Research Journal of Botany

ISSN 1816-4919

Academic Journals Inc.

www.academicjournals.com

Research Journal of Botany 2 (4): 186-194, 2007
ISSN 1816-4919

An Improved Micropropagation Protocol for Bael- A Vulnerable Medicinal Tree

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Abstract: An efficient and rapid *in vitro* clonal propagation of the endangered medicinal tree *Aegle marmelos* (L.) Corr. (Rutaceae) by enhanced axillary shoot proliferation from mature single node was designed. The explants showed marked seasonal variation in their response under *in vitro* conditions. Explants collected in October (72.8%) and November (78.6%) showed maximum response. Multiple shoots were formed on Murashige and Skoog (MS) medium supplemented with 0.5 mg L^{-1} 6-Benzyladenine (BA). An average of 6.2 shoots/explant could be obtained after 45 days of culture. The number of shoots was increased at the third subculture with an average of 16.3 shoots per explant. The effect of subsequent subcultures (upto 20 cycles) on shoot formation was also studied. Subculturing was carried out every 45 days on fresh shoot multiplication medium. Continuous culture in the same medium resulted in distorted and vitrified shoots. Transfer of cultures to half strength MS medium devoid of ammonium ions and cytokinin (BA) for a single cycle before going to the shoot multiplication medium could solve this problem. *In vitro* rooting was inconsistent in medium with different auxins (Indole 3-butyric acid-IBA, Indole 3-acetic acid-IAA and α-naphthalene acetic acid-NAA) at varying concentration and combinations. But *in vitro* raised shoots could be rooted *ex vitro* by pulse treatment with naphthoxy acetic acid (NOA) and IBA and then in chlorogenic acid followed by planting in moist sand. This treatment resulted in 83.9% survival of plantlets. The method standardised could be used for large scale planting material production and conservation of this important endangered medicinal tree.

Key words: *Aegle marmelos*, collection season, hyperhydricity, large-scale propagation, *ex vitro* rooting

INTRODUCTION

Aegle marmelos (L.) Corr. (Rutaceae) is an armed spiny medicinal tree sparsely distributed throughout India on the plains and in hilly tracts up to 1300 m elevation (Anonymous, 2003). This plant is listed in earliest ayurvedic (an ancient Indian system of preventative health care) medicinal texts viz. *Charaka samhita* (600 BC) and *Sushruta samhita* (500 BC) and has been widely used in ayurveda for a variety of ailments. All parts of the tree like root, bark, leaves and fruits are highly medicinal. It is astringent, cooling, carminative, laxative, restorative and stomachic and is used in dysentery, diarrhoea, flatulence, fever, vomiting and colic. The leaves are astringent, laxative, febrifuge

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and expectorant and are useful in ophthalmia, deafness, inflammations, diabetes and asthmatic complaints. The tender fruit is bitter, astringent, antilaxative, digestive and promotes digestion and strength, overcomes vata, colics and diarrhoea. The ripe fruits are astringent, sweet, aromatic, cooling, febrifuge, laxative and tonic and are good for the heart and brain (Sivarajan and Balachandran, 1994; Warrier *et al*., 1994). Antidiabetic property (Kamalakkanan and Prince, 2003; Kar *et al*., 2003), antidiarrhoeal activity (Shoba and Thomas, 2001), antiulcer activity of seeds (Goel *et al*., 1997), antifungal activity of leaves (Rana *et al*., 1997) and antitumour and antimutagenic activity (Annapurani and Priya, 1999; Lampronti *et al*., 2003) of this plant are clinically evaluated.

This plant is conventionally propagated through seeds. Seeds have short viability and are prone to insect attack. Vegetative propagation through root suckers is slow, difficult and cumbersome (Anonymous, 2003). Based on the studies conducted on seed propagation, the germination percentage of seed was very low under natural and controlled conditions (Raghu, 2006). Indiscriminate collection resulted in the disappearance of this plant from the wild sources and the species is reported to be vulnerable in the Western Ghats of Kerala, Tamil Nadu and Karnataka states of India (Ravikumar and Ved, 2000). Root being the major medicinally useful part, destructive harvesting poses a serious threat to the sustenance of the tree. Propagation through tissue culture is a viable alternative in this species. Several workers have reported *in vitro* propagation of *A. marmelos* using axillary bud multiplication (Arumugam and Rao, 1996; Islam *et al*., 1994; Hossain *et al*., 1994a; Varghese *et al*., 1993; Ajithkumar and Seeni, 1998), nucellar calli (Hossain *et al*., 1994b) and from leaf explants (Islam *et al*., 1993). The present study was undertaken to determine a suitable collection season of explants and reproducible and reliable large-scale micropropagation system using mature nodes.

