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In vitro nodal explants propagation of *Aegle marmelos* (L.) Correa

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Abstract

Aegle marmelos (L.), Correa is an ancient sacred tree of India, belonging to family Rutaceae. It is one of the most important tree with religious importance and several medicinal uses. Charaka Samhita, one of the most respected works in Ayurveda, provides plenty of information on its medicinal attributes. This tree when normally propagated using seeds, exhibit morphological and biochemical variations within the population due to its heterozygous nature. Developing, *in vitro* technique for rapid clonal propagation from elite tree will help in preserving genotype qualities in raised population. In the present study, efforts were made to find out the suitable media composition for rapid clonal propagation of *Aegle marmelos* tree using nodal segments as a explant material. All the experiments were carried out in Murashige and Skoog's (MS) media using different phytohormone concentrations to record callogenic and morphogenic responses. In the establishment stage, 33 individual phytohormone treatments (Auxin/Cytokinin) and 20 combination treatments (Auxin + Cytokinin) were used. For multiplication purpose 25 combination treatments (Auxin + Cytokinin) and for elongation 12 combination treatments (Auxin + Cytokinin) were used. Rooting was carried out in half strength MS media with IBA in 3 different concentrations. Finally, rooted micro shoots were hardened using autoclaved potting mixture of Sand: Soil: Vermicompost in 1:1:1 ratio. Plantlets that survived were ultimately transferred to *ex-vitro* conditions taking due care.

Key word: *Aegle marmelos*, Phytohormones, Morphogenesis, Subculture, Establishment, Multiplication, Elongation, Rooting, Hardening.

Introduction

Among the many medicinally important trees, *Aegle mermelos* is an important species found throughout the deciduous forests of India. It is a medium sized deciduous thorny tree with its roots, bark, leaves and fruits of high medicinal value and is cited as one of the red-listed medicinal species of South India (Ravi kumar & Ved, 2000). Almost all the ancient Ayurvedic treatises like Charaka Samhita (600 BC), Sushruta Samhita (500 BC) etc. make special reference of its rare therapeutic values. Even in Siddha and Unani, its unique healing powers were revealed. In Charaka Samhita, Charaka prescribed tender fruits of *Aegle mermelos* with buttermilk for diarrhea. In case of diarrhea with blood, Sushruta prescribed tender fruits mixed with jaggery, honey and oil. One can find many such references in ancient and in most recent works proving the importance of this red listed tree. Among the many confirmed medicinal properties, this tree acts as a potent anti-helminthic, hypoglycaemic, cardiac stimulant, anti-diarrhoeal and antiviral agent (Khare, 2004). Its bark, roots, leaves, unripe and ripe fruits are all used as ingredients in many ayurvedic preperations like Bilva Taila, Dasamoolaarishta, Gangaadhara Choorna, Amritaarishta, Mahaanaaraayana taila, Chyawana praasa, Pushyaarvuga choorna. The root of this tree being the major medicinally useful part, afforestration causes a serious threat for its survival in nature.

Aegle mermelos is usually propagated by seeds and root suckers though many problems are associated with these methods. Progeny raised from this tree seeds are usually not uniform besides remaining viable for short duration and prone for insect attacks. Vegetative propagation by root suckers and by conventional methods of budding and soft wood grafting are very slow, seasonal,

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difficult and labour intensive. Due to all these drawbacks, micropropagation methods alone following tissue culture techniques can provide some hope for rapid mass multiplication and germplasm conservation of this rare endangered medicinal tree, though several limitations such as low shoot proliferation, excessive phenolic exudation, basal callusing, vitrification and shoot tip necrosis come in its way.

Several workers attempted to raise this hardy tree through *in vitro* techniques namely Arya et al, (1981), Arya & Shekhawat (1986) Hossain *et al*, (1993, 1994, 1994a) Islam *et al.* (1993, 1994, 1995, 1996a, 1996b), Arumugam & Rao (1996, 2000), Ling & Iwamasa (1997), Ajith kumar & Seeni (1998), Islam (2006), Prematilake *et al* (2006), Pranati & Behera (2007), Raghu *et al*, (2007), Das *et al* (2008), Rajesh Pati *et al*, (2008), Neha *et al.* (2010), Rekha Warrier *et al* (2010), Ramanathan *et al* (2011), Puspasree & Thirunavoukkasu (2011), Kuldeep & Narendra (2011). In the current report, efforts were made to find out, which is the best suitable explant material and best media formulation for rapid clonal propagation of *Aegle marmelos*.

Materials and methods

Plant Material: Two mature *Aegle marmelos* trees were identified after conducting structured and detailed studies on their morphological parameters. These selected trees growing within the premises of Sri Sathya Sai Institute of Higher learning, were physiologically active, well irrigated and beyond the reach of pollution zone. Various explants collected from them, like stem bits, shoot tips, nodal segments, leaf parts, floral and fruit parts were used to develop a suitable protocol for fast propagating this tree species following *in vitro* techniques.

Preparation and sterilization of explants: All the collected explant materials were carefully handled, while washing and surface sterilization process, causing no damage to the materials. In the first level, explants were washed using clean tap water for 5-6 times. The materials were submerged in clean tap water for each wash and the container with materials was shaken gently for about 4-5 minutes before decanting the wash water. In the second level, two to three drops of Tween-20 a detergent and a wetting agent was added to the water and used to rinse the material for about 4-5 minutes time. After this step, to remove all the detergent effect, clean tap water rinsing was done for about 3-4 times. In the third level, Mercuric chloride 0.1%, prepared using double distilled water was used to surface sterilize the explant materials for about 4-5 minutes. After this wash, $HgCl_2$ solution was carefully decanted and materials were thoroughly washed 2- 3 times using sterile double distilled water to remove all the traces of chemical used. Finally, 80% ethanol was used to sterilize the materials, inside the laminar airflow hood. This final rinse of alcohol was given for about 2-3 minutes and after decanting the alcohol, materials were washed 2-3 times using autoclaved double distilled water (DDW) and then used for inoculating on different media compositions.

