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Short communication

Callus induction and plantlet regeneration in *Aegle marmelos* (L.) Corr. using cotyledon explants

M.S. Hazeena* and G.R. Sulekha

Department of Plantation Crops and Spices, College of Agriculture, Kerala Agricultural University, Vellayani, Thiruvananthapuram 695522, Kerala, India.

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Abstract

A protocol was developed for callus induction and shoot regeneration from cotyledon explants of *Aegle marmelos* (L.) Corr., a medicinal tree. Murashige and Skoog (MS) medium supplemented with benzyl adenine (2.2 µM) and 2,4-dichlorophenoxy acetic acid (2.26 µM) recorded the highest growth score for callus induction and proliferation. Shoot regeneration response from the callus was best on MS medium containing 8.8 µM benzyl adenine and 2.85 µM indole-3- acetic acid. Callus derived shoots were rooted *in vitro* on MS medium supplemented with 12.3 µM indole-3- butyric acid. The plantlets were acclimatized in sand and transferred to the field.

Keywords: Medicinal trees, callus induction, MS medium

Aegle marmelos (L.) Corr. commonly known as *bael* is an important fruit tree belonging to the family Rutaceae with extensive medicinal uses in the indigenous medicine systems of India. The leaves are astringent, febrifuge, expectorant, and are reported to have hypoglycaemic and antiasthmatic properties (Nambiar et al., 2000). Leaf extract of *A. marmelos* is used as an antispermatogenic (Sur and Pramani, 1999) to cure jaundice (Gupta and Sharma, 1999). It also enhances the wound healing activity (Jaswanth et al., 2001). The unripe and ripe fruits are useful for curing diarrhoea, dysentery, and stomachalgia (Warrier et al., 1996). *A. marmelos* root is one of the ingredients of the popular ayurvedic preparations such as *Dasamula* and *Vilvadi lehya*.

Generally, *A. marmelos* is propagated by seed. The seeds, however, have short viability and are prone to insect attacks. Vegetative propagation through root suckers is slow, difficult, and cumbersome (Ray and Chatterjee, 1996). Plant regeneration *via in vitro* methods has been reported in *A. marmelos* from different explants, i.e., cotyledonary node (Nayak et al., 2007), root segments (Bhati et al., 1992), nucellus (Hossain et al., 1994), and single-node segments (Ajithkumar and Seeni, 1998). The present study demonstrates an efficient protocol for callus induction and plantlet regeneration from cotyledon explants of *A. marmelos*.

A 60 year old tree of *A. marmelos* (L.) Corr. with a large trunk having ~50 cm diameter at breast height and regular flowering and fruiting characters from a homestead in Thiruvananthapuram, Kerala served as the source of explants. Seeds were obtained from freshly harvested fruits. The hard shell of the fruit was broken and seeds taken out. They were washed thoroughly in running tap water for 15 min. to remove the mucilagenous sheath. Seeds were then washed with 1% Tween 20, followed by washing with double-distilled water. Surface sterilization was carried out by treating with $HgCl_2$ (0.1%) for 10 min. The seeds were

*Author for correspondence: Phone : 91-471-2722460; Email: <haze_ameen@yahoo.com>.

dissected so that the cotyledon inside seed coat was longitudinally cut into two equal halves and then inoculated on to the culture media after cutting and excluding the embryo axis.

The nutrient medium for callus induction consisted of salts and vitamins of Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of 6-benzyl adenine (BA; 0.44 and 2.2 µM), 2,4-dichlorophenoxy acetic acid (2,4-D; 0.45 µm, 2.26 µm), and naphthalene acetic acid (NAA; 5.37 and 10.74 µM; Table 1). The medium contained 3% (w/v) sucrose and 0.8% (w/v) agar. All cultures were maintained at 24± 2°C with 60% relative humidity under fluorescent lights (intensity 2000 lux) with 16/8 h light/ dark cycles.

