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In vitro propagation of *Aegle marmelos* (L.) corr., a medicinal plant through axillary bud multiplication

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ABSTRACT

A protocol for rapid *in vitro* propagation from nodal explants of the medicinal tree species *Aegle marmelos* (L.) corr. of family Rutaceae has been described. High frequency bud break were induced on Murashige and Skoog's (1962) basal medium supplemented with 0.5 mg benzyladenine (BA)/l. After 10 days of culture, nodal explants with multiplied buds started callusing with restricted growth and defoliation. When the same nodal explants ware transferred into the same basal medium supplemented with 0.5 mg BA/l with different concentrations of either kinetin (KN) or gibberellic acid (GA_3) or in combinations has shown healthy shoots with expanded shoot length. Excised shoots (2 cm - 3 cm long with 2 to 3 nodes) when grown on 1/2 MS basal medium with 2.5 mg/l indole-3-butyric acid (IBA) and 0.5% activated charcoal (A.C.)/l has shown rhizogenesis. After excision, in the second passage, the nodal explants also showed bud break when sub cultured on MS basal medium supplemented with 0.5 mg BA/l. These shoots also successfully rooted on the same above said medium.

Keywords: *Aegle marmelos*; Organogenesis; Rhizogenesis; Callus Culture; Plant Growth Regulators; Shoot Multiplication

1. INTRODUCTION

Aegle marmelos (L.) corr. (family-Rutaceae) is a forest cosmopolitan as well as an important medicinal plant. Different parts of *A. marmelos* have been investigated by several workers and found to contain coumarins, alkaloids, triterpenes, sterols and essential oils [1,2]. The essential oil has shown a broad spectrum of anti-bacterial and anti-fungal activities [3]. Bael has a wide therapeutic value in the treatment of diabetes, anaemia, fractures, healing of wound, swollen Joints, high blood pressure, Jundice, diarrhoea, troubles during pregnancy and typhoid. Pulp of the ripe fruit is taken during summer to keep the body and mind cool and to sharpen intellect and concentration of mind [4]. Roasted or dried pulp of ripe fruit is a purgative, astringent, digestive, tonic and stomachic. The leaves also help in controlling pollution by absorbing foul gases from the atmosphere and keep it clean and salubrious. Due to large-scale and unrestricted exploitation to meet increasing demands by the pharmaceutical industries coupled with limited cultivation and insufficient attempts for its reforestation, the stock of this important medicinal plant species has been markedly depleted. In recent years there has been an increased interest in *in vitro* culture techniques which offer a viable tool for mass multiplication and germplasm conservation of rare, endangered, aromatic and medicinal plants [5-8], as well as forest trees where the lifecycle takes a longer period [9,10].

In general, micropropagation through axillary shoot proliferation has proven to be a handy tool [11] An appreciable level of success has been achieved by many investigators in the case of many tropical medicinal plants through micropropagation as in case of *Hemidesmus indicus* ([12], *Cleistanthus collinus* [13], *Gmelina arborea* [14], *Plumbago zeylanica* [15] and *Holarrhena antidysenterica* [16]. Although there are techniques have been reported for *in vitro* propagation of *Aegle marmelos* from *in vitro* grown seedling parts, *i.e*. from roots [17], from hypocotyl [18], cotyledonary node [19] and excised leaf explants [20] but the works have more been concentrated on *in vitro* grown seedling explants. So in the present study trial has been taken to establish a protocol for regeneration and mass propagation of *A. marmelos* from mature tree explants *i.e*. from nodal explants through axillary bud multiplication.

2. MATERIALS AND METHODS

2.1. Explants Source and Surface Decontamination Methods

Young shoots from the crowns of mature tree (20 years

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old) growing in the B.J.B. College garden were collected. Healthy nodal segments measuring 1.0 - 2.0 cm were cut, defoliated and with dormant axillary buds were used as explants. The nodal segments were initially rinsed thoroughly in running tap water for 14 minutes followed by immersing in a 5% (v/v) solution of detergent (Labolene, Qualigens, India) for 10 min. Then it was surface sterilized with 0.1% (w/v) mercuric chloride for (4 - 5 min) subjected to repeated washings in sterile distilled water. These surface-decontaminated explants were taken for inoculation.