Culture Medium adopted: Murashige and Skoog medium (1962), was used for designing all the experiments at different stages of growth. This medium which is rich in H_3BO_3, $MnSO_4$ $4H_2O$ and $ZnSO_4$ $7H_2O$ is considered as the highest salt medium containing 19 times highest total ionic concentration than in White's Medium. In order to save the time for preparing the medium, concentrated stock solutions of MS components were prepared and stored in deep freezer. Each time when medium was prepared, required quantities of major, minor, Iron and organic stocks were added to the double distilled autoclaved water containing 20% sucrose (Qualigens, Glaxo Fine Chemicals, India) and mixed well. Growth regulators stock solutions were later added in required quantities as per the need and pH was adjusted to 5.6 using 1N NaOH and 1N HCl. Ultimately, after adjusting final volume by DDW, 9.5 gm/L of Agar agar (Hi-media, Pvt, Mumbai, India) was added in case of semi solid medium preparation and boiled before dispensing to desired culture vessels. After dispensing, medium was autoclaved at 121^0C and 105 Kpa for 15-20 minutes and allowed to cool at room temperature.

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Different Media formulations used: All the explants after surface sterilization were aseptically implanted on suitable media formulations depending on their growth stages. For establishment purpose, five cytokines (Ad, AS, BAP, DPU, KN) and six auxins (CPA, 2,4-D, IBA, NAA, NOA, PIC) were individually tested for explants response by adding to the MS medium. All these growth regulators were taken in three different concentrations of 1mg/L, 2mg/L and 3mg/L totaling to 33 treatments. In the same establishment step, 4 Cytokinins Ad/AS/BAP/KN were added to the MS medium in 2mg/L concentration along with one of these auxins CPA/IBA/NAA/NOA/PIC in 1mg/L concentration, were also evaluated for explants responses. In total, 20 combination treatments were tested for explants establishment purpose.

For proliferation of microshoots, established explants were sub-cultured on a medium supplemented with Cytokinin (2mg/L) + Auxin (1mg/L) combination. A total of 25 combination treatments were tested for explants response. Even tender coconut milk was added as a supplement to the MS medium instead of growth regulators in the concentrations of 5, 10, 20, 30 mg/L and was tested for multiplication purpose. For achieving elongation of micro shoots, cytiokinins Ad/AS/DPU/KN in concentration of 2mg/L were added along with one of these auxins CPA/IBA/NAA in concentration of 1 mg/L. In total, 12 combination treatments were tested for the elongation purpose. For rooting of elongated microshoots, 4 varieties of ½ strength MS medium were tested. In all these medium preparations, IBA in concentrations of 8/10/15 mg/L was added. Finally, hardening was carried out using Sand:Soil:Vermicompost potting mixture.

Maintenance of cultures: All the cultures were maintained under 14 hr light and 10 hr dark cycle (unless required otherwise for specific explants) in a culture room at 23 ± 2^0 C with 55 ± 5 % relative humidity. White cool fluorescent lights controlled by a timer were used to provide about 1000 Lux intensity light for obtaining morphogenesis. 15 replicates were maintained for each treatment and cultures thus developed were regularly monitored for callusing, direct or indirect differentiation of shoots/roots at regular intervals.

Data Analysis: Data taken at regular intervals was analysed using ANOVA (Analysis of variance) technique.

Results

Explant materials: Series of experiments were simultaneously conducted using different explants on different nutrient media formulation. In the process, different explants took different course of development before attaining the micro shoot form. In case of nodal explants, it was direct morphogenesis in most of the cases, though indirect morphogenesis interfered sometimes in the presence of some strong callus inducing growth regulator. In case of explants like, leaf bits, floral parts, fruit parts, it was indirect morphogenesis, allowing callus to be the intermediate and predominant stage of development.

In all the experiments, leaching of phenolic compounds from the explants was the major issue of concern. To tackle this serious problem of oxidative browning, explants after surface sterilization were allowed to remain in a sterile DDW for about an hour and then given a final thorough rinse using fresh sterile DDW, prior to inoculating them on to a suitable media substrate. Occasionally, quick transfer of explants material to fresh medium was also done, at 2 to 3 times of short intervals to cut down the deleterious effect of phenolic exudates. Season and time of collection of the explants material also played a major role in minimizing the problem. Materials collected between August-December months and during the early hours of the day responded well with less phenolic exudates problem. Even the position of nodal segments in the shoot had some effect in cultures. In all the experiments, nodes collected from first 3-4 positions did not show any responses. In all the trials, terminal shoot tips and first few nodes failed to establish. From these observations, it appears that nodal segments are suitable and fast responding materials for adventitious shoots development. The results provided in this report are

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Table 1: Establishment stage: Individual phytohormone treatments