Observations were recorded on the number of cultures initiating callus from the explant. Callus Index (CI) was computed by multiplying percent cultures initiating callus with growth score (G), which was assessed by visual rating (poor=1, medium=2, good=3, and profuse=4). The mean score was expressed as growth score (G). Subculturing was done at 2 to 3 weeks interval to attain callus proliferation. After the third subculture, the callus tissue (10 to 15 mg fresh weight) was transferred on to MS basal medium containing different combinations of cytokinins and auxins for shoot initiation and proliferation. The effect of BA (0.44 to 8.8 µM), kinetin (4.64 and 9.29 µM), 2,4-D (0.45 and 2.26 µM), NAA (5.37 and 10. 74 µm) and indole-3-acetic acid (IAA; 2.85 and 5.7 µM) on regeneration of shoots from callus were evaluated (Table 2). Observations were taken on the percent of cultures developing shoots, number of shoots, length of the longest shoot, and average length of shoots.

Well developed shoots (3 to 5 cm long) were excised and transferred to rooting media with varying levels of indole-3-butyric acid (IBA; 2.46 to 12.30 µM), IAA (5.70 to 14.27 µM), and NAA (5.37 µM). Shoots with *in vitro* developed roots recovered from culture tubes were washed to remove agar and transplanted to plastic containers with sand, Soilrite, and sand:Soilrite (2:1) as potting media. The plants were irrigated and acclimatized for 4 to 6 weeks in a mist chamber before transfer to the field. Each treatment consisted of 12

Table 1. Effect of plant growth regulators on callus induction from cotyledon explants of *Aegle marmelos.*

Plant growth regulator (µm)		Cultures initiating callus (%)	Growth score (g)	Callus index (CI)
BA	0.44	-	-	-
	2.2	-	-	-
BA + 2,4-D				
	0.44 +0.45	67	1.75	117
	0.44 + 2.26	100	2.83	283
	2.2 + 0.45	67	1.00	67
	2.2 + 2.26	100	3.50	350
2,4-D				
	0.45	50	1.33	67
	2.26	83	2.40	120
NAA				
	5.37	83	1.40	117
	10.74	100	1.33	133
NAA + BA				
	5.37 + 0.44	50	1.00	50
	5.37 + 2.2	33	1.50	50
	10.74 + 0.44	100	1.67	167
	10.74 + 2.2	100	1.17	117

Means of 12 replications per treatment, each experiment repeated thrice, data scored after 4 weeks of culture; - no response; CI = G * Cultures initiating callus (%).

Table 2. Effect of plant growth regulators on shoot proliferation via indirect somatic organogenesis from cotyledon explants of *Aegle marmelos.*

Plant growth regulator (µM)		% cultures developing shoots	Number of shoots/ callus	Length of longest shoot (cm)	Average length of shoots (cm)
BA	0.44	67	19.50^{hi}	3.23^{abc}	2.33^{ab}
	2.2	100	21.33^{ijk}	3.32^{abc}	2.00^{ab}
BA + 2, 4-D					
	0.44 +0.45	83	13.40^{gh}	2.56^{abc}	1.64^{ab}
	2.2 + 0.45	100	20.16^{hij}	2.88^{abc}	2.08^{ab}
BA + NAA					
	0.44 + 5.37	83	8.80^{efg}	7.10^{e}	6.26^{d}
	0.44 + 10.74	100	6.17^{cde}	7.62^{e}	6.03^{d}
	2.2 + 5.37	100	9.83^{fg}	7.53^{e}	5.97^{d}
	2.2 + 10.74	100	7.50^{de}	8.33^{e}	6.58^{d}
BA + IAA					
	4.4 + 2.85	100	31.67^{kl}	2.95^{abc}	1.75^{a}
	4.4 + 5.7	100	29.83^{jk}	3.12^{abc}	2.12^{ab}
	8.8 + 2.85	100	41.17^{l}	4.05^{cd}	2.45^{ab}
	8.8 +5.7	100	35.17^{l}	3.87^{abc}	2.3^{ab}
Kinetin					
	4.64	83.	4.40^{bc}	4.02^{cd}	2.80^{bc}
	9.29	50	3.67^{a}	2.43^{abc}	1.10^{ab}
Kinetin + NAA					
	4.64 + 5.37	67	7.50^{ef}	2.25^{abc}	1.43^{ab}
	4.64 + 10.74	67	4.00^{ab}	2.63^{abc}	1.78^{ab}
	9.29 + 5.37	33	4.50^{bcd}	2.20^{abc}	1.25^{ab}
	9.29 + 10.74	33	2.00^{a}	1.95^{abc}	1.75^{ab}
NAA					
	5.37	-	-	-	-
	10.74	-	-	-	-
p			<0.001	<0.001	<0.001

Means of three independent experiments with each treatment replicated 12 times. Observations were taken after 6 weeks of culture. The mean values followed by the same superscript do not differ significantly in DMRT at 5% level of significance; - No response.

replications and each experiment repeated thrice. The data were analyzed using analysis of variance (ANOVA) and the means compared with Duncan's Multiple Range test (DMRT).

