Bingham, 1988).

The morphogenic differentiation of the cotyledon explant of five different trees was found to be determined by the growth regulators formulation. There are two types of differentiation such as development of callus subsequent to adventitious bud directly from the surface of the explants. But degree of these differentiation greatly varied with media formulation.

Present investigation demonstrated successful plant regeneration directly from cotyledon explant of *A. marmelos*. The formation of adventitious shoot from the cotyledon of *A. marmelos* was independent to the presence of embryonic axis which indicates that full potential for plant regeneration was present within the cotyledon itself. The reports on plant regeneration from the cotyledon explant of apple (Kouider *et al.*, 1985) and of water melon (Dong and Jia, 1991) confirm the result of present study. Plant regeneration from cotyledon needed proper concentration of cytokinins. Among the three types of cytokinins (BA, Kn and 2ip), BA was found to be more effective to include adventitious regeneration. Among the different concentration of BA, 2.0 mgl^{-1} gave better result. Shoot proliferation suppressed with the increase or decrease of BA, Kn and 2ip concentration. The superior effect of BA on adventitious bud proliferation from cotyledon explant has been reported by Kouider *et al.* (1985) for apple; Kim *et al.* (1988); Niedz *et al.* (1989); Dirks and Van Buggenum (1989) for water melon. However, contrary to these Dong and Jia (1991) reported that higher concentration of BA (7.0 mgl^{-1}) gave the best result in shoot regeneration from cotyledon of water melon.

Presence of low concentration of NAA, IAA (0.1-0.2 mgl^{-1}) and GA_3 (0.5-1.0 mgl^{-1}) in the media markedly promoted shoot regeneration from cotyledon. BA (1.0 mgl^{-1}) + NAA (0.2 mgl^{-1}) + GA_3 (0.5 mgl^{-1}) was found to be the best formulation for the highest frequency of shoot regeneration (56.50%) and BA (1.0 mgl^{-1}) + NAA (0.2 mgl^{-1}) + GA_3 (1.0 mgl^{-1}) then other hormonal supplement from cotyledon of *A. marmelos*. Promotive effect of GA_3 in combination with BA-NAA on shoot regeneration from cotyledon of melon was also reported by Kathal *et al.* (1986) and Niedz *et al.* (1989).

The use of cotyledon explant has several advantages (Gogala and Camloh, 1988). Microbial contamination of such explant has never been a serious problem. Nevertheless, cotyledons have been shown to posses high morphogenic potential (Rao *et al.*, 1981a; Singh *et al.*, 1981 and Fagekas *et al.*, 1986). Rao *et al.* (1981a) initiated cotyledon culture of two tropical fruits with very limited success. Tissue culture methodology for plant regeneration from cotyledon explant is well established for herbaceous, ornamentals, fruits or vegetable crops specially for those belong to family cucurbitaceae and cruiciferae. However, to date, investigators have made relative slow progress on plant regeneration protocols for tree species (Rubos and Pryke, 1984 and Mante *et al.*, 1989).

The morphogenic competence of 5 trees tested in the present study was not same with regard to shoot regeneration potentiality from cotyledon explant. Optimum hormonal concentrations were not same for all trees. These trees reacted differently with different levels and types of cytokinin. Tree 5 showed the highest degree of shoot regeneration capacity and tree 2 (table 3) and tree 1 (table 15) were the second highest with regards to percentage of regeneration. In respect of the number of shoot regeneration per culture. Tree 5 was also best but tree 4 was the second highest The items, tree and treatment were significant for the percentage of shoot producing explant. In respect of number of shoots per culture where the main

item tree was non-significant. A real treatment effect was also noted for number of shoots per culture where treatment effect was highly significant.

Tree specificity to response in *in vitro* culture has been well established fact for wide range of plants for example in soybean (Ranch *et al.*, 1985; Parrott *et al.*, 1989); alfalfa (Chen and Marowitch, 1987); in melon (Niedz *et al.*, 1989); in cucumber (Kim *et al.*, 1988); in *Brassica* sp. (Fagekas *et al.*, 1986).

Early reports of Arya *et al.* (1981) have proven the applicability of hypocotyl segments as explant in case of *Aegle marmelos* tissue culture. Hence in the present experiment hypocotyl segments were also selected as explants (Table 4 and 10). Avoiding an intervening callus phase by inducing direct organogenesis in this case may on the other hand produce a multitude of plants without risking clonal fidelity. Direct organogenesis from hypocotyl has also been reported in geranium (Qurshi and Saxena, 1992) and *Phaseolus* (Malik and Saxena, 1992). Since differentiation occurs on hypocotyl the number of manipulations required to induce regeneration (Arya *et al.*, 1981).

Shoot proliferation from the hypocotyl explants of 5 trees took place through the formation of shoot primordia only at the cut end towards the apical region. Adventitious buds initially grew as globulas embryoid like protrutious eventually grew to adventitious buds and formed multiple shoots during the period of culture. The result showed that maximum number of hypocotyl explants produced shoots in 2.0 mgl^{-1} BA. The highest number of shoots per culture was recorded at same BA concentration Among the three cytokinins, the higher level (2.0mgl^{-1}) of BA showed the best shoot differentiation. The number of shoot per culture increased with the increase of BA concentration. Similar results have been reported by Islam (1991) in chickpea.

In general, the direct multiple shoot regeneration of hypocotyl was found to be accentuated in the presence of auxins (NAA or IAA) and GA_3 together with BA. But BA-NAA-GA_3 combinations were proved to be more effective then BA-NAA, BA-IAA and BA-GA_3 combinations. Similar results have been reported by Anil *et al.* (1986b) in *Cicer arietenum* L.

Observation on comparative studies with different concentrations of cytokinins and in combinations of auxins (NAA and IAA) and GA_3 on direct regeneration of hypocotyl explants of 5 trees of *Aegle marmelos* shows that the treatment concentration not only produced pronounced variation in days to shoot initiation, frequency of shoot proliferation and shoot growth, but also had a great effect on number of shoot per culture. Maximum frequency of regenerating explants and number of shoots per culture was recorded in trees. The trees interacted significantly with growth regulator level. The items tree and treatment were significant for both percentage of shoot forming explants and number of shoots per culture. However, the items BA, BA+IAA, Kn, 2ip and TXE were non-significant in both cases. Tree specificity with regard to shoot regeneration was also observed by Lutova and Zabelina (1988); Li *et al.* (1988) and Hammat *et al.* (1989). Significant interaction was also observed between genotypes of soybean and two cultural protocols used for both embryoid formation and shoot regeneration. It was concluded that factors in addition to genes controlling regeneration ability influence tree × cultural protocols (Kris and Bingham, 1988).