Phytohormone Supplied (Auxin / Cytokinin)	Average no of days taken for bud burst	Average length of auxiliary shoots, after 40 days	Average number of leaves, after 40 days
Ad (1mg/L)	9.07	1.40	3
Ad (2 mg/L)	8.2	1.51	3
Ad (3 mg/L)	7.93	1.95*	3
AS (1mg/L)	7.47*	1.4	3
AS (2 mg/L)	7.27*	1.55	2.93
AS (3 mg/L)	6.53*	1.85*	3
BAP (1mg/L)	8.93	1.17	3.47
BAP (2 mg/L)	8.73	1.42	4.4
BAP (3 mg/L)	8.47	1.45	5.47
DPU (1mg/L)	9.8	0.97	2.73
DPU (2 mg/L)	9	1.5	3
DPU (3 mg/L)	8.93	1.74*	3
KN (1mg/L)	8.13	1.4	3
KN (2 mg/L)	7.87*	1.64*	3
KN (3 mg/L)	6.8*	1.76*	3
CPA (1mg/L)	11.6	0.76	2.47
CPA (2 mg/L)	11.13	0.85	2.4
CPA (3 mg/L)	9.87	0.86	2.53
2,4-D (1mg/L)	15.67	0.48	2.2
2,4-D (2 mg/L)	17.13	0.27	1.27
2,4-D (3 mg/L)	18.2	0.04	0
IBA (1mg/L)	10.67	0.98	2.6
IBA (2 mg/L)	10.73	1.18	2.53
IBA (3 mg/L)	10.33	1.34	2.47
NAA (1mg/L)	12	0.93	2.67
NAA (2 mg/L)	12.2	1.12	2.47
NAA (3 mg/L)	10.8	1.20	2.87
NOA (1mg/L)	12.2	1.05	2.67
NOA (2 mg/L)	11.27	1.3	2.67
NOA (3 mg/L)	10.93	1.30	2.53
PIC (1mg/L)	11.13	0.91	2.93
PIC (2 mg/L)	10.07	0.96	2.93
PIC (3 mg/L)	9.8	1.03	2.93
NO Phytohormone	17.13	0.42	2.07
CD Calculated	0.7941	0.11695	0.275932

the outcome of periodic observations recorded during the course of *in vitro* organogenesis using nodal segments as explant material.

Establishment stage: This step is very crucial in order to obtain an aseptic culture of the selected plant material. Batches of nodal segments were inoculated for this purpose in different media formulations until adequate number (fifteen for each treatment) of them had survived without contamination. Thirty three Individual phytohormone treatments, both Auxins and cytokinins in concentrations of 1 mg/L, 2 mg/L and 3 mg/L as well as twenty combined treatments of Cytokinin in 2mg/L + Auxin in 1 mg/L were tested (Table. 1 & 2).

Data in the form of days taken for bud burst, length of auxiliary shoots formed, number of leaves emerged, number of nodal points appeared, all these characteristics became basis for Analysis of variance statistical analysis to analyse and sort out the individual and combined treatments performances by making use of Critical Difference (CD), an error of sum of squares obtained from ANOVA statistical tool. In individual treatments, when the data was recorded on number of days taken for bud burst was considered, a total of nine groups were formed in such a way that within the group the treatments were statistically not significant i.e. they were homogenous ($P > 0.05$), and between the groups the treatments were statistically significant i.e.

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heterogeneous ($P < 0.05$). In the first group three treatments involving AS (3 mg/L), KN (3 mg/L), AS (2 mg/L) took just 7 days for bud burst. The second group involving AS (1 mg/L), KN (2 mg/L), Ad (3 mg/L), KN (1 mg/L), Ad (2 mg/L) took about 8 days for bud burst and the third group including BAP (3 mg/L), BAP (2 mg/L), BAP (1 mg/L), DPU (3 mg/L), DPU (2 mg/L), Ad (1 mg/L) took about 9 days for bud burst. The fourth, fifth and sixth groups including 16 treatments; PIC (3 mg/L), DPU (1 mg/L), CPA (3 mg/L) PIC (2 mg/L), IBA (3 mg/L), IBA (1 mg/L), IBA (2 mg/L), NAA (3 mg/L),

NOA (3 mg/L), PIC (1 mg/L), CPA (2 mg/L), NOA (2 mg/L), CPA (1 mg/L), NAA (1 mg/L), NAA (2 mg/L), NOA (1 mg/L) took about 10, 11, 12 days for bud initiation and the last three groups involving control and 2,4 D (1, 2, 3 mg/L) treatments took almost 15-18 days showing poor bud initiation responses.

When data of length of auxiliary shoots attained after 40 days was analysed, twelve groups with significant differences were formed. The first group including just one phytohormone treatment Ad (3 mg/L) showed maximum growth of emerged shoot measuring 1.9 cm in size. Following this, the second group including treatments of AS (3 mg/L), KN (3 mg/L), DPU (3 mg/L) showed auxiliary shoot length of 1.7 to 1.8 cm. The third group including AS (2 mg/L) and KN (2 mg/L) treatments showed 1.5 to 1.6 cm size and the fourth group including treatments of BAP (2 mg/L), BAP (3 mg/L), DPU (2 mg/L), Ad (2 mg/L) treatments showed 1.4 to 1.5 cm size. The fifth, sixth and seventh groups involving twelve phytohormone treatments including Ad (1 mg/L), KN (1 mg/L), AS (1 mg/L), IBA (3 mg/L), NOA (3 mg/L), NOA (2 mg/L), NAA (3 mg/L), IBA (2 mg/L), BAP (1 mg/L), NAA (2 mg/L), PIC (3 mg/L), NOA (1 mg/L), all showed auxiliary shoot length of 1-1.4 cm. In groups 8-12 including twelve treatments of IBA (1 mg/L) DPU (1 mg/L), PIC (1, 2 mg/L) NAA (1 mg/L), CPA (1, 2, 3 mg/L), 2, 4 – D (1, 2, 3 mg/L) and control, the treatments had poor responses on nodal explants though the presence of phytohormone in media triggered callus formation. Axillary shoots in these (8-12) group treatments, showed a result of maximum growth of 0.98 cm size and minimum growth of 0.04 cm in length.