The results indicated that explants dedifferentiated and initiated calli at the cut surfaces within 8 to10 days of inoculation and proliferated within 4 weeks of culture. Highest callus index (350) was recorded in the medium supplemented with 2.2 µM BA and 2.26 µM 2,4-D, followed by the treatment combination of 0.44 µM BA and 2.26 µM 2,4-D (Table 1). This result is consistent with the earlier report where a combination of BA and 2,4-D was found to be the best for callus induction from embryonic tissues of *A. marmelos* (Islam et al., 1995). There was, however, no callus formation from explants cultured on media fortified with BA.

Callus morphology varied with different growth regulators used in the culture medium. Yellowish green compact calli which turned slightly brownish on further subculturing was induced on medium with BA and 2,4,-D. In some of these treatments, callogenesis and adventitious bud initiation took place simultaneously (Fig 1a). Media with NAA alone and in combination with BA developed creamy white friable slow growing

callus. Rhizogenesis from callus characterized by thick spongy roots was seen in media supplemented with NAA alone.

The compact callus issue was sectioned and transferred on to MS medium fortified with auxins and cytokinins in various combinations and concentrations for shoot initiation and proliferation (Table 2). Green adventitious shoot buds developed on the compact masses was subcultured on to the same media at 2 to 3 weeks interval for shoot proliferation. Highest regeneration frequency and number of shoots per callus (41) were observed on MS media supplemented with 8.8 µM BA and 2.85 µM IAA (Fig. 1b). Similar results where combination of cytokinin (BA) with auxins (IAA and NAA) was found to enhance sprouting response in *A. marmelos* has been reported earlier too (Nayak et al., 2007).

Subculture of regenerated shoots into same medium exhibited further shoot proliferation with minimal basal callus. Earlier studies by Arumugam et al. (2003) demonstrated that BA suppressed callus formation but induced shoot bud regeneration. During the present study, low regeneration frequency of shoot buds was observed in the medium supplemented with combination of kinetin and NAA. BA in general was better than kinetin for shoot regeneration.

Well developed elongated shoots (3 to 5 cm) were excised and transferred to MS medium with varying concentrations of auxins IAA, IBA and NAA. IBA (12.3 µM) was the best in respect of response