Present study also demonstrated plant regeneration from leaves of 5 trees of *Aegle marmelos*. Adventitious shoot regeneration on first 1-4 distal leaf explant of *A. marmelos* was found to be depended on proper growth regulator formulation, physiological age of the explant and culture environment. Shoot regeneration occurred in presence of 0.5-2.0 mgl^{-1} BA, 1.0-5.0 mgl^{-1} Kn and 2ip. However, the lower concentration of cytokinins suppressed shoot regeneration. Among the 9

different concentrations of cytokinins, 2.0 mgl^{-1} BA was the optimum for frequency of shoot regenerating explants and the number of shoot per culture. These results are in agreement with Marilyn Daykin *et al.* (1976) who regenerated shoot from petunia leaves in present of BA only. In present investigation it was found that the shoot regeneration prospensity of explant increased when auxins (NAA or IAA) and GA_3 were added to the medium with BA. Low NAA, IAA or GA_3 concentration with BA accentuated shoot proliferation but BA-IAA combination was better than BA-NAA or BA-GA_3. Among the different combinations and concentrations of BA and auxin or BA-GA_3, 2.0 mgl^{-1} BA with 0.1 mgl^{-1} IAA was found optimum combinations. Same concentrations of BA and NAA were also effective than other growth regulator levels. The results in the present study are in agreement with the observation of Calvo and Segura (1989) for *Lavandula latifolia* leaves. Roest and Bokelmann (1975) regenerated shoots of *Chrysanthemum* from pedicels on a similar optimum medium of BA and IAA.

In the use of plant tissue culture technologies for plant breeding, leaves may be the preferable explant as the leaf disc method has been used for genetic transformation of several species (Horsch *et al.*, 1985; Klee *et al.*, 1987; Horsch *et al.* 1987). The method has obviated the need to develop a protoplast to plant regeneration system and transgenic plants can be obtained more efficiently, often within 4-6 weeks of subculture (Klee *et al.*, 1987). A method of producing shoots, ultimately from single cells may help to retain clonal fidelity (Broertjes and Keen, 1980). Nevertheless, adventitious organogenesis specially from single cells or from small number initial can induce high levels of somaclonal variation and are useful in raising valuable non-chimeral solid mutant (Larkin and Scowcroft, 1981a; Evans and Sharp, 1986). Recently plant regeneration has been achieved from leaves in a number of fruits species (James *et al.*, 1987) with frequencies depending on genetic trait and explant source (Thorpe, 1980). Organogenesis from the leaf

tissues of woody dicots has been reported previously (Liu *et al.*, 1983; James *et al.*, 1984; Kim *et al.*, 1985; Simola, 1985; Strivastava *et al.*, 1985; Charles *et al.*, 1986). Islam *et al.* (1993) obtained plants directly from *in vitro* grown leaf explants in *A. marmelos*.

Among the 5 trees, tree 5 produced more direct multiple shoots from leaf in all treatments compared to other trees. In the present investigation significant variation among the trees and treatments for multiple shoot regeneration from leaf was also noted.

The results of the present experiment present a reproducible and efficient plant regeneration system through organogenesis from nucellar explants of 5 trees of *A. marmelos*. The nucellar of *A. marmelos* grew to the white mass of tissue within 90-130 days after pollination which were found amenable for culture. These observations are in agreement with the reports of Singh (1963). The excised nucellus when cultured with proper growth regulator induced to develop organogenic callus or to regenerate shoot directly. The morphogenic response of nucellar tissues were completely depended on growth regulator supplement in culture media. The nucelli of *A. marmelos* when cultured in presence of cytokinins and cytokinins-auxins in MS medium induced small amount of callus, accompanied with a numerous small spherical and elongated pseudobulbils which developed later into complete plantlets or into shoots only. Plantlets formation was more frequent when these pseudobulbils were cultured onto fresh medium. These observations are also in consonance with Kochba and Spiegel-Roy (1973); Kochba and Spiegel-Roy (1976) for *Citrus* nucellus. Among the different cytokinins, BA-auxins, BA-GA_3 and BA-NAA-GA_3 concentrations or combinations supplements tested 1.0 mgl^{-1} BA + 0.1 mgl^{-1} NAA was the best combinations for optimum regeneration. Nevertheless, BA-NAA combinations were better than cytokinins alone or BA-IAA or BA-GA_3 and BA-NAA-GA_3. Kochkba and Spiegel (1976)

observed embryo induction in 'Shamouti, nucellar callus when incubated in the medium having 1.0 mgl^{-1} Kn + 1.0 mgl^{-1} IAA. In the present study, low concentrations of cytokinins (0.5-1.0 mgl^{-1}) although initiated lower percentage of regenerating explants but in higher concentration, this cytokinin inhibited nucellar tissue growth. Similar effect of BA or Kn on nucellus culture of *Citrus* spp. also reported by Kochba and Spiegel-Roy (1977) and Tisserat and Murashige (1974). Direct shoot regeneration capacity of nucellars explant was increased with the addition of low concentration auxins or GA_3 with BA. BA-NAA combination was the best formulation for shoot regeneration (Hossain *et al.*, 1993). Direct development of somatic embryos from the nucellar tissues of *Citrus* spp. without an intermediate callus stage was also reported by Button and Bornman (1971); Button and Kochba (1977) and Ling *et al.* (1990). Nucellar derived plants are free of virus and other disease causing micro-organisms due to the absence of vascular connection between the surrounding maternal tissue and the nucellus (Button and Kochba, 1977). Litz (1984b) demonstrated the practically of using ovule cultures to simulate *in vitro* somatic embryogenesis from the nucellus of monoembryonic mango. *In vitro* plant regeneration from nucellar explants has been described (Rangan *et al.*, 1960; Maheshwari and Rangaswamy, 1965; Rangan, 1982). The nucellus culture technique has been used to recover virus-free citrus cultivars in Spain (Navarro, 1976; Navarro *et al.*, 1980). *A. marmelos* is polyembryonic and the plantlets achieved was through shoot bud organogenesis. Nucellus culture *in vitro* may, therefore, facilitate rapid clonal propagation of *A. marmelos* and is potentially useful for breeding of horticulturally useful disease free plants.