MATERIALS AND METHODS

Explant Collection, Collecting Season and Surface Sterilization

A 25 year old tree growing in the Herb Garden of the Institute was the source of the explant. Stem cuttings with four to five nodes were collected from the tip of the lower branch. After leaf excision, the stem was cut into single node pieces, thoroughly washed under running tap water and then in dilute detergent teepol (0.2 mL in 100 mL) for 10 mins. The explants were surface sterilized with 0.1% mercuric chloride and Tween-20 (0.2 mL in 100 mL) for 5 min followed by three washing in distilled water. The explants were then taken to the laminar airflow chamber and surface sterilized with 0.1% (w/v) mercuric chloride for 3 min followed by three washing in sterile distilled water. Under aseptic conditions the nodes were inserted vertically into the culture medium consisting of full strength Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) medium with 3% (w/v) sucrose, 0.7% (w/v) agar and growth regulators.

In *A. marmelos* under natural conditions, new shoots and leaves appear at the end of April and the flower buds appear almost simultaneously during May-July, followed by fruit setting. During September-December the plant goes through the vegetative phase. Defoliation appears during March-April. To determine the most suitable month for culture establishment, explants were collected every month for one year.

Culture Establishment, Shoot Multiplication and Large-Scale Propagation

Disinfected single nodal segments were cultured on MS basal medium for culture initiation. After 45 days of incubation the contamination free cultures showing bud break were used for multiplication experiments in MS medium supplemented with low concentrations of BA (0.1-1.0 mg L^{-1}) and Kinetin (0.1-1.0 mg L^{-1}) (Table 1). The pH of the medium was adjusted to 5.8 before autoclaving at

Table 1: Effect of cytokinins and progressive subcultures on the number and length of shoots in shoot cultures of *A. marmelos*

Cytokinins	Shoot induction		Subculture 1		Subculture 2		Subculture 3	
(mg L^{-1})	Number	Length (cm)	Number	Length (cm)	Number	Length (cm)	Number	Length (cm)
Kinetin								
0.1	0.2±1.6^{a}	0.2±0.7^{a}	0.0	0.0	0.0	0.0	0.0	0.0
0.3	1.1±1.2^{b}	0.6±0.9^{b}	1.8±0.5^{a}	0.6±0.3^{a}	1.8±0.5^{a}	1.9±0.2^{c}	1.7±0.4^{a}	1.7±0.3^{b}
0.5	1.6±0.3^{bc}	1.2±1.1^{c}	2.3±0.6^{b}	1.1±0.5^{b}	2.7±0.4^{b}	2.2±0.8^{d}	3.1±0.6^{b}	2.3±0.2^{c}
0.8	2.1±0.9^{c}	1.5±0.6^{d}	2.4±0.5^{b}	1.5±0.4^{c}	2.7±0.9^{b}	2.5±0.1^{e}	2.9±0.2^{b}	2.5±0.9^{c}
1.0	2.5±1.1^{cd}	1.8±0.8^{e}	2.9±0.3^{c}	1.9±0.9^{cd}	3.4±0.6^{c}	2.7±1.1^{e}	3.3±0.9^{bc}	2.8±0.8^{d}
BA								
0.1	1.2±0.2^{b}	0.1±0.6^{a}	1.8±0.2^{a}	0.3±1.3^{a}	2.7±0.5^{b}	0.5±0.3^{a}	3.1±0.6^{b}	0.6±0.2^{a}
0.3	2.3±0.8^{c}	0.9±0.7^{b}	2.4±0.5^{b}	1.1±0.4^{b}	2.9±0.9^{bc}	1.5±0.2^{b}	3.8±0.7^{c}	1.6±0.6^{b}
0.5	6.2±0.4^{g}	1.6±0.9^{d}	8.4±0.6^{e}	2.1±0.3^{d}	12.7±0.8^{e}	2.2±0.6^{d}	16.3±0.6^{f}	2.4±0.7^{c}
0.8	5.9±1.0^{f}	1.2±0.8^{c}	6.4±0.7^{d}	1.7±0.6^{c}	10.1±0.2^{d}	1.7±0.8^{b}	11.9±0.6^{e}	1.5±0.7^{b}
1.0	5.6±0.3^{f}	1.2±0.7^{c}	6.6±0.5^{d}	1.6±0.2^{c}	9.9±0.1^{d}	1.7±1.3^{b}	10.4±0.4^{d}	1.6±0.8^{b}