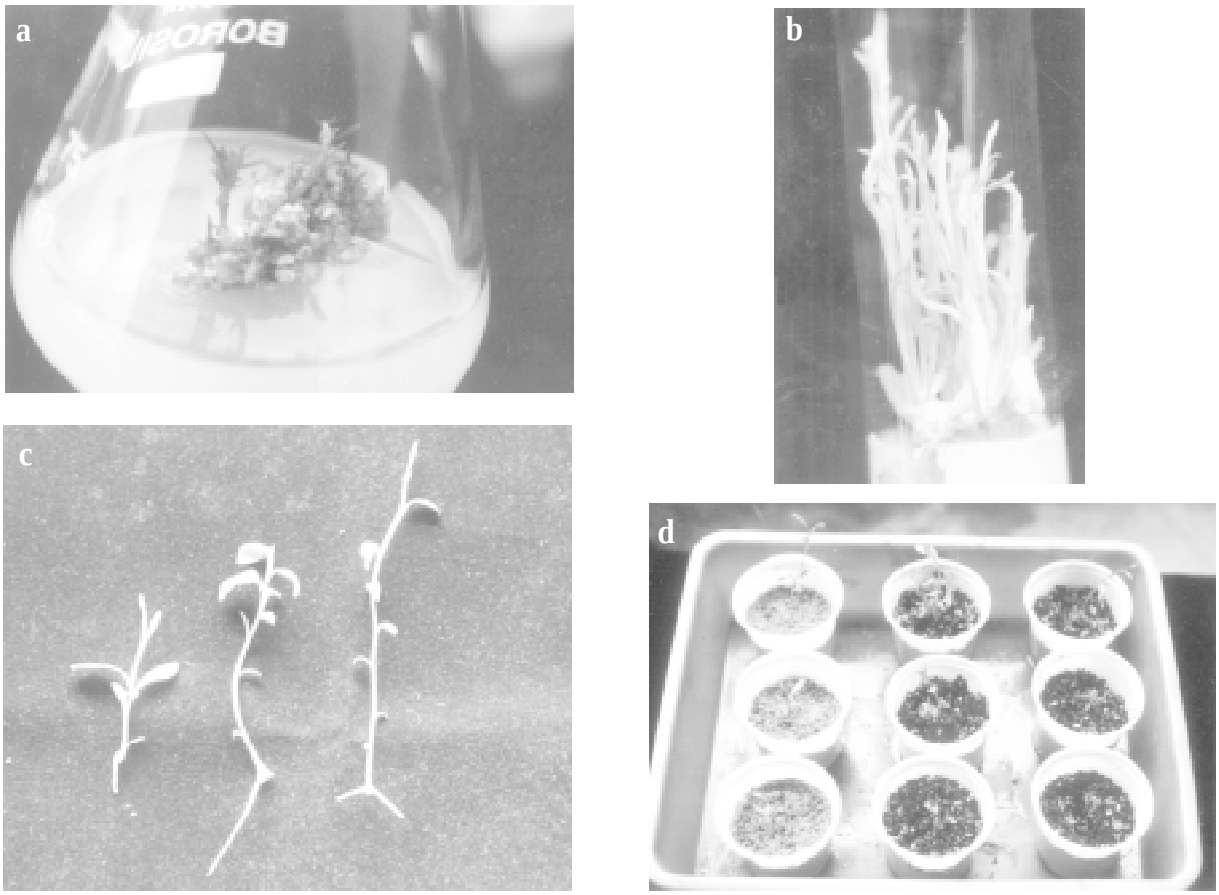

Figure 1 (a) Shoot regeneration from callus on MS media supplemented with 2.2 µM BA and 2.26 µM 2,4-D *(b)* Shoot proliferation on MS medium supplemented with 8.8 µM BA and 2.85 µM IAA *(c) In vitro* rooting on MS media supplemented with 12.3 µM IBA *(d)* hardening of *in vitro* rooted plants

Table 3. Effect of plant growth regulators on *in vitro* rooting of *Aegle marmelos.*

Plant growth regulator (µM)	Root initiation (%)	Number of days for root initiation	No of roots/ shoot	Length of root (cm)
IBA				
2.46	50	7^{a}	1.0	2.16
4.92	50	19^{de}	3.0	1.16
7.38	50	12^{abc}	1.0	2.56
9.84	33	14^{abc}	1.0	0.90
12.30	67	8^{ab}	3.3	1.16
IAA				
5.70	17	11^{abc}	1.0	0.80
8.56	17	14^{abc}	1.0	1.20
11.41	50	12^{abc}	1.7	2.63
14.27	17	23^{d}	1.0	1.10
NAA				
5.37	17	23^{d}	1.0	0.70
p		0.034	0.16	0.064

Means of three independent experiments with 12 replications per treatment. Mean values followed by the same superscript are not significantly different according to DMRT at 5%.

percentage, rapidity of root initiation, and root proliferation (Table 3; Fig 1c). IBA has been reported to give better results compared to other auxins for *in vitro* rhizogenesis of *A. marmelos* shoots earlier too (Arumugam et al., 2003). In most cases, root initiation took place within 2 weeks. Delayed rooting response, even after 3 weeks in root induction medium observed in certain cases can be attributed to the persisting effect of BA. With regard to the number of roots, single root formation was observed in majority of treatments. *In vitro* single root formation can be due to the aborescent character and has been reported in tree species including *A. marmelos* (Ajithkumar and Seeni, 1998). The *in vitro* rooted plants were planted out on small plastic containers with sand, Soilrite, and Soilrite:sand (2:1) as potting media (Fig. 1d). Sand was superior to other potting media and recorded good survival rate of 75% after 6 weeks of acclimatization.

Overall, the present study demonstrates a plant regeneration system through indirect organogenesis from cotyledon explants of *A. marmelos*, which may be useful in future research to find out somaclonal variations for genetic improvement and enhanced production of this valuable medicinal tree.

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