Adventitious bud regeneration ability of nucellar explants of the 5 trees of *A. marmelos* differed significantly. Analysis of variance showed that the items tree and treatment were significant for the percentage of explant induced bud regeneration and the number of shoots per culture. These indicated that real genetic

difference existed among the trees and each tree interacted differentially with different growth regulator levels tested. The degree of shoot regeneration was the highest for tree 5 and the lowest for tree 3. Tree specificity in morphogenic competency was also observed by Wehner and Locy (1981) and Zelcer *et al.* (1984) in different crop plants.

Nucellus holds a great potential for applied research in horticulture which has already been augmented specially in citriculture and vitriculture. Demonstration of whole plant regeneration from single cell of nucellar tissue without the intervenation of callus phase or plant regeneration from isolated nucellar protoplast, has opened up excellent opportunity for breeding, development of solid mutant and transgenic plant. Nevertheless, nucellar clones retain maternal clonal purity and virus free because of lack of vascular connection of this tissue with parent plant. These unique characters of nucellar clones provides excellent opportunity of using in large scale micropropagation. Therefore, plant regeneration from nucellar tissues of *A. marmelos* which has been described in the foregoing section may provide opportunity of large scale multiplication of elite trees and genetic in improvement of this crop.

Present investigation demonstrated that induction of organogenic callus from embryo cultures of *A. marmelos*. Morphogenesis of the cultured embryos varied with the concentrations and combinations of auxins and cytokinins. Callus proliferation was noticed in all media formulation. However, there was a wide range of variation among them. Calli found in media with higher concentration of auxins (5.0 mgl^{-1}). Supplemention of lower concentrations of cytokinins and GA_3 with auxins in the culture media showed promotive effect on the frequency of callusing but decreased the potentiality to adventitious bud regeneration.

Among the different concentrations and combinations of auxins and cytokinins, 5.0 mgl^{-1} 2,4-D + 2.0 mgl^{-1} Kn was found best deducing the highest

percentage of explant to develop calluse and fresh weight of calli per explant (g). The embryos are known to be good source for initiating callus which possesses high regenerative capacity, as has been shown in Zea maize (Duncan *et al.*, 1985), barley (Goldstein and Kronstad, 1986; Daphne and Breiman, 1989), peach (Hamerschlag *et al.*, 1985), *Trifolium* (Maheswaran and Williams, 1985), *Calotrips* (Roy and De, 1990), *Camellia* (Vieitez and Braciela, 1990), oak and linden (Chalupa, 1990) and *Limnanthes* (Southworth and Kwiatkowski, 1991).

The callus proliferation ability of embryo explants of the 5 trees of *A. marmelos* differed significantly. Analysis of variance showed that the items tree and treatment were highly significant for percentage of callusing and fresh weight of calli per culture. The item T×E was non-significant. The egree of callus proliferation was the highest for tree 5 and the lowest for tree 3. Tree specificity in morphogenic competency was also observed by Wehner and Locy (1981); Zelcer *et al.* (1984) and Kim *et al.*, (1988) in different crop plants.

Present investigation also demonstrated successful callus induction from cotyledon explants of *A. marmelos*. Response of callusing greatly varied with explant source (trees) and treatment concentrations and combinations. Calli found in media with higher concentration of auxins (5.0 mgl^{-1}) with cytokinins showed promotive effect on percentage of callusing and fresh weight of calli per culture (g). The highest percentage of callusing (65.86%) and fresh weight of calli per culture (1.15 g) were recorded at 5.0 mgl^{-1} 2,4-D + 2.0 mgl^{-1} Kn. Similar results were also reported by Gonzales *et al.* (1985) for cotyledon explant of chest nut and by Miller and Chandler (1990) for cotyledon explant of strawberry.

Morphogenic competence of 5 trees of *A. marmelos* with regard to callus proliferation from cotyledon explants markedly differed, as the items tree and treatment (E) were highly significant. The interaction item tree×treatment was non-

significant for percentage of callusing and fresh weight of calli per culture. Nevertheless, optimum hormone requirement was different from tree to tree. Morphogenic competence of the tree 5 was the highest, whereas, tree 3 was the lowest. Tree specificity for callusing was well documented, in soybean (Ranch *et al.*, 1985; Parrott *et al.*, 1989); in white clover (Mohapara and Gresshoff, 1982); Bhojwani *et al.* (1988); in melon (Niedz *et al.*, 1989).

Early reports of Arya *et al.* (1981) have proven the applicability of hypocotyl segments as explant in case of *A. marmelos* tissue culture. Many of the problems of inducing callus from plant tissues may be overcome by using parts of freshly germinated seedlings, ensuring that tissue fragments composed of callus with high growth potential (Yeoman and Forche, 1980). Hence in the present experiment to induce callus only young parts such as hypocotyl segments were selected as explants.

Earlier there is only one report for *A. marmelos* tissue culture concerning with the induction of callus and subsequent plant regeneration (Arya *et al.*, 1981). They induced callus from hypocotyl and stem segments of aseptically grown seedlings in MS medium employed with auxins (NAA or IBA) and Kn.

In the present work, the different concentrations of auxins and cytokinins or GA_3 were used either alone or in combinations. It was found that callus proliferation strictly depended on exogenous growth regulator supplementation. In the absence of exogenous growth regulator the explants failed to induce callus. Callus proliferation observed from explants in all the concentrations and combinations of auxins and cytokinins or GA_3. Maximum percentage of callusing (79.71%) and highest fresh weight of calli per culture (1.65 g) were recorded at 5.0 mgl^{-1} 2,4-D + 2.0 mgl^{-1} Kn. Between the two auxins and 2,4-D showed more promotive effect on callus growth through increasing fresh weight of callus. The adddition of kn for the increased the degree of callusing. Many workers observed

2,4-D as the best auxin for callus induction as common in monocot and even in dicot (Gonzalez *et al.*, 1985; Wang *et al.*, 1987; Chee, 1990). Arya *et al.* (1981) and Hossain, 1992 observed the best callus growth on hypocotyl explant of *A. marmelos* in NAA (5.0 mgl^{-1}) supplemented MS medium.