2.2. Culture Medium and Shoot Multiplication

Murashige and Skoog's (1962) basal medium [21] was used for culture. This medium was supplemented with various concentrations of only benzyladenine (BA) and Kinetin (KN) or in combinations with 2% (W/V) sucrose and gelled with 0.8% (W/V) agar (Bacteriological Grade, Himedia, Mumbai, India). Using 1N NaOH or 1N HCl, the pH of the medium adjusted to 5.8 before autoclaving. Routinely 15 ml each of molten medium was dispensed into sterile 25 × 150 mm culture tubes and tightly plugged with cotton. After that autoclaving was done at a temp of 121˚C and 15 Ib steam pressure for 15 min. The cultures were incubated at 25˚C ± 2˚C, 55% - 60% RH and 16-h photoperiod supplied by a bank of cool-white fluorescent tubes (PPF 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$). After 2 weeks, the % of bud break, numbers of days required for bud break, number of shoots per explants and mean shoot length were measured. The culture tubes showing callusing and defoliation of leaves are also marked.

2.3. Elongation of Shoots

Nodal explants were transformed into the elongation medium (MS + different concentrations of BA, KN and GA_3 either or in combinations) to restrict defoliation and callusing with healthy shoot growth. The cultures were sub cultured thrice at fortnightly intervals by transferring to fresh medium. The evaluations were made in terms of number of shoots per explants inducing healthy growth, shoot length after 4 and 6 weeks of culture and number of nodes per shoot.

2.4. Rooting

Shoots (2 cm or longer) excised and transferred into rooting medium. Rooting medium was MS (1962) basal medium supplemented with various concentrations of IBA, IAA with 0.5%, and 0.2% (W/V) activated charcoal. Rooting response also varied in terms of % of response, length and number of roots depending on the medium and source of material.

Autoclaving, incubation conditions were same for proliferation, differentiation and rooting medium as described above. For each treatment, 10 replicates were used and all the experiments were repeated three times.

All data were taken by Mean ± SE and significance of mean is tested by Duncan (1955) multiple range tests [22].

2.5. Hardening

After 4 weeks of culture the rooted plantlets were thoroughly washed with tap water and dipped in an antifungal solution 0.1% (w/v) Bavistin solution for 5 minutes, then washed and planted in plastic pots (5 cm diameter) containing autoclaved artificial soil (1 part vermiculite: 1 part perlite by v/v) and were supplied sterile distilled water for 4 weeks. Established plants were repotted in polythene bags (of the size 20 cm × 10 cm) filled with autoclaved sand, soil/red earth and farmyard manure (v:v:v) in the proportions of 1:1:1 and then to bigger earthen pots (25 cm diameter) containing a non-sterile sand: soil: compost mixture (1:3:1) and kept under shade (40% shade) in the garden for another 3weeks and watering was done as and when required. When the plants were established they were transferred to open light conditions in the botanical garden.

3. RESULTS AND DISCUSSION

The stem segments ware cultured on MS medium supplemented with different conc. of BA and KN. Multiple shoot buds appeared to arise directly from the node within 2 - 5 days of culture (**Figure 1(a)**). The frequency of bud producing explants increased with the increase of culture period and became 86.6 ± 3.33 at the end of 6th day of culture. The number of days taken for bud break is different with different growth regulators and it's number increased with the increase in concentration of growth regulators used. The percentages of bud break, numbers of shoots and mean shoot length decreased with increase of concentrations of different growth regulators. Among different treatment combinations 0.5 mg BA/l was found to be the best for maximum bud break, number of shoots and mean shoot length (**Table 1**). Cultures of nodal explants with multiple shoots after 1 week and within the second week started callusing at the base of leaf and node and also started defoliation (**Figure 1(b)**). These primary cultures were then transferred into elongation/ induction medium for healthy growth of shoots by restricting callusing and defoliation. The induction medium was MS basal medium [21] supplemented with different concentrations of BA, KN and GA_3 (**Table 2**).

The better response of explants in the medium containing benzyladenine with gibberellin and kinetin was shown because kinetin prevents senescence/defoliation by prevention of formation of hydrolases e.g. nucleases and proteases and causing immobilization of nutrients or their transport to cytokinin treated areas. Gibberellins

Table 1. Effect of plant growth regulators on axiliary bud multiplication of nodal explants of *Aegle marmelos* in terms of number of shoots, length of shoot and callusing.