Data were recorded 45 days following transfer of the shoots to MS medium after each subculture. Treatment means followed by same superscripts within column are not significantly different from each other ($p<0.05$); comparison by LSD multiple range test

121°C for 20 min. The cultures were incubated at 24±2°C with cool white fluorescent lamps, 35-40 μmol m^{-2} sec^{-1} irradiance (Philips India Ltd., Mumbai), at a photoperiod of 10-12 h. For checking the adventitious shoot regeneration from root, *in vitro* derived roots were cultured on MS basal medium alone or with 0.5 mg L^{-1} BA.

Shoots differentiated upon the primary cultures served as the source of explants for large-scale propagation. *In vitro* differentiated shoots (2.0-3.0 cm in length) were separated into clumps (three or four shoots per clump) and placed in the MS medium containing 0.5 mg L^{-1} BA for 45 days. Continuous culture in the same medium resulted in distorted and vitrified shoots. Hence, the cultures were transferred to half strength MS medium devoid of ammonium ions (NH_4^+) and BA. To test the shoot multiplication rate during subculture passages, the process was repeated up to 20 passages each at 45 days interval. During large-scale propagation, the shoots were inoculated on 25 mL agar gelled multiplication medium in jam bottles capped with polypropylene caps.

In vitro and *ex vitro* Rooting

For *in vitro* root induction isolated shoots of 2-3 cm were transferred to quarter, half and full strength MS medium with or without auxin such as IBA, IAA and NAA under different concentrations (0.5-5.0 mg L^{-1}). The *in vitro* rooted plants were taken out from the medium, washed under tap water to remove all traces of media and then individual plants were transferred to polythene bags containing soil, sand and farmyard manure (1:1:1) and kept in humid chamber in the nursery for maintaining humidity. After one week the plants, which showed new growth, were transferred to pots containing same matrix or to the field for further growth.

For *ex vitro* rooting, shoots were subjected to pulse treatment with 100 mg L^{-1} NOA and 100 mg L^{-1} IBA for 2 min and then in 100 mg L^{-1} chlorogenic acid for 5 min. The pulsed shoot tips were planted into cups filled with moist sand. The cups were kept in humid chamber in the green house conditions for 6 weeks and then transferred to the field.

Experimental Design and Data Analysis

All experiments were repeated thrice with 12 replicates each. Standard errors of means were calculated and statistically significant mean differences were determined by the Least Significant Difference (LSD) test.

RESULTS AND DISCUSSION

Culture Establishment and Multiplication

The explants collected during different months of the year showed marked variations in their morphogenic response (Fig. 1). Explants collected during November (after the fruit setting period) showed maximum response (78.6%). The response gradually declined during the subsequent months and reached to 12-13% during March-April (leaf fall time). During May, the time of new leaf emergence, the axillary bud response was little evoked (Fig. 1). Influence of the seasonal growth of the mother plant was evident in morphogenesis of this tree, like *Holarrhena antidysenterica* (Kumar *et al*., 2005), Sandalwood (Sanjaya *et al*., 2006), Teak (Tiwari *et al*., 2002) etc. Under culture conditions, however once the shoots are established, recurrent plantlet regeneration is possible throughout the year.

Shoot development could not be induced from nodal explants on growth regulator free medium. All the explants responded on medium containing either of the two cytokinins. Nodal explants started responding within two weeks (Fig. 3A) and attained multiple axillary shoots on MS medium supplemented with BA and Kinetin in another four weeks. Explants cultured in MS medium supplemented with 0.1, 0.3, 0.5, 0.8 and 1.0 mg L^{-1} BA and kinetin induced multiple shoots after 45 days of culture (Table 1). As compared to the BA treatment, relatively lower responses were observed in media supplemented with Kinetin. This observation is contradictory to the report by Ajithkumar and Seeni (1998) who found both BA and kinetin were equally effective for this plant. Of the different concentrations of the cytokinins tested, 0.5 mg L^{-1} BA elicited multiple shoots with an average of 6.2±0.4 shoots per explant and an average shoot length of 1.6±0.9 cm (Table 1). The shoots were excised and nodal segments were further subcultured on the same but fresh medium. A significant enhancement of the morphogenic response was evident and the average shoot number increased to 8.4±0.5. The shoots were excised again and subcultured on the fresh medium. A 2-fold multiplication rate of shoots was achieved and an average of 16.3±0.9 shoots per explant was obtained after a third subculture (Table 1 and Fig. 3B).