The morphogenic competence of 5 trees tested in the present study was not same with regard to callus proliferation from hypocotyl explants. Optimum growth regulator supplements were not same for all trees. These trees reacted differentially with different levels and types of growth regulators. Tree 5 showed the highest frequency (66.80%) of callusing and fresh weight of calli per culture (1.35 g). Tree 3 showed the lowest (54.32%) frequency of callusing and fresh weight of calli per culture (1.01 g). Tree specificity in response to callus formation has been well establish fact for wide range of plants (Parrott *et al.*, 1989; Mahapara and Gresshoff, 1982).

In the present study, the leaf explants of *A. marmelos* frequently developed callus at their out edge in auxin-cytokinin supplemented medium. The media with 5.0 mgl^{-1} 2,4-D + 2.0 mgl^{-1} Kn was more effective for callus induction than other concentration and combinations. These results are in conforming with finding of Ohyama and Oka (1980); Mroginski and Kartha (1981).

Two different patterns of bud differentiation from leaf explant of *A. marmelos* were observed. One was through meristemoids directly formed on the epidermal layer without intermediate callus formation. The other was through callus usually formed at the basal cut ends of the leaf explants. These observations are in agreement with the results of Mroginski and Kartha (1981).

Tree specificity with regard to callus proliferation from leaf explants of 5 trees was observed, when the leaf explants of these trees were cultured onto 11 different media composition. Tree-5 showed the highest degree of callus

proliferation and fresh weight of calli per culture. Whereas, tree 4 was the lowest for percentage of callusing and tree 3 for fresh weight of calli per culture.

The callus proliferation ability of leaf explants of the 5 trees of *A. marmelos* differed significantly. Analysis of variance showed that the items tree and treatment were significant, however, the interaction item T×E was non-significant. It can be concluded that factors in addition to genes controlling the proliferation ability, influenced tree×culture protocol (Kris and Bingham, 1988).

Successful callus proliferation from nucellar tissues of *A. marmelos* has been demonstrated in the present investigation. Media with auxins alone (5.0 mgl^{-1}) or in combination with cytokinins (1.0 - 2.0 mgl^{-1}) or in combination with cytokinins (1.0 - 2.0 mgl^{-1}) were used for callus induction. Among the different combinations Kn—NAA and BA—2,4-D were found to be more efficient than other combinations for callus induction. Maximum response was obtained in medium when 2.0 mgl^{-1} Kn + 5.0 mgl^{-1} NAA were added. Ling *et al.* (1990) initiated embryonic callus from nucellus of *Citrus spp.* by using BA+2,4-D. Embryogenic callus initiation from mango nucellar tissues with the help of 2,4-D was also reported by Litz *et al.* (1982, 1984b) which supports the result of present study. Hossain *et al.* (1993) developed a protocol for organogenesis from nucellar explants from the fertilized ovules of immature fruits of *A. marmelos*. Analysis of variance showed that the items tree and treatment were significant in respect of percentage of callusing and fresh weight of calli per culture and the item T×E was non-significant in both characters.

Present investigation demonstrated that multiple shoot regeneration could be possible from embryo derived callus culture of *A. marmelos*. The morphogenesis of the cultured callus varied with the concentrations and combinations of cytokinins alone or in combination with auxins and GA_3 present in the media. The

physiological state of the explant plays an important role in the regeneration of plants. Among the three cytokinins tested BA was the best in inducing multiple shoots than Kn or 2ip. Superior effect of BA on multiple shoot regeneration has been described by Ohyama and Oka (1980) for hypocotyl of *Broussonetia kaziooki*; Ishikawa (1975) in *Cryptomeria japonica*; Kouider *et al.* (1985) in apple embryo.

Present study showed that addition of auxins (NAA and IAA) and GA_3 with BA in the culture media increased multiple shoot regeneration from embryo derived callus. On the other hand the addition of auxins and GA_3 with Kn in the culture media decreased multiple shoot regeneration from embryo derived callus. Among 10 different concentrations and combinations, 1.0 mgl^{-1} BA + 0.5 mgl^{-1} GA_3 was the best combination in which 60.99% of calli developed shoot buds. Number of shoots per callus was also high in this formulation. Similar results were also demonstrated by Niedz *et al.*, 1989; Roy and De, 1990.

Multiple shoot regeneration ability of embryo derived callus of the 5 trees of *A. marmelos* differed significantly. Analysis of variance showed that the items tree and treatment were highly significant for number of shoots per culture. Number of shoot regeneration was the highest for tree 5 and the lowest for tree 4. Genotypic variation in morphogenic competency was also observed by Wehner and Locy (1981); Lutova and Zabelina (1988) in different crop plants.

Present study demonstrated successful plant regeneration through callus formation from cotyledon explants of *A. marmelos*. Nevertheless, plant regeneration from cotyledon derived callus needed proper combination and concentration of hormonal supplement. Among the different concentrations and combinations of growth regulators used in the present study, low concentration of BA (1.0 or 2.0 mgl^{-1}) in combination with lower concentration of NAA (0.1 or 0.2 mgl^{-1}) and GA_3 (0.5 mgl^{-1}) gave the better result in multiple shoot regeneration.

Although BA alone showed less promotive effect but found to be better than Kn alone. Among the different concentrations and combinations of growth regulators, 1.0 mgl^{-1} BA + 0.5 mgl^{-1} GA_3 gave better result (36.23% with 39.77 shoots per culture). Promotive effect of GA_3 in combination with BA on multiple shoot regeneration from cotyledon derived callus was also reported by Kathal *et al.* (1986) and Niedz *et al.* (1989) in melon. In *A. marmelos* multiple shoot regeneration has been reported from hypocotyl and cotyledon derived calli (Arya *et al.*, 1981; Islam *et al.*, 1993). Tissue culture methodology for plant regeneration from cotyledon explants is well established for fruit crops especially for those belonging to the Cucurbitaceae and Cruciferae (Dong and Jia, 1991). However, investigators have made relatively slow progress on plant regeneration protocol fro tree species (Rao *et al.*, 1981; Mante *et al.*, 1989; Kouider *et al.*, 1985).

The morphogenic competence of 5 trees of *A. marmelos* with regard to shoot regeneration from cotyledon derived callus explants markedly differed, as the items tree, treatment and T×E interaction were significant for the percentage of shoot producing callus and the number of shoots per explant. The morphogenic competence of the tree 5 was the highest and tree 4 was the lowest in respect of percentage of organogenic callus and number of shoots per callus.