When data of number of leaves emerged after 40 days in each individual treatment case was analysed, eight groups with significant variation were formed. The first, second and third group involving BAP (1, 2, 3 mg/L) treatments showed leaves between 5 - 3 in number. In fourth and fifth group including treatments of Ad (1, 2, 3 mg/L), AS (1, 2, 3 mg/L), KN (1, 2, 3 mg/L), DPU (1, 2, 3 mg/L), CPA (1, 2, 3 mg/L), IBA (1, 2, 3 mg/L), NAA (1, 2, 3 mg/L), NOA (1, 2, 3 mg/L) and PIC (1, 2, 3 mg/L) leaves formed were 2-3 in number. In case of sixth, seventh and eight group including control and 2,4–D (1, 2, 3 mg/L) treatments leaves formed were 0 – 2 in number.

Comparing all the above results of individual treatments (Table, 1), it appears that all Cytokinin treatment of AS, Ad, BAP, DPU, KN in concentrations of (1,2 and 3 mg/L) are favourable in achieving direct morphogenesis using nodal segments while in Auxin individual treatments, PIC, CPA and IBA in 3 mg/L the responses were bit slower. In case of individual treatments of NAA (1, 2, 3 mg/L), NOA (1, 2, 3 mg/L), CPA (1, 2 mg/L), PIC (1, 2 mg/L), IBA (1, 2 mg/L), Callus intervention before auxiliary shoots formation was certain. Lastly, 2, 4 –D (1, 2, 3 mg/L) treatments had much damaging effect on nodal segments and showed poor responses much lower than the control cultures without any plant growth regulator supplied.

Further, when these establishing explants of individual treatments were sub-cultured on the MS media supplemented with twenty different combination treatments of Cytokinin (2 mg/L) + Auxin (1 mg/L), the explants got well established within forty days revealing different levels of morphogenesis. Length of auxiliary shoot, number of nodes and number of leaves that emerged has all formed the basis for deciding the role of these combination treatments. When the data of 40 days old auxiliary shoot length was used, five

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Table 2: Establishment stage: Combination phytohormone treatments

Phytohormones Supplied (Auxin + Cytokinin)	Average length of Axillary shoot, after 40 days	Average number of nodes, after 40 days	Average number of leaves, after 40 days
Ad (2 mg/L) + CPA (1 mg/L)	2.60	4.2	5.8
Ad (2 mg/L) + IBA (1 mg/L)	2.87	5.26	7.33
Ad (2 mg/L) + NAA (1 mg/L)	2.99	4.67	5.67
Ad (2 mg/L) + NOA (1 mg/L)	2.88	4.73	5.67
Ad (2 mg/L) + PIC (1 mg/L)	2.75	3.93	5.33
AS (2 mg/L) + CPA (1 mg/L)	3.27	3.6	4.53
AS (2 mg/L) + IBA (1 mg/L)	3.87*	5.13	8.13
AS (2 mg/L) + NAA (1 mg/L)	3.35*	5.33	7.2
AS (2 mg/L) + NOA (1 mg/L)	3.22	4.67	7.53
AS (2 mg/L) + PIC (1 mg/L)	2.97	4.2	6.87
BAP (2 mg/L) + CPA (1 mg/L)	2.89	6.07*	8.87*
BAP (2 mg/L) + IBA (1 mg/L)	2.92	7.33*	11.4*
BAP (2 mg/L) + NAA (1 mg/L)	2.93	7.93*	12.13*
BAP (2 mg/L) + NOA (1 mg/L)	2.87	7.6*	12.06*
BAP (2 mg/L) + PIC (1 mg/L)	2.87	6.67*	9.13*
KN (2 mg/L) + CPA (1 mg/L)	3.32	5.8	5.93
KN (2 mg/L) + IBA (1 mg/L)	3.66*	5.4	6.07
KN (2 mg/L) + NAA (1 mg/L)	3.34*	6	7.07
KN (2 mg/L) + NOA (1 mg/L)	3.2	4.6	7.13
KN (2 mg/L) + PIC (1 mg/L)	3.38*	4.47	7.07
CD CALCULATED	0.155907932	0.497086	0.6398625

heterogeneous groups with significant differences were formed (with $P < 0.05$). The first group including AS (2 mg/L) + IBA (1 mg/L) treatment showed maximum shoot length of 3.8 cm. The second group including KN (2 mg/L) + IBA (2 mg/L) treatment showed auxiliary shoot length of 3.6 cm. The third group including seven combination treatments KN (2 mg/L) + PIC (1 mg/L), KN (2 mg/L) + NAA (1 mg/L), KN (2 mg/L) + CPA (1 mg/L), KN (2 mg/L) + NOA (1 mg/L), AS (2 mg/L) + NAA (1 mg/L), AS (2 mg/L) + CPA (1 mg/L), AS (2 mg/L) + NOA (1 mg/L) attained 3.2 to 3.4 cm length. The fourth group including nine combination treatments of Ad (2 mg/L) with IBA (1 mg/L), NOA (1 mg/L), NAA (1 mg/L), BAP (2 mg/L) with NOA (1 mg/L), PIC (1 mg/L), CPA (1 mg/L), IBA (1 mg/L), NAA (1 mg/L) and Ad (2 mg/L) + NAA (1 mg/L) all showed 2.8 to 2.9 cm of auxiliary shoot growth. The fifth group with two combination treatments of Ad (2 mg/L) with CPA (1 mg/L) and PIC (1 mg/L) showed slowest growth of all. Axillary shoots in these combination treatments attained 2.7 cm size in forty days time.

When data of number of nodes notably formed after 40 days of sub culturing was analysed, eight heterogeneous groups with significant differences were formed. The first six groups showed 5 – 8 nodes formation within 40 days of sub-culturing. In BAP (2 mg/L) combination treatments along with NAA, NOA, IBA, PIC, and CPA all in 1 mg/L concentration, 6-8 nodes were prominently noticed though they were closely arranged in the developing auxiliary shoot. In KN (2 mg/L) combination treatments along with NAA, CPA, IBA (1 mg/L), AS (2 mg/L) combination treatments with IBA, NAA in (1 mg/L) and Ad (2 mg/L) + NOA (1 mg/L) treatment, the number of nodes formed were between 4-6 and were clearly separated. In the last two groups including eight combination treatments of cytokinins AS, Ad and KN in 2 mg/L concentration along with one of these auxins NOA, PIC, CPA in 1 mg/L concentration, showed auxiliary shoots with 3-4 nodes.