Contrasting results were reported by earlier workers regarding the type, concentration and combination of plant growth regulators required for axillary bud multiplication of *A. marmelos*. Most of the earlier workers used cotyledonary node explants. Arumugam and Rao (1996) suggested that higher concentration (3.0 mg L^{-1}) of BA was best for shoot multiplication from cotyledonary node cultures. Islam *et al*. (1994) reported that combination of BA with IAA or gibberellic acid gave better results than BA alone in cotyledonary node explants. Varghese *et al*. (1993) suggested the combination of Kinetin and NAA while Hossain *et al*. (1994a) employed a combination of BA (2.0 mg L^{-1}) and IAA (0.2 mg L^{-1}) for axillary bud multiplication from cotyledonary node explants. A synergistic combination of 2.5 mg L^{-1} BA and 1.0 mg L^{-1} IAA was best for shoot induction (12.1 shoots) in the

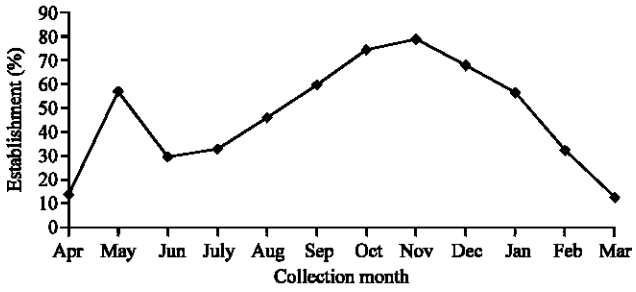

Fig. 1: Effect of explant collection months on culture establishment

observation led by Ajithkumar and Seeni (1998). The present study showed that lower concentration of BA was sufficient for shoot multiplication in this plant. At higher concentrations of BA or kinetin, the proliferated shoots were stunted and vitrified in nature. Increasing the concentration of BA (above 1.0 mg L^{-1}) in the culture medium also resulted in callus formation at basal parts of the growing shoots. The use of comparatively lower concentration of growth regulator in present protocol is an important factor worth mentioning, as it minimizes the risk of producing genetically altered individuals (Edson *et al*., 1996).

Large-scale Propagation

Shoot clumps consisting of three or four shoots were found to be better than single shoots for large-scale propagation. The continuous shoot proliferation, without any obvious decline on subsequent subculturing (third subculture onwards) was noticed in the same multiplication medium (MS + 0.5 mg L^{-1} BA) up to 20 cycles (Fig. 2). Repeated subculturing after 6-8 cycles in the same medium resulted in under developed, distorted and vitrified shoots. This may be hyperhydricity due to the uncontrolled uptake of ammonium ions by cells and continuous culture on the BA supplemented medium. Hyperhydricity of shoot and node cultures is most often encountered in the mass propagation of woody species. Hyperhydricity is influenced by ammonium and nitrate ions in the media and it could be overcome by omitting ammonium ions in the MS macronutrients (George, 1996). There were some reports that the ammonium became toxic to many species in *in vitro* at continued subculture in MS full strength medium (Bonga and Von Aderkas, 1992). Shoots placed continuously on a medium containing BA may also be responsible for this phenomenon. In *Sequoia sempervirens* hyperhydricity of the cultures due to continuous culturing in BA containing medium was reported. This could be overcome by placing them alternatively on growth regulator free medium at monthly intervals and the cultures could be maintained healthy and proliferative upto 13 years (Franclet, 1991). Transfer of the cultures to ammonium ion and cytokinin free half strength MS medium for a subculture cycle solved this problem in *A. marmelos*. Again the cycles can be continued with the same multiplication medium (MS + 0.5 mg L^{-1} BA). Better growth was achieved when shoot cultures were grown in jam bottles with polypropylene caps (Fig. 3D). The type of container that is used for culture can have a considerable impact on the growth rate of cultures, on the quality of shoots and plantlets produced and on the degree of vitrification. For woody species the best shoot growth and rooting was obtained in