Successful plant regeneration through callus formation from hypocotyl explants of *A. marmelos* was also achieved in the present work. Different concentrations of cytokinins and auxins on GA_3 were used either alone or in combinations. Among the 10 different concentrations and combinations, 1.0 mgl^{-1} BA + 0.5 mgl^{-1} GA_3 was found to be the best for shoot induction and the ratio of BA-NAA or BA-IAA was 2 : 1. These observations support classical cytokinin-auxin balance hypothesis of Skoog and Miller (1957). Combination of BA and NAA have successfully been employed to induce plant regeneration in wide range of species (Mroginski and Kartha, 1981; Rubluo *et al.*, 1984; Kameya and

Widholm, 1986). There is only two reports for *A. marmelos* tissue culture concerning with the induction of callus and subsequent plant regeneration (Arya *et al.*, 1981; Hossain, 1992) which supported the findings of the present investigation.

Multiple shoot regeneration ability of hypocotyl callus of the 5 trees of *A. marmelos* different significantly. Analysis of variance showed that the items tree and treatment were significant and interaction item T×E was non-significant.

Present study demonstrated plant regeneration from leaf derived callus of *A. marmelos*. Among the different concentrations and combinations of growth regulators used in the present study, low concentration of NAA or IAA with BA accentuated shoot proliferation but BA-NAA, BA-IAA combinations were better than other combinations. The results in the present study are in agreement with the observation of Calvo and Segura (1989) for *Lavandula latifolia* leaves). Roest and Bokelmann (1975) regenerated shoots of *Chrysanthemum* from pedicels on a similar optimum medium of BA and IAA.

Early reports of Islam *et al.* (1992; 1993) have proven the applicability of leaf segments as explant in case of *A. marmelos* tissue culture. A reproducible and efficient plant regeneration system through organogenesis from leaf explants was developed by these authors. Organogenesis from leaf tissues of other woody dicots has been reported previously (Liu *et al.*, 1983; James *et al.*, 1984; 1986; Kim *et al.*, 1985; Simola, 1985; Strivastava *et al.*, 1985; Charles *et al.*, 1986). If an intermediary callus phase is involved, as of regeneration via organogenesis, the frequency of genetic changes is increased (Hu and Wang, 1983). When differentiated tissues, such as those derived from leaf are used for plant regeneration through an intermediary callus phase, the possibility of somaclonal variation increases affording an opportunity to breeders to exploit variations for improvement of fruit crops, such as *A. marmelos*.

The quantitative estimation of calli reveals that the shoot regeneration ability of callus cultures were highly affected by different treatment concentrations and combinations. It was observed in the present study that leaf callus of 5 different trees interacted differently with different growth regulator concentrations and combinations as the items tree and treatment for percentage of organogenic calli and number of shoots per callus were significant. Tree 5 thrived well at all tested growth regulators level. Analysis of variance in this case showed that in most cases a real difference existed among the trees and treatments.

The results of the present experiment present a reproducible and efficient plant regeneration system through organogenesis from nucellar callus of *A. marmelos*. The morphogenic responses of nucellar callus tissues were completely depended on growth regulator supplement in culture media. The nucellar calli of *A. marmelos* when cultured in presence of cytokinins (BA and Kn) and auxins (NAA and IAA) or GA_3 in the MS medium induced adventitious buds. These observations are also in consonance with Kochba *et al.* (1978); Kochba and Spiegel-Roy (1976) of *Citrus* nucellus. Among the different growth regulator concentrations and combinations tested, 1.0 mgl^{-1} BA + 0.5 mgl^{-1} GA_3 was the best combination for percentage of organogenic calli and number of shoots per callus. Similar results have been reported from nucellar derived callus in the same species (Hossain *et al.*, 1993; 1994). Nucellus derived plants are supposed to be true-to-type because nucellar tissues are of maternal origin (Rangaswamy, 1981). Moreover, nucellus-derived plants generally are free of virus (Navarro, 1976; Navarro *et al.*, 1980) and other disease-causing micro-organisms, due to the absence of vascular connections between the surrounding maternal tissue and the nucellus (Button and Kochba, 1977). Efficient recovery of *A. marmelos* plants through the process described will facilitate storage and interactional exchange of germplasm.

The multiple shoot regeneration ability of nucellar callus explants of the 5 trees of *A. marmelos* differed significantly. Analysis of variance showed that the items tree and treatment were significant for the percentage of organogenic calli and the number of shoots per callus. These indicated that real genetic difference existed among the trees and each tree interacted differently with different growth regulators tested. Morphogenic competence of the tree 5 was the highest. Whereas, tree 4 was the lowest.

The shoot regenerated from different explants of 5 trees were needed to induce roots for their ultimate establish in the field. So, experiments were conducted with basal MS medium supplemented with different concentrations of IBA. Percentage of root induction, number of roots per culture and length of root were highly influenced by the concentrations of IBA. Among different concentrations of IBA only 30 mgl^{-1} IBA was found to be the best for root induction where optimum percentage of shoot (87%) induced roots with maximum number (6.57 roots per shoot) of roots per shoot and length of root (3.50 cm) was achieved. These findings showed similarities to Normah (1988) in mangosteen (*Garcinia mangostana*). Roy and De (1986) and Mante *et al.* (1989) also reported promotive effect of IBA on rooting percentage and faster growth of roots in *Calotropis gigantea* and *Prunus* sp. respectively.

Microcuttings cultured for root initiation in media having higher concentration (40 mgl^{-1}) of IBA induced root and callus proliferation simultaneously. Callus proliferation at the base of microcuttings made them difficult to survive in the field condition. Islam *et al.* (1993) reported higher frequency of rooting of microcuttings of *A. marmelos* in presence of 30 mgl^{-1} IBA which supported these findings.