MS (1962) basal medium with plant growth regulators mg/l	Number of days required for bud break	Percentage of bud break after 5 days	Number of shoots per explants	Mean shoot length after 2 weeks
MS (Control)	NR	NR	NR	NR
BA 0.5	3	86.6 ± 3.33 a	10.0 ± 0.30 a (+)	0.870 ± 0.005 a
BA 1.0	4	83.3 ± 3.33 a	8.0 ± 0.30 b (+)	0.616 ± 0.008 c
BA 1.5	4	76.6 ± 3.33 ab	7.1 ± 0.26 c (+)	0.593 ± 0.006 c
BA 2.0	5	70.0 ± 0.00 bc	5.5 ± 0.33 d (+)	0.520 ± 0.115 d
KN 0.5	2	63.3 ± 3.33 c	4.7 ± 0.13 e (+)	0.506 ± 0.003 d
KN 1.0	2	66.6 ± 3.33 bc	4.3 ± 0.08 e (+)	0.523 ± 0.005 d
KN 1.5	3	60.0 + 5.77 c	3.3 + 0.03 f (+)	0.530 + 0.005 d
KN 2.0	4	43.3 ± 3.33 d	2.0 ± 0.03 g (+)	0.506 ± 0.003 d
BA 0.5 + KN 0.5	3	70.0 ± 5.77 bc	5.5 ± 0.05 d (+) Delayed callusing and defoliation	0.700 ± 0.011 b

Data (Mean ± SE) of 3 independent experiments each with 10 replicates. Means followed by the same letter within the columns are not significantly different. ($P < 0.05$) as tested by the multiple range test of Duncan (1955). NR—Not responded, (+) callusing and defoliation of leaves in explants. Shoots measuring < 0.5 cm not taken into account for calculation of shoot length.

Table 2. Effect of different growth regulators for inducing healthy and elongated shoots growth by restricting callussing and defoliation of multiple axiliary bud of *A. marmelos.*

MS (1962) basal medium with plant growth hormone mg/l	Number of shoot per explants remains attached after 1 week culture	Percentage of shoot induced healthy growth after 2 weeks *i.e*. with shoot length > 0.5	Shoot length after 4 weeks	Number of nodes per shoot
BA 0.5 + KN 0.1	3.3 ± 0.33 a	70 ± 1.15 c	2.8 ± 0.16 a	2.67 ± 0.33 a
BA 0.5 + KN 0.25	2.3 ± 0.33 b	76 ± 0.88 b	2.1 ± 0.16 b (dried up shoots)	2.33 ± 0.33 a
BA 0.5 + GA_3 0.5+ KN 0.1	3.6 ± 0.33 ab	87 ± 0.57 a	2.8 ± 0.20 a	2.67 ± 0.33 a
BA 0.5 + GA_3 0.5 + KN 0.25	2.3 ± 0.33 b	64 ± 0.57 d	2.1 ± 0.16 b (Some shoots dried up)	1.67 ± 0.33 a
BA 0.5 + GA_3 0.5	3.3 ± 0.33 ab	71 ± 0.57c	2.7 ± 0.14 a	2.0 ± 0.57 a

Data (Mean ± SE) of 3 independent experiments each with 10 replicates. Means followed by the same letter within the columns are not significantly different. ($P < 0.05$) as tested by the multiple range test of Duncan (1955).

participate in cambium activity and differentiation of tissues by stimulating RNA, protein and DNA synthesis, which ultimately stimulates axillary bud elongation. But the application of higher concentration of GA_3 and KN gave decreased response.

The stimulating effect of BA on bud break and multiple shoots formation has been reported earlier for several medicinal and aromatic plant species including *Chlorophytum borivilianum* [23], *Withania somnifera* [24], *Litchi chinensis* [25] and *Anacardium occidentale* [26]. Reduction in the number of shoots generated from each node at higher concentrations of BA than the optimum level was also reported for several medicinal plants [24].

It was found the induction medium with BA 0.5 mg/l + GA_3 0.5 mg/l + KN 0.1 mg/l to be the most appropriate cocktail where maximum number of shoots per explants remains attached and out of this maximum percentage of shoots induced healthy growth with maximum shoot length, maximum number of number of nodes per shoot and expanded leaves (**Figure 1(c)**).

Excised shoots 2 cm or more were rooted on different strength of MS with different concentrations of IBA, IAA and activated charcoal. The percentage of rooting, root length is presented in **Table 3**.

Highest response was obtained from 1/2 MS + IBA 2.5 mg/l + 0.5% A.C. medium with maximum root length growth (**Figure 1(d)**). Different strength of MS basal medium with charcoal was used because explant on full strength MS medium without activated charcoal did not respond to the rhizogenesis. The shoots in the rooting medium devoid of charcoal dried up may be due to oxidation. Charcoal was used to avoid oxidation of polyphenols and reduce the light effect though it is a good absorbent of light (light enhances polyphenol oxidation which leads the blackening and death of explants). Previous investigators have also obtained comparable results on the effect of temp and light on rooting cv. Bridal pink [27,28] and lower concentration of MS salts in rooting [29].