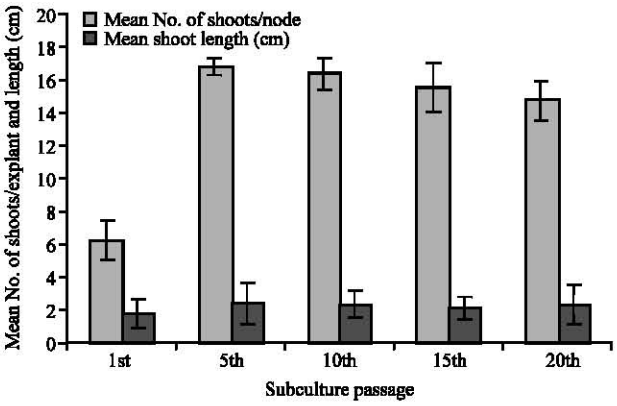

Fig. 2: Frequency of shoot multiplication in *in vitro* derived nodal explants of *A. marmelos* during the subsequent subculture passages at 45 days interval in MS medium containing 0.5 mg L^{-1} BA and 3% (w/v) sucrose. The results are the mean±standard error (n = 5)

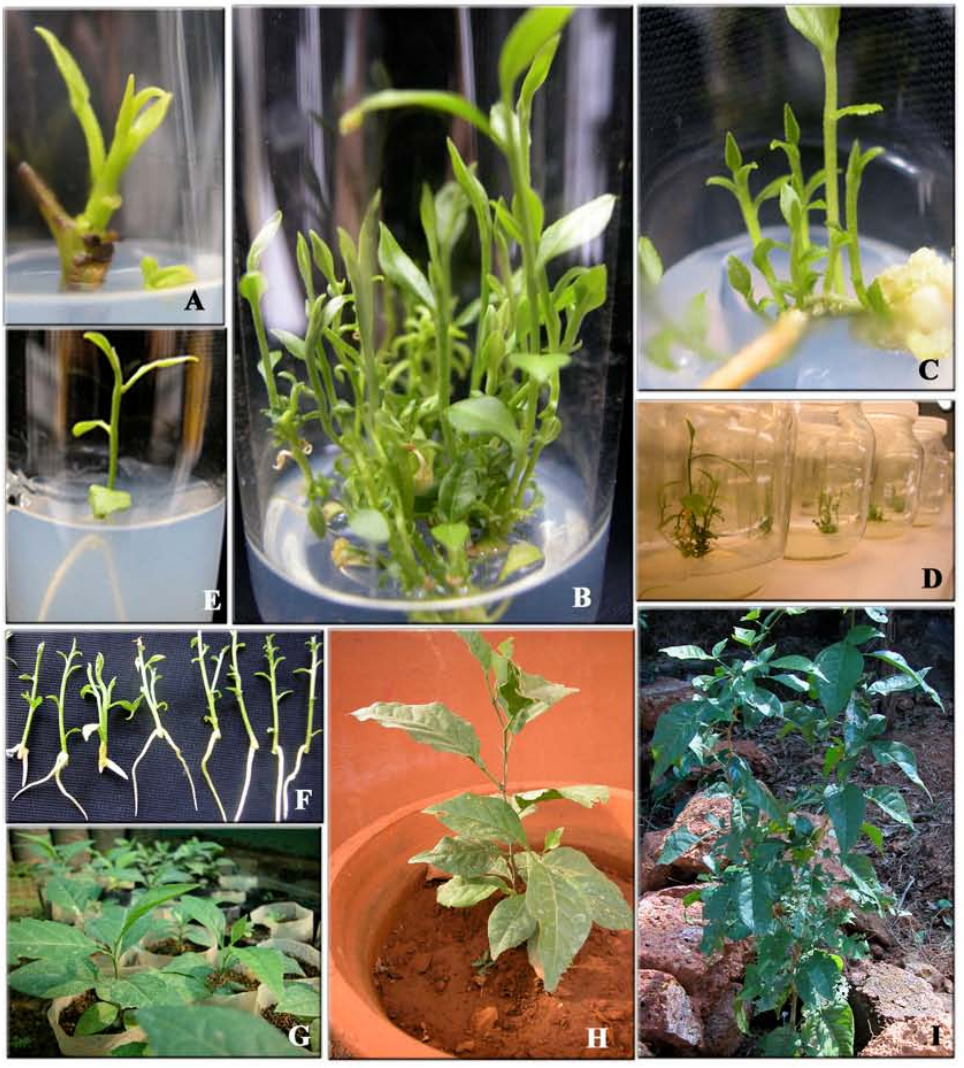

Fig. 3: A-I. Large scale micropropagtion of *Aegle marmelos*. A: Culture initiation on MS medium with 0.2 mg L^{-1} BA; B: Multiple shoot formation on MS with 0.5 mg L^{-1} BA; C: Multiple shoot formation on root explant; D: Large scale propagation; E: Roots produces in quarter strength MS basal medium; F: *Ex vitro* (Pulse) rooted plants; G: Acclimatized plantlets in the nursery; H and I: One year old tissue cultured plants in the pot and field

the largest vessels (Bonga and Von Aderkas, 1992). It was striking to note that the micropropagated shoots could be subcultured up to 20 cycles without loss of vigour to produce shoots free from morphological and growth abnormalities (Fig. 2).