When the data for number of leaves emerged was analysed using statistical tool of ANOVA, eight groups were formed with significant differences. In the first four groups when BAP was

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used along with one of the selected auxin, more leaves were formed. In BAP (2 mg/L) + NAA (1 mg/L) and BAP (2 mg/L) + NOA (1 mg/L), 12 leaves developed. In BAP (2 mg/L) + IBA (1 mg/L) around 11 leaves were formed. In BAP (2 mg/L) + CPA (1 mg/L) and BAP (2 mg/L) + PIC (1 mg/L) combinations, 8 – 9 leaves were formed. In Fifth and sixth group including treatments of AS (2 mg/L) along with IBA, NAA, NOA in 1 mg/L concentration, Ad (2 mg/L) + IBA (1 mg/L) and KN (2 mg/L) along with NAA, PIC, NOA in 1 mg/L concentration, leaves appeared were 7-8 in number. In seventh, eighth and ninth groups KN (2 mg/L) along with IBA, CPA in 1 mg/L concentration, Ad (2 mg/L) along with PIC, NAA, NOA, CPA in 1 mg/L concentration and AS (2 mg/L) + CPA (1 mg/L), leaves appeared were 4-6 in number.

So, comparing the results of establishment step in combination treatments (Table 2), sub- culturing of the established explants in media supplemented with cytokinins AS, KN helped in the fast growth of developing the auxiliary shoot with distinct nodal regions. In case of Ad involved media treatments of shoot development, nodes developed and leaves emerged. In case of BAP supplied media treatments, though the shoot development was compact, it showed more number of leaves emerging from closely formed nodal points. Callus formation was also noticed in all the combination treatments and was more in treatments involving auxins like NOA, NAA and IBA. The callus formed was densely packed and brownish white in colour.

The second step of *in vitro* culture, the multiplication stage is mainly recognized by formation, growth and proliferation of adventitious/auxiliary shoots from the established explants material. Usually, the media used in this particular step helps in rapid proliferation of shoot mass and it is achieved by sub culturing the shoot clumps avoiding basal callus. For achieving multiplication in *Aegle marmelos*, twenty five media formulations involving five cytokinins: Ad, AS, BAP, KN, DPU all in 2 mg/L concentration and five auxins: CPA, IBA, NAA, NOA, PIC in 1 mg/L concentration in different combinations were used. In all these media formulations, results were recorded under three headings (Number of multiple shoots formed, Average length of multiple shoots attained and Average number of leaves appeared on each shoot) after 60 days of repeated sub culturing formed basis for comparison.

When the data of number of multiple shoots formed was used for testing Analysis of Variance, seven groups with significant differences ($P < 0.05$) were formed. In the first group, media supplemented with BAP (2 mg/L) + NOA (1 mg/L) and BAP (2 mg/L) + NAA (1 mg/L); the results obtained were remarkable showing up to 30-33 multiple shoots. In the second group, KN (2 mg/L) + NAA (1 mg/L) and BAP (2 mg/L) + IBA (1 mg/L) media treatments, about 25 multiple shoots formed. In third and fourth groups, treatments of BAP (2 mg/L) + PIC (1 mg/L), AS (2 mg/L) + NOA (1 mg/L), BAP (2 mg/L) + CPA (1 mg/L) and AS (2 mg/L) + NAA (1 mg/L), showed 18 – 21 multiple shoots formation. In the fifth group, treatments of AS (2 mg/L) + CPA (1 mg/L), KN (2 mg/L) + IBA (1 mg/L), Ad (2 mg/L) + IBA (1 mg/L), Ad (2 mg/L) + NOA (1 mg/L) all showed multiple shoots between 15 – 17 in no. Good results were also noticed in last two groups involving thirteen MS media formulations with 8 – 14 shoots formation. In all these groups, multiple shoots formed were distinct, uniform and appear to be emerging from undifferentiated expanding mass of basal callus tissue.

When the readings for multiple shoots length attained in 25 different media compositions where analysed, using ANOVA statistical tool, eight distinct groups with significant differences were formed. Maximum multiple shoots length of about 4.1 cm after 60 days of repeated sub- culturing was noticed in KN (2 mg/L) + PIC (1 mg/L) treatment. In the second group, MS media treatments including KN (2 mg/L) + NAA (1 mg/L) and AS (2 mg/L) + NOA (1 mg/L) showed shoots length of 3.5 – 3.7 cm. In the third group, shoots attained 3.4 cm length in KN (2 mg/L) + IBA (1 mg/L), BAP (2 mg/L) + IBA (1 mg/L) treatments. In fourth and the fifth groups, media treatments of BAP (2 mg/L)

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Table 3: Multiplication Stage: Combination phytohormone treatments