Plantlet with six or seven fully expanded leaves and

Table 3. Rooting responses of *Aegle marmelos* in various concentration of IBA, IAA and activated charcoal.

Treatment	% of rooting of shoots from nodal explants	Root length		Number of roots per shoot
		After 2 weeks		
1/2 MS + IBA 2.5 mg/l + 0.2% A.C.	20.3 ± 0.88 b	1.2 ± 0.03 b	1.2 ± 0.03 b	1
1/2 MS + IBA 2.5 mg/l + 0.5% A.C.	31.6 ± 0.88 a	1.4 ± 0.03 a	1.4 ± 0.03 a	1 With very small callus at the base
1/2 MS + IBA 2.5 mg/l + IAA 2.0 mg/l + 0.5% A.C.	30.0 ± 0.57 a	1.3 ± 0.03 a	1.3 ± 0.03 a	1 With small callus at the base
1/4 MS + IBA 2.5 mg/l + 0.5% A.C.	14.6 ± 0.33 c	1.4 ± 0.01 a	1.4 ± 0.01 a	1

A.C.—Activated Charcoal. Data is mean ± SE of three independent experiments, each with 10 replicates. Means followed by the same letter within column are not significantly different ($P < 0.05$) as tested by the multiple range test of Duncan (1955).

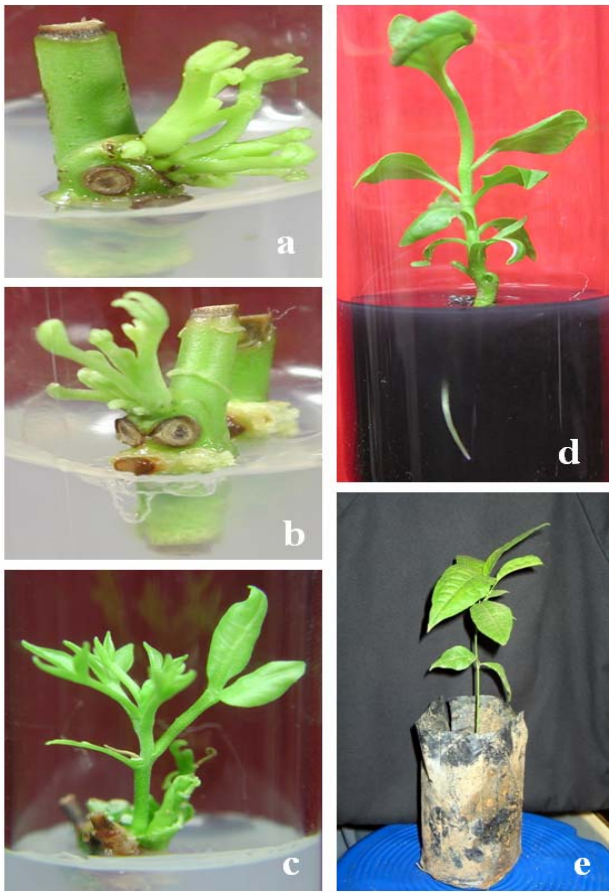

Figure 1. *In vitro* morphogenesis of nodal explants of *Aegle marmelos* and their derived plantlets. (a) Nodal explants showing bud break; (b) Nodal explants with multiple shoots starting callusing at the base of leaf and node within 1 week of culture; (c) Nodal explants with healthy shoot length growth in the induction medium; (d) Excised shoot showing well developed root and (e) Fully developed plantlets after 7weeks of hardening.

well developed roots were successfully hardened on vermiculite for 4 weeks and subsequently transferred to a mixture of sand:soil:farmyard manure (1:1:1) in poly bags (**Figure 1(e)**). Then these are transferred into bigger pots. The percentage of survival was 100%. All plants had normal leaf development and did not show any detectable differences in morphological or growth characteristics compared to the donor plants.

Trials for mass *in vitro* propagation of *A. marmelos* from a single node segments of a 20 years old tree which resulted 12.1 shoots of up to 5.2 cm length in 48% of the explants after 7 weeks of culture [30], 70% - 90% cuttings rooted and 85% of plants were established after hardening. In the present study 86.6% ± 3.33% response were obtained producing 10.0 ± 0.30 number of shoots out of which 3.6 ± 0.33 number of shoots gave 2.67 ± 0.33 cm shoot length after 2 week of primary culture. The rooted shoots gave 100% establishment after hardening and also in advantage, did not show any morphological differences to that of mother plant.

The nodal segments are the best explants as they could develop more multiple shoots [31]. The results obtained are highly reproducible, thus the method described is useful for clonal propagation of selected germplasm through axially bud multiplication.

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