Agar has been used in the culture media as gelling agent which increased water loss due to evaporation associated with reduced sugar concentration (Conger,

1981). Agar is also a source of numerous mineral elements (Dodds and Roberts, 1985). Nevertheless, *in vitro* root proliferation and growth variously affected by source and concentration of agar. In the present investigation optimum agar concentration for the best root induction and root length was 7.0-7.5 gl^{-1}. However, root induction was suppressed at higher and lower concentration of agar. High agar concentrations reduced the relative humidity and the most of the roots from the higher agar concentration were damaged at the time of transplanting and thus there was no improvement in establishment of plant in *ex vitro* condition. Similar results were observed in jackfruit (Rahman and Blake, 1988) and rose (Rahman *et al.*, 1992). Reduced growth and less number of roots on the medium containing higher concentration of agar might be due to restricted diffusion of macronutrients (Romberger and Tabor, 1971; Gasper *et al.*, 1987 and reduced availability of organic matter and water (Stoltz, 1971; Skirvin, 1984 and Debergh, 1983). It is evident from the preceding results that concentrations of agar in the medium can affect the culture growth in several ways.

Carbohydrates not only function as a carbon source in metabolism but they also play an important role in the regulation of the external osmotic potential in the culture media (Brown *et al.*, 1979; Brown and Thorpe, 1980). Sucrose at 2-4% generally incorporated as a carbon and energy source (Murashige and Tucker, 1969). Different concentration of sucrose with constant hormonal concentration may regulate xylem and phloem differentiation in several callus tissues (Fadia and Mehta, 1973) and also help in the development of organized shoots or roots (Rowal and Mehta, 1982 and Miah *et al.*, 1986). In tobacco, a constant concentration of auxins (0.3 mgl^{-1}) with 1% sucrose level suppressed shoot differentiation, but at 3% sucrose level shoots were initiated, while at 6% level only roots were developed (Rowal and Mehta, 1982). A constant concentration of 5% sucrose found to be optimum in *Citrus* tissue culture (Murashige and Tucker, 1969). In the present

study, 30 mgl^{-1} sucrose concentration favoured optimum root proliferation response and root growth. At the lowest (10.0 - 25.0 gl^{-1}) and the highest (35.0 - 80.0 gl^{-1}) concentrations, proliferation rates were reduced and the root elongations was inhibited. These results are in conformity with the findings of Rahman *et al.* (1992) who reported that lower and higher sucrose level inhibited root elongation in rose cultures. In the present investigation 3% sucrose was found to be optimum for root differentiation. Higher concentrations of sucrose (4-8%) showed bushy and dwarf roots development. However, positive effect of increased levels of sucrose on *in vitro* rooting has been reported for walnut (Driver and Kuniyuki, 1984).

The *in vitro* adventitious root induction capacity of the microcuttings were also affected by pH of the medium. The present investigation revealed that higher and lower pH level gradually decreased adventitious root induction. P^H 7.0 was found to be the optimum at which maximum number of roots per cutting (11.60) and root length (3.42 cm.) were obtained. Skirvin (1984) has stated that most fruit tissue cultures are grown at pH 5.6-7.0. More acidic pH (4.0-5.0) gave softer gel which might have adverse effect on adventitious root induction. Williams *et al.* (1985) found pH (4.0) best for two woody species, cherries rooted at pH 5.3-5.8 (Sauer, 1983); plum at pH 5.5-5.9 (Nemeth, 1986) and rose at pH 4.5-7.5 (Rahman *et al.*, 1992). All these results indicated the species specific rooting response to different pH of the medium.

Microcuttings showed variable response to root initiation and root length depending on different media composition. Among the 5 media composition, MS medium gave more roots and higher roots length. White's medium, showed the lowest performance in root initiation and root length. MS allowed 5 to 7 times active growth in wide range of plant species including monocot and dicot (Conger, 1981 and Gautheret, 1985).

Present study showed that the continuous production of microcuttings from shoot cultures obtained from different explants was possible without affecting shoot growth. Continuous production of microcutting was the high for germinated embryo explant. Microcutting production rate of cotyledon, nucellus hypocotyl and leaf explants were not so high when compared with embryo explant. Among the different explant sources, percentages of rooting of shoots were found to be the highest for those shoots that were obtained from embryo explant and lowest for those obtained from leaf explants.

Percentage of root forming microcuttings, number of root per cutting and root length of the 5 trees of *A. marmelos* differed significantly. Analysis of variance showed that the items tree and treatment were significant in all cases. These indicated that a real genetic difference existed among the trees and each tree interacted differently with different factors (IBA, agar, sucrose, pH, media composition and source of explants) tested.

Microclones of *A. marmelos* irrespective to the explant sources thrived well in the soil showed vigourous and uniform growth. Increased vigour of microclones under field condition has also been reported for apple (Zimmerman, 1986) and thornless blackberry (Swartz *et al.*, 1983).

The linear regression (b) is considered to be a definite and measurable response to the treatment (E). Trees which have relatively the same amount of performance over a wide range of treatments, would have b values less than unity and would be east responsive to change in the treatment. In the present investigation, the distribution of five b values were heterogeneous as revealed by regression analysis and hence all these trees had different response to different treatments. The standard error of regression is a measure of "stability of response" exhibited by each tree. Since, the regression represents very definite and

measurable response to the treatment (Finlay and Wilkinson, 1963; Lerner, 1958; Eberhart and Russel, 1966).

In all cases crossing of regression lines (Figs. 1-11) was very marked and in most cases it turned out that significant difference could still be detected even in the poor as well as in good environment or treatment (E).

Chapter V

Summary

SUMMARY

Different explants of 5 trees like embryo, cotyledon, hypocotyl, leaf and nucellus of *Aegle marmelos* Corr. locally known as 'Bael' were taken as experimental materials to took for direct and indirect regeneration. Following the procedure presented in the foregoing chapters plant regeneration could be possible from all these explants of 5 trees. However, the morphogenic competence of these explants and trees were not same. Among these explants morphogenic potentiality of embryo explant was the highest and leaf explants was the lowest. The morphogenic competency of Tree 5 was found to be higher than other trees.

The embryo explants when cultured in MS media supplemented with different cytokinins, auxins-cytokinins and auxin-cytokinin-GA_3 concentrations and combinations, underwent different types of morphogenic changes: (a) normal plant differentiation, (b) direct multiple shoot regeneration without intervening callus phase and (c) callus proliferation. However, nature of differentiations of the embryo explants was depended on hormonal supplement present in the culture media. Seed specificity to the morphogenic response of the embryo was observed as the embryos were collected from seeds of open pollinated fruits. Among the different growth regulators concentrations and supplements 1.0 mgl^{-1} BA + 0.5 mgl^{-1} GA_3 was more effective in induction of multiple shoots without intermediate callus stage. BA, Kn or 2ip alone was less effective. Lower concentrations of auxins or GA_3 in combination with cytokinins increased direct shoot proliferation but supported callus growth. Occasional shoot proliferation was observed from root tip and leaf base still they were attached with parent shoot. Shoot regeneration ability of the embryo explants varied with their physiological age. In general, the

morphogenic potentiality of the immature embryo was higher than the mature embryo.