Phytohormones Supplied (Auxin + Cytokinin)	Average number of multiple shoots formed after 60 days	Average shoot length attained after 60 days	Average number of leaves on each shoot after 60 days
Ad (2 mg/L) + CPA (1 mg/L)	13	2.88	4.27
Ad (2 mg/L) + IBA (1 mg/L)	16.33	3.29	5.27
Ad (2 mg/L) + NAA (1 mg/L)	14	3.25	8.13*
Ad (2 mg/L) + NOA (1 mg/L)	15.53	2.97	5.67
Ad (2 mg/L) + PIC (1 mg/L)	13.27	2.78	5.47
AS (2 mg/L) + CPA (1 mg/L)	17.27	2.93	6.13
AS (2 mg/L) + IBA (1 mg/L)	13.4	2.57	5.73
AS (2 mg/L) + NAA (1 mg/L)	17.87	3.16	6.93
AS (2 mg/L) + NOA (1 mg/L)	18.93	3.60*	7.33
AS (2 mg/L) + PIC (1 mg/L)	14.06	2.26	7.6
BAP (2 mg/L) + CPA (1 mg/L)	17.87	3.29	9.2*
BAP (2 mg/L) + IBA (1 mg/L)	24.6*	3.37*	9.93*
BAP (2 mg/L) + NAA (1 mg/L)	32.6*	3.3	10.87*
BAP (2 mg/L) + NOA (1 mg/L)	32.8*	2.18	6.8
BAP (2 mg/L) + PIC (1 mg/L)	21.53*	2.64	6.8
KN (2 mg/L) + CPA (1 mg/L)	14.07	3	4.53
KN (2 mg/L) + IBA (1 mg/L)	17.07	3.37*	7.47
KN (2 mg/L) + NAA (1 mg/L)	24.87*	3.71*	8.2*
KN (2 mg/L) + NOA (1 mg/L)	12.93	3.21	6.27
KN (2 mg/L) + PIC (1 mg/L)	13.33	4.10*	7.73
DPU (2 mg/L) + CPA (1 mg/L)	10.2	3.02	5.67
DPU (2 mg/L) + IBA (1 mg/L)	12.4	3.06	5.33
DPU (2 mg/L) + NAA (1 mg/L)	12.2	2.30	5.2
DPU (2 mg/L) + NOA (1 mg/L)	13.6	3.02	4.53
DPU (2 mg/L) + PIC (1 mg/L)	8.53	2.52	5.67
CD Calculated	2.017113355	0.155722446	0.61478830

along with NAA/CPA in 1 mg/L concentration, Ad (2 mg/L) along with IBA/NAA/NOA in 1 mg/L concentration, KN (2 mg/L) along with NOA/CPA in 1 mg/L concentration, AS (2 mg/L) +NAA (1 mg/L) all showed multiple shoots of 3-3.3 cm length. In sixth, seventh and the eighth groups, nine treatments showed more of callus growth and multiple shoots measured 2 – 3 cm in length.

When data of average number of leaves formed on each shoot after 60 days of repeated sub-culturing was used for testing Analysis of Variance, nine groups with significant difference were formed. The first three groups involving BAP as main cytokinin in 2 mg/L concentration along with NAA, IBA and CPA all in 1 mg/L concentration showed 11, 10 and 9 leaves formation respectively. In the fourth group, KN (2 mg/L) + NAA (1 mg/L), Ad (2 mg/L) + NAA (1 mg/L) showed 8 leaves formation. In the fifth group, KN (2 mg/L) along with the PIC/IBA in 1 mg/L concentration and As (2 mg/L) + PIC (1 mg/L), showed 7- 7.5 leaves formation. In the sixth group, BAP (2 mg/L) along with PIC/NOA (1 mg/L) and AS (2 mg/L) with NOA/NAA (1 mg/L) showed 6.5 – 7 leaves and the last 3 groups, with 13 media treatments showed average of 6- 4 leaves on developed multiple shoots.

Comparing all the results in multiplication stage (Table, 3), it appears that sub-culturing of established explants in some of the combination treatments will certainly trigger multiple shoots formation. In few other media treatments both morphogenesis and callogenesis simultaneously got initiated. In combination treatments where BAP was main cytokinin supplement, multiple shoot formation dominated. In KN, AS, Ad, DPU involved treatments also multiple shoot formation was noticed. In all the experiments auxins like NAA,

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Table 4: Elongation Stage: Combination phytohormone treatments		
Phytohormones supplied (Auxin + Cytokinin)	Average length of the shoots, after 40 days	Average number of leaves, after 40 days
Ad (2 mg/L) + CPA (1 mg/L)	4.16	11*
Ad (2 mg/L) + IBA (1 mg/L)	4.26	9.53*
Ad (2 mg/L) + NAA (1 mg/L)	4.3	7.27*
AS (2 mg/L) + CPA (1 mg/L)	4.28	4.6
AS (2 mg/L) + IBA (1 mg/L)	4.33	5
AS (2 mg/L) + NAA (1 mg/L)	4.59*	4.73
KN (2 mg/L) + CPA (1 mg/L)	3.96	4.93
KN (2 mg/L) + IBA (1 mg/L)	4.58*	7.8*
KN (2 mg/L) + NAA (1 mg/L)	5.47*	5.33
DPU (2 mg/L) + CPA (1 mg/L)	4.17	4.13
DPU (2 mg/L) + IBA (1 mg/L)	4.45*	4.27
DPU (2 mg/L) + NAA (1 mg/L)	5.06*	5.53*
CD Calculated	0.189397898	0.459409429

NOA, IBA, PIC and CPA induced callus formation.

Coming to the elongation step which is again a very important stage in *in vitro* propagation, requires special focus as the multiple shoots formed in previous stage are not capable of self supporting themselves. In this particular stage, precautions were taken to prepare the multiple shoots in to individual plantlets by nourishing them with required suitable growth regulators. Twelve combination treatments were tested and their responses in the form of average length of the axillary shoots attained and average number of leaves for each plantlet produced after 40 days of sub-culturing were all analysed using ANOVA statistical tool.