Adventitious Shoot Regeneration

Single shoots were regenerated from the root segments in MS growth regulator free medium. Multiple shoots were induced from the root segments (1.0-2.0 cm) on MS medium supplemented with 0.5 mg L^{-1} BA (Fig. 3C). Islam *et al.* (1996) had achieved the same results from the enlarged apical regions of the root tip of intact seedlings on MS medium supplemented with 2 mg L^{-1} BA and

0.1 mg L^{-1} IAA. Direct organogenesis from the root explant and its multiplication in lower concentrations of BA in our study might be due to the higher levels of endogenous growth regulators in this species.

Rooting and Field Establishment

The multishoot clumps as such were subcultured at an interval of every 45 days and the shoot tips of about 2-3 cm were harvested for rooting experiments. *In vitro* rooting trials attempted with different concentrations of auxins (IBA, IAA and NAA) did not produce consistent rooting (data not shown). Rooting is more difficult when explants from mature trees are used (Bonga and Von Aderkas, 1992). However, one or two roots were observed in less than 30% of cultures in ¼ strength MS medium without any growth regulators and with full strength MS medium supplemented with IBA at higher concentration (10 mg L^{-1}) after 10 weeks of culture (Fig. 3E). But the number of roots did not affect the subsequent establishment of rooted plants in the field. This is in agreement with reports by Ajithkumar and Seeni (1998) in the same plant. This discrepancy in rooting response might be due to differential accumulation of BA during different cycles of subculture. This may be in connection with flavonoid accumulation during the shoot multiplication phase. Shoot exposure to BA during different subculture cycles showed no flavonoid accumulation, which is favorable to auxin-induced rooting (Bonga and Von Aderkas, 1992). Earlier reports available on *in vitro* rooting of *A. marmelos* indicated the use of higher levels of auxins (IBA, IAA and NAA) ranging from 4 to 25 mg L^{-1} (Arumugam and Rao, 1996; Islam *et al*., 1994; Varghese *et al*., 1993; Hossain *et al*., 1994a; Ajithkumar and Seeni, 1998).

When the shoots obtained from *in vitro* cultures are rooted in the external environment, rooting and hardening took place concurrently and the plants were fully acclimatized before transplanting. *Ex vitro* rooting through pulse treatment, consisting of initial treatment in NOA and IBA for about 2 h followed by chlorogenic acid treatment for 6 min yielded uniform rooting (80-85%) on subsequent transfer to suitable substrate (Fig. 3F). Arya *et al*. (2002) and Martin *et al*. (2004) reported the rooting of plants after pulse treatment using the same auxins in *Celastrus paniculatus*. The shoots that failed to root in *in vitro*, responded to this method. The type of planting substrate also played a significant role in the induction of roots from the pulse treated shoots. Maximum rooting was observed in moist sand, within 30 days. Coir pith either alone or with sand (1:1) showed comparatively lesser rate of rooting *ex vitro* rooting is more advantageous over *in vitro* rooting and subsequent planting out, as the latter requires utmost care while planting out, more time and labor. *ex vitro* rooting is a more reliable and cost effective method, as a separate *in vitro* rooting stage could be avoided and the hardening could be simultaneous with rooting. The cups were covered with polythene bags and kept under green house conditions for 6 weeks and then transferred to the field conditions with 98% establishment (Fig. 3G and H). The micro plants planted in the field for 12 months showed uniform morphology and the growth indicated stability of micropropagated plants free from visible abnormalities (Fig. 3I).

This study reports successful development of an efficient, cost effective and easy to handle micropropagation protocol for the vulnerable medicinal tree *A. marmelos*. This study also ensures stability of plants raised through a number of subculture passages. This protocol provides a successful rapid technique that can be used for the propagation and *ex situ* conservation of this species and helps for minimising the pressure on wild populations and contributes to the conservation of the valuable flora of the Western Ghats.

ACKNOWLEDGMENT

The authors are grateful to Sir. Dorabji TATA Trust, Mumbai, India for providing financial assistance.

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