Auxins alone was less effective in callus proliferation. Higher concentrations of auxins (5.0 mgl^{-1}) in combination with lower concentrations of cytokinins (1.0-2.0 mgl^{-1}) suppressed adventitious bud induction but influenced callus proliferation. Among the different auxins and auxins-cytokinins concentrations and combinations, 5.0 mgl^{-1} 2,4-D + 2.0 mgl^{-1} Kn was optimum for percentage callusing and fresh weight of calli per culture (g). Undifferentiated hard, green to greenish yellow calli composed of small compact cells obtained from embryo explants were subcultured onto MS medium supplemented with various concentrations and combinations of cytokinins and auxins or GA_3 for inducing shoot regeneration. Higher concentrations of cytokinins and lower concentrations of auxins or GA_3 suppressed callus proliferation but supported multiple shoot initiation. Among the 10 different concentration and combination of cytokinins or GA_3, 1.0 mgl^{-1} BA + 0.5 mgl^{-1} GA_3 was the best combination in which calli developed shoot buds.

Tree specificity was found in *in vitro* response of embryo explants of 5 trees of *A. marmelos*. Most of the trees showed optimum shoot regeneration within different growth regulators concentrations and combinations whereas, tree 5 preferated more degree of the morphogenic potentiality.

The cotyledon explant of 5 trees of *A. marmelos* when cultured in MS medium supplemented with various cytokinins and auxins or GA_3 concentrations and combinations showed various types of morphogenic differentiation. However, degree of plant regeneration was depended on proper hormonal supplement, physiological state of explant, types tree and culture environment. Among the cytokinins tested in different concentrations, 2.0 mgl^{-1} BA was the best concentration for direct shoot regeneration. BA in combination with lower

concentration of auxins and GA_3 showed promotive effect on direct shoot proliferation. Among the treatment combinations, 1.0 mgl^{-1} BA + 0.5 mgl^{-1} GA_3 was found to be the best formulation where maximum number of shoots per culture and percentage of shoot forming explants were recorded. BA, Kn and 2ip with higher concentration of auxins supported callus initiation from cut surface of the explant and suppressed shoot proliferation. Shoot regeneration potentiality from cotyledon explant was also varied with trees. Among the 5 trees, shoot regeneration capacity of tree 5 was maximum. Frequent shoot regeneration occurred from the primary callus proliferated at the cut surface of explant.

The hypocotyl explant induced multiple shoot formation directly or by passing callus stage when incubated in MS medium containing different concentrations and combinations of cytokinins, auxins and GA_3. Shoot regeneration potentiality varied greatly with types of growth regulators. Cytokinins alone was less effective. Auxins or GA_3 in combination with cytokinin increased direct shoot proliferation but supported callus growth. Higher concentration of auxins alone or with lower concentration of cytokinins induced to developed callus. However, degree of callus proliferation, morphological nature and organogenic potentiality of the callus were found to vary with kinds of growth regulator supplement and nature of explant. Calli proliferated from hypocotyl in low concentration of auxins were hard green and showed organogenic response. Whereas, calli proliferated in higher concentration of auxins were spongy white to greenish and did not showed any organogenic activity. Among the different concentrations of auxins tested 5.0 mgl^{-1} was optimum for callus proliferation. Callus proliferation from hypocotyl explant was accentuated when low concentrations of cytokinins were added with auxins. Kn-auxin combinations in this respect were better than BA-auxin and 2ip-auxin. Among the 11 different

growth regulator supplements 2.0 mgl^{-1} Kn + 5.0 mgl^{-1} 2,4-D was proved to be the best for long term maintenance of callus culture.

In the efforts of induce shoots from unorganized hypocotyl callus, 2.0 mgl^{-1} BA + 0.5 mgl^{-1} GA_3 was found to be the best growth regulator supplement. BA 2.0 gml^{-1} + NAA 0.2 mgl^{-1} + GA_3 1.0 gml^{-1} was found more effective combination for direct shoot regeneration from hypocotyl explant.

The leaves of *in vitro* grown shoot of *A. marmelos* also showed potentiality to regenerate shoots. However, growth regulator formulations, explant, tree and culture environment were the major factors which influenced successful shoot regeneration from leaf explant. Among the cytokinins, BA alone (0.5-2.0 mgl^{-1}) or in combination with NAA, IAA (0.1-0.2 mgl^{-1}) or GA_3 (0.5 mgl^{-1}) was more effective growth regulator supplement for shoot proliferation. BA 2.0 mgl^{-1} + NAA 0.2 mgl^{-1} and BA 0.2 mgl^{-1} + IAA 0.2 mgl^{-1} were the optimum growth regulator supplements for direct shoot regeneration from the leaf explant. Frequent shoot regeneration was observed from primary callus of the leaf explant. Higher concentrations of auxins with cytokinins accentuated callus proliferation from the basal cut edge of the explant and suppressed shoot proliferation. Shoot regeneration in this also occurred frequently from the primary callus. The morphogenic competence of 5 trees of *A. marmelos* was different with regard to shoot regeneration from leaf explant. The tree 5 gave the highest number of shoots per explant.

The nucellus explants of 5 trees of *A. marmelos* showed potentiality to regenerate shoots directly or via callus. Preliminary investigations showed that, 60 days after pollination, nucellus was present as a thin mass of watery tissue lined on the ovule wall. Nucellus was found as a small mass of white tissue 80 days after pollination, but was unsuitable for excision, well developed and abundant nucellus was found in ovules collected 90-120 days after pollination. At this stage, the

developing cotyledons with the embryonic axis was not more than 1.5 mm in diameter, and was much smaller than the nucellar tissue, ovules of this stage were used as explant source.

Direct shoot proliferation occurred from the nucellar tissues in presence of low concentrations of cytokinin. Direct shoot regeneration capacity increased with higher concentration of cytokinin in combination with lower concentration of auxin or GA_3. Among the growth regulators tested at different concentrations and combinations, 1.0 mgl^{-1} BA + 0.1 mgl^{-1} NAA was the optimum hormonal supplement for induction of greater degree of shoot regeneration from the nucellar explants.