When the data of Average length of shoots was analysed, five heterogeneous groups with significant differences were formed. In the first group, KN (2 mg/L) + NAA (1 mg/L) combination treatment showed maximum development of 5.5 cm plantlets growth. In the second group, DPU (2 mg/L) + NAA (1 mg/L) combination treatment showed average shoots length of 5 cm. In the third group AS (2 mg/L) + NAA (1 mg/L), KN (2 mg/L) + IBA (1 mg/L) and DPU (2 mg/L) + IBA (1 mg/L) treatments showed 4.5 – 4.6 cm plantlet growth. In the forth group, 6 combination treatments including AS (2 mg/L) + IBA/CPA in 1 mg/L concentration, Ad (2 mg/L) + NAA/IBA/CPA in 1 mg/L concentration and DPU (2 mg/L) + CPA (1 mg/L) treatments, all showed 4.1 – 4.3 cm shoot length. In the fifth group, KN (2 mg/L) + CPA (1 mg/L) treatment attained average shoot length of 4 cm.

When the data for number of leaves developed on each shoot-let was used, seven groups of significant differences were formed. In the first five groups involving combination treatments of Ad (2mg/L) along with CPA/IBA/NAA in 1 mg/L concentration, KN (2 mg/L) along with IBA/NAA in 1 mg/L combination and DPU (2 mg/L) + NAA (1mg/L) treatments all showed leaves varying from 5 – 11. In last two groups 6 combination treatments, AS (2 mg/L) along with IBA/NAA/CPA in 1 mg/L concentration, DPU (2 mg/L) along with IBA/CPA in 1 mg/L concentration and KN (2 mg/L) + CPA (1 mg/L), all showed just 4- 5 leaves formation.

Comparing all these results (Table, 4), it appears that the elongation of shoots seems to be better in treatments involving cytokinins KN first, DPU second, AS third and Ad in the fourth position though all the 12 combination treatments enhanced the elongation process of shoots. In the final stage of rooting, the elongated plantlets of *Aegle marmelos* were sub-cultured in half strength MS medium supplemented with three different concentrations of IBA Phytohormone. Visible results were recorded under the headings; Days taken for root initiation, No of roots formed, Root length achieved after forty five days and Root width attained, all have formed the basis for identifying effective rooting media. ½ strength MS

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liquid media was also used along with semisolid media for comparison.

Table 5: Rooting Stage: Combination phytohormone treatments

Phytohormones Supplied	Average number of days for root initiation	Average number of roots , after 30 days	Average length of roots after 45 days	Average width of roots after 45 days
½ MS IBA (8 mg/L)	32.87	1.2	1.88	1.06
½ MS IBA (10 mg/L)	27.93	1.67	2.37	1.59
½ MS IBA (15 mg/L)	21.6	2	2.65	1.65
½ MS liquid medium with IBA (8 mg/L)	21.67	1.8	2.53	1.96
CD Calculated	1.756088	0.287975307	0.214223891	0.13846

When the data recorded for days taken for root initiation were analysed using ANOVA statistical tool, three concentrations of IBA, 15mg/L, 10 mg/L & 8 mg/L showed significant differences. In 15 mg/L IBA supplemented media, root induction triggered at around 21 days time. In 10 mg/L IBA concentration media, it took around 28 days and in 8 mg/L IBA supplemented media, root initiation was formed after 32 days. In ½ strength MS liquid media with IBA 10 mg/L concentration, rooting was quicker and took just 21 days for root initiation. When the data of number of roots formed was considered, significant difference among the three concentrations was noticed. In ½ MS IBA (15 mg/L) media, on an average 2 roots were formed. In ½ MS IBA (10 mg/L) treatment and in ½ MS liquid medium with IBA (10 mg/L) treatment, average of 1.6-1.8 roots formation was noticed. In ½ MS IBA (8 mg/L) media roots formation was about 1.2 on average. When data for root length and root width attained after 45 days was analysed, similar differences were noticed in all three concentrations. Root length attained in ½ MS IBA (15 mg/L) was 2.64 cm and width attained was 1.96 mm. In ½ MS IBA (10 mg/L) and in ½ MS liquid media with IBA (10 mg/L), length attained was 2.3 – 2.5 cm and root width was 1.5-1.6 mm. In ½ MS IBA (8 mg/L) medium, roots attained just 1.88 cm in length and measured 1mm in width (Table, 5).

Finally, necessary precautions were taken to successfully transfer *in vitro* rooted *Aegle marmelos* plantlets in to *in vivo* climatic conditions. Fully developed plantlets were first transferred to distilled water to avoid dehydration and then carefully transferred to smaller pots filled with sterile mixture of Sand:Soil:Vermicompost in 1:1:1 ratio. Light, temperature and humidity were all regulated for the next one month duration and were gradually withdrawn once the plantlets got adapted for *in vivo* climatic conditions. During this crucial period, plantlets were irrigated only with knops solution containing all essential macro and micro nutrients. Successfully survived plants were ultimately transferred to bigger pots containing soil + manure and maintained in green house conditions until they became completely autotrophic and independent.

Discussion

In vitro propagation of *Aegle marmelos* was a challenging task due to various reasons like excessive phenolic exudation, low shoot proliferation, basal callusing, vitrification and difficulty in rooting. Different explants materials were used to test the regeneration potential of each of them following tissue culture methods. Of all the explants used, nodal explants proved to be the best suited material for the fast adventitious plantlets development. Furthermore, it was also evident that the nodal explants collected in respect to the factors like season, time of excision, position in the twig, are all equally very important in deciding the fate of the cultures.

From the studies made, we report the following observations, 1. The nodes collected between the months of August to December flourished well due to the fact that they were freshly formed and free of contaminants. 2. The nodes collected in the morning hours responded

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well due to their active state and less of phenolic compounds release. 3. The nodes which are slightly matured leaving the first 3 – 4 tender ones responded well.