Different concentrations and combinations of auxin and cytokinin were used for callus induction. When MS medium was supplemented with 5 mgl^{-1} auxin, the explants produced callus but the growth of callus was very poor and the frequency of callusing was low. Therefore, a low concentration of cytokinin or GA_3 was added to increase the frequency of callusing. Maximum response was obtained in medium when 5.0 mgl^{-1} NAA and 2.0 mgl^{-1} Kn were added. The growing callus consisted of a hard, compact, irregular-shaped with green weight ranging from 0.32-0.72 g. per culture. Such calli obtained from each explant were then cut into smaller pieces and were transferred to fresh multiple shoot proliferating medium containing different concentrations of BA or Kn in combination with NAA, IAA or GA_3. BA 1.0 mgl^{-1} + 0.5 mgl^{-1} GA_3 was the best hormonal supplement for multiple shoot regeneration from nucellar callus. Number of shoots per culture and fresh weight of callus (g) were the highest for tree 5 and the lowest for tree 3. A real difference among trees and treatment combination was also noted in these cases.

Rooting experiment with various concentration of IBA revealed that 30 mgl^{-1} was optimum IBA supplement for maximum frequency of rooting and root length (cm). Among different agar levels in the rooting medium 7.5 gl^{-1} significantly

increased the rooting frequency and root length in microcuttings of initial culture. Sucrose 30 gl^{-1} gave significantly more roots and root length. Microcuttings also showed variable response to root induction and root length depending on pH level. pH 7.0 was the best for root initiation and root length. Among 5 different basal media for rooting, MS medium gave the best performances and whites' medium was the least in respect of number of roots per cutting and root length. Rooting potentiality of microcuttings markedly varied with source of explants from which they were originated. In general, rooting frequency of microcuttings derived from immature embryo, germinated embryo and nucellus were higher than the microcuttings derived from mature embryo, cotyledon, hypocotyl and leaf. Microcuttings derived from germinated embryo yielded more rooting. Higher frequency of rooting was also recorded for immature embryo and nucellus initiated cultures. Rooting frequency was the lowest for microcuttings derived from leaf explant cultures. Regenerated plantlets were successfully established in soil under natural environment following a few days indoor acclimatization.

Chapter VI

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Chapter VII

Appendix

APPENDIX

(A) MS medium (Murashige and Skoog, 1962) :

Components	Concentrations (mgl^{-1})
Macro-nutrients:	
KNO_3	1900.00
NH_4NO_3	1650.00
KH_2PO_4	170.00
$CaCl_2.2H_2O$	440.00
$MgSO_4.7H_2O$	370.00
Micro-nutrients:	
$FeSO_4.7H_2O$	27.80
Na_2-EDTA	37.30
$MnSO_4.4H_2O$	22.30
H_3BO_4	6.20
$ZnSO_4.4H_2O$	8.60
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Organic-nutrients:	
Glycine	2.00
Nicotinic acid	0.50
Pyridoxine-HCl	0.50
Thiamine-HCL	0.10
Inositol	100.00
Sucrose	30000.00

p^H adjusted to 5.6 before autoclaving.

(B) LS medium (Linsmaier and Skoog, 1965) :

Components	Concentrations (mgl^{-1})
Macro-nutrients:	
KNO_3	1900.00
NH_4NO_3	1650.00
KH_2PO_4	170.00
$CaCl_2.2H_2O$	440.00
$MgSO_4.7H_2O$	370.00
Micro-nutrients:	
$FeSO_4.7H_2O$	27.80
Na_2-EDTA	37.30
$MnSO_4.4H_2O$	22.30
H_3BO_4	6.20
$ZnSO_4.4H_2O$	8.60
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Organic-nutrients:	
Thiamine-HCl	0.10
Inositol	100.00
Sucrose	30000.00

p^H adjusted to 5.6 before autoclaving.

(C) B_5 medium (Gamborg *et al.*, 1968) :

Components	Concentrations (mgl^{-1})
Macro-nutrients:	
KNO_3	2500.00
$CaCl_2.2H_2P$	440.00
$MgSO_4.7H_2O$	370.00
$(NH_4)_2SO_4$	134.00
$NaH_2PO_4.H_2O$	150.00
Micro-nutrients:	
$FeSO_4.7H_2O$	27.80
Na_2-EDTA	37.30
$MnSO_4.4H_2O$	2.00
H_3BO_4	3.00
$ZnSO_4.4H_2O$	2.00
KI	0.75
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Vitamins :	
Nicotinic acid	1.00
Pyridoxine-HCl	1.00
Thiamine-HCl	10.00
Inositol	100.00
Sucrose	20000.00

p^H adjusted to 5.6 before autoclaving.

(D) White's medium, 1943 :

Components	Concentrations (mgl^{-1})
Macro-nutrients:	
$Ca(NO_3)_2.4H_2O$	288.00
KNO_3	80.00
KCl	65.00
$NaH_2PO_4.4H_2O$	19.00
$MgSO_4.7H_2O$	7.37
Na_2SO_4	200.00
Micro-nutrients:	
$FeSO_4.7H_2O$	02.50
$MnSO_4.4H_2O$	06.70
H_3BO_3	01.50
$ZnSO_4.4H_2O$	02.20
KI	0.75
Sucrose	20000.00

p^H adjusted to 5.6 before autoclaving.

(E) N_6 medium (Chu *et al.*, 1975) :

Components	Concentrations (mgl^{-1})
Macro-nutrients:	
KNO_3	2830.00
$(NH_4)_2SO_4$	463.00
KH_2PO_4	400.00
$CaCl_2.2H_2O$	166.00
$MgSO_4.7H_2O$	185.00

Micro-nutrients:

$FeSO_4.7H_2O$	27.80
Na_2-EDTA	37.30
$MnSO_4.4H_2O$	4.40
H_3BO_3	1.60
$ZnSO_4.4H_2O$	1.50
KI	0.80

Organic-nutrients:

Glycine	2.00
Nicotinic acid	0.50
Pyridoxine-HCl	0.50
Thiamine-HCL	0.10
Sucrose	30000.00

p^H adjusted to 5.6 before autoclaving.