The observations made by Bhajaj (1997), pointed out that the position of nodal explants is very crucial in determining the growth and differentiation of cultures. Kuldeep & Narendra (2011), mentioned that the nodal segments of 4-8th position in any freshly appeared twigs are the best ones for the shoot initiation and not the terminal tender ones. Raghu *et al*. (2007), Rajesh Pati *et al.* (2008), also stressed the point of seasonal response of explants material. According to their view, nodal explants collected during September – December months are most active and successful in cultures. Considering all these observations, it appears that the position of nodes, time of collection, size and number of buds on explants, are all very important in deciding culture responses.

In establishment stage, cytokinin treatments of AS, Ad, BAP, DPU, KN in concentrations of 1, 2, 3 mg/L and auxin treatments of PIC, CPA and IBA in 3 mg/L concentration, induced direct adventitious shoot development. Only in the treatments of NAA (1, 2, 3 mg/L), NOA (1, 2, 3 mg/L), CPA (1, 2 mg/L), PIC (1, 2 mg/L) and IBA (1, 2 mg/L), callus interference delayed the process. Nodal explants also failed to show any positive response and gradually showed evidence of decline in 2, 4 – D (1, 2, 3 mg/L) treated MS media formulations. When the established nodal segments got further sub-cultured on combination treatments of MS medium, pronounced axillary shoot growth was observed in media containing cytokinins like AS or KN. On the contrary, NOA, NAA and IBA presence, induced callus formation and expansion. In support to these results, Pranati & Behera (2007), while culturing cotyledonary nodes of *Aegle marmelos* on MS medium used BAP and KN to induce adventitious shoot development. In the same lines, Rekha Warrier *et al*. (2010), used BAP treated MS medium for establishing nodal segments and BAP treated WPM for proliferation purpose. Kuldeep & Narendra (2011), also made an attempt to induced callus growth in cultures by using MS media supplemented with BAP/2,4-D.

With respect to the multiplication stage, BAP involving combination treatments showed greater proliferation rate than in treatments containing AS, Ad, KN and DPU phytohormones. In all these combination treatments multiple shoots formed after 60 days of sub-culturing are as follows; In BAP involved media, 17 to 32 multiple shoots. In AS involved treatment, 13 to 17 multiple shoots. In Ad supplied media, 13 to 16 multiple shoots. In KN treated media, 12 to 17 multiple shoots and lastly in DPU involved media, 8 to 14 multiple shoots were formed. Many authors to achieve success in multiplication stage tried using various combination treatments involving Cytokinins + Auxins.

Arya & Shekhawat (1986), to achieve proliferation used MS medium supplemented with various concentrations of selected auxins and cytokinins. Ajithkumar & Seeni (1998), stressed the importance of cytokinins and auxins in the stages of multiplication. Islam *et al*. (2006), by culturing cotyledon explants of *Aegle marmelos*, found that regeneration of the plantlets was highest on MS medium fortified with BAP 2 mg/L + NAA 0.2 mg/L. Islam *et al*. (2007), while culturing node derived callus to regenerate plantlets used different concentrations and combinations of BAP with 2, 4-D, NAA and IBA. Raghu *et al*. (2007), used low concentrations (0.1- 1 mg/L) of BAP and KN phytohormones to induce multiple shoots development from nodal segments. Das et al. (2008) induced multiple shoots formation by using combination treatments of BAP + NAA and attained about 22.7 multiple shoots.

In recent years Neha *et al*. (2010), sucessfully cultured nodal segment on MS media provided a range of plant growth regulators to attain the multiple shoot-lets. They obtained about 8 plantlets/explants by sub-culturing nodal segments on medium supplemented with KN (2 mg/L). Puspasree & Thirunavoukkarasu (2011), used MS medium containing BAP (1 or 1.5 mg/L) + IAA (0.2 mg/L) composition to attain proliferation. Kuldeep & Narendra (2011), in their

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experiments used MS media with KN (2mg/L) + NAA (0.5 mg/L) and BAP (2mg/L) +IAA (1mg/L) to attain multiple shoots. Puspashree *et al*. (2012), also used MS medium supplemented with various concentrations of only BAP and KN for multiplication purpose. In their experiments, about 10 plantlets per explants were attained in BA (0.5 mg/L) treated MS medium.

For elongation, 12 combination treatments involving cytokinins like KN, DPU, AS and Ad were used. Results obtained in all these formulations produced good length in plantlets with good number of nodal regions and leaves. In all the experiments carried out GA3 was not used and still plantlets got elongated well. Hossain *et al*. (1994a), in this stage of morphogenesis used GA3 in the concentration of 1 mg/L to enhance the growth of the plantlets. Puspashree *et al*. (2012) used MS medium with different concentrations of BA, KN and GA_3 either individually or in combination.

For rooting under conditions employed, ½ strength MS semi solid medium and ½ strength liquid medium with various concentrations of IBA, helped in inducing roots formation. In MS ½ strength liquid medium with IBA 8 mg/L concentration and in MS ½ strength semi solid medium with IBA in 15 mg/L concentration, about 2 roots formed measuring 2.5 cm in length in just 21 days time. Once the roots were formed, rooted plantlets were successful acclimatized to the *in vivo* conditions following suitable measures. ½ strength MS media supplemented with IBA in different concentrations was also used by Arya & Shekhawat (1986), Ajithkumar & Seeni (1998), Pranati & Behera (2007), Rajesh Pati et al. (2008), Hossain *et al*. (1994a), Kuldeep & Narendra (2011), Puspasree & Thirunavoukkarasu (2011). Some acheived success by using lower concentrations of IBA (0.5 – 2 mg/L) and some by using higher concentrations of IBA (max 30 mg/L) hormone. Comparing through all the results, it appears ½ strength liquid MS medium fortified with different concentrations of IBA is equally good as ½ strength semi solid medium IBA treatments. Further, it was also observed that post rooting handling of plantlets is very crucial and important to successfully transfer the developed plantlets from *in vitro* to *in vivo* conditions.

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