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Full Length Research Paper

Direct organogenesis of *Aegle marmelos* (L.) Corr. from cotyledon explants

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Cotyledon segments from *in vitro* seedlings of *Aegle marmelos* were cultured on Murashige and Skoog (MS) medium supplemented with different concentration and combination of growth regulators. Highest regenerative response was observed in the medium containing 1.5 mg/l BA + 0.2 mg/l IAA in which 80% of the explant produced shootlets. After 2 sub-cultures in the same media composition, the shoot bud clusters that developed from cotyledon segments had started elongation of shootlets to a reasonable length of 3 - 5 cm. Elongated shoots could be rooted onto half-strength MS medium supplemented with 4 mg/l IBA with 0.2% activated charcoal.

Key words: Cotyledon segments, growth regulator, shoot bud induction.

INTRODUCTION

Aegle marmelos Corr. commonly known as 'Bael' tree is a sacred tree of family Rutaceae. It is native to northern India, but is found widely throughout the Indian peninsula and in Ceylon. All parts of the tree are used in Ayurvedic preparation for various ailments. The unripe dried fruit is astringent, digestive and stomachic used to cure diarrhoea and dysentery (Watt, 1889). The ripe fruit is a good and simple cure for dyspepsia and the unripe and half-ripe fruits improve appetite and digestion (Jain, 1968; Jauhari et al., 1969). The roots and the bark of the tree are used in the treatment of fever and to control pain in the abdomen. They are also useful in the disorders of vata, pitta and kapha (Kirtikar and Basu, 1935). The leaves are made into a poultice and used in the treatments of ophthalmic. The rind of the ripe fruit is also sometimes used as a medicine (Dastur, 1962).

Due to its high medicinal value this plant is being exploited to a larger extent by the drug and pharmaceutical industries. Hence, there is an urgency to develop appropriate technique for mass propagation of this valuable species so as to domesticate it for future use. Although *A. marmelos* is propagated through seeds, plantation raised through seeds exhibited wide variations within the population. Micropropagation of elite genotype may play an important role in solving the problem through rapid multiplication. We report here an efficient propagation method for large-scale cultivation of this valuable tree species.

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Abbreviations: BA, N_6-Benzyl adenine; **IBA,** indole-3-butyric acid; **KN,** kinetin; **IAA,** indole-3-acetic acid; **BAP,** benzylaminopurine; **NAA,** α–napthalene acetic acid; **MS,** Murashige and Skoog (1962) medium.

MATERIALS AND METHODS

Explant preparation

Ripe fruits were collected from a 20 year old tree of *A. marmelos* grown in the garden of B. J. B. College, Bhubaneswar (20^0 17' 45" N latitude and 85^0 49' 15" E longitudes). Seeds were removed from the fruits with the help of scalpel and washed in tap water and agitated in a liquid detergent solution for 15 - 20 min followed by agitation in 0.1% $HgCl_2$ for 7 - 8 min for surface sterilization. Finally the seeds were thoroughly rinsed in sterile double distilled water for at least four times under aseptic conditions and the sterile seeds were aseptically inoculated on to basal Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) devoid of any hormones and incubated in an incubator for germination. Segments of cotyledon (0.5 to 1.0 cm) were aseptically excised from 10 days' old

Figure 1. *In vitro* propagation of *Aegle marmelos* through direct shoot regeneration from cotyledon explants. Formation of shoot buds from cotyledon explant (1 week old).

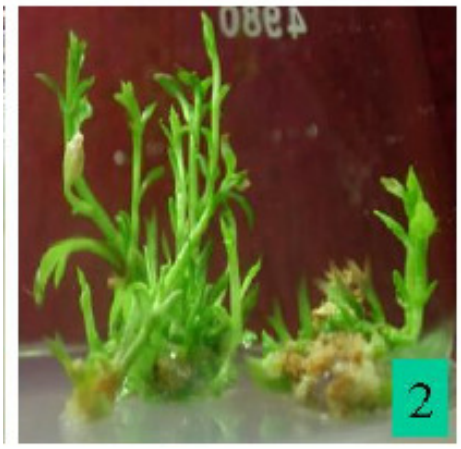

Figure 2. *In vitro* propagation of Aegle marmelos through direct shoot regeneration from cotyledon explants. Clusters of shoot buds (5 weeks old).

aseptically grown seedlings and cultured on different nutrient media. All operations during inoculation including surface sterilization were carried out inside the laminar airflow cabinet (Thermadyne, India).

Culture medium and culture condition for shoot formation and rooting

MS salt composition, sucrose (30 g/l) and bacteriological grade agar-agar (8 g/l) were used throughout the study. For shoot induction, growth regulators, BA alone or in combination with IAA, were added to the MS basal medium. For root induction, half-strength MS basal medium in addition to activated charcoal supplemented with either IAA or IBA and the pH was adjusted 5.8. All the cultures were incubated in a culture room maintained at 25° 2°C under 16 h/8 h light/dark cycle, 45 µmol m^{-2} s^{-1} irradiance level provided by cool white fluorescent tubes (Philips, India) and with 55 - 60% relative humidity. Each treatment consisted of 10 replicates and repeated thrice.

Hardening

Rooted plantlets were planted in the polybags containing sand + soil (1:1) and maintained in a temperature controlled green house. Temperature was set to 28 ± 2□C. All data are represented by mean ± SE of three independent experiments each with 20 replicates and significance were tested by the multiple range test of Duncan (1955) at $p<0.05$.

RESULTS

Cotyledonary segments maintained on MS medium supplemented with growth regulators showed swellings at different points along the cut surface of the explants within the first week of inoculation. In the subsequent 1 - 2 weeks depending on the media combinations, most of these swellings were developed into shoot buds (Figure 1) while few entered in to callusing. Cotyledon segments failed to show any morphogenetic response on growth regulator-free MS medium. Among the different concentrations and combination tested, MS medium enriched with BA and IAA stimulated the best response in terms of shoot bud formation. In such cultures, clusters of shoot buds ranging 20 - 80 numbers were recorded, which appeared as tiny tube-like structures that resembled embryoids. Such embyroids like structure continued to grow and visible morphological changes were observed and recognizable as monopolar structures. The growth of the monopolar structures was asynchronous, about 50% of them elongated and attained reasonable length of 1 cm in four weeks time. After 2 sub-cultures in the same media composition, the shoot bud clusters that developed from cotyledon segments had started further elongation of shootlets. The shootlets produced on the medium containing BA and IAA gave shoot length elongation of maximum number of shootlets when sub-cultured on the same medium. Growths of the shoot buds were well pronounced in this media composition and after 7 weeks of culture they attained a reasonable length of 3 - 5 cm (Figure 2). Shootlets, which were above 2 cm long, were taken into consideration. Among different combinations tested BA (1.5 mg/l) + IAA (0.2 mg/l) showed highest response where in 81 ± 0.58% cultures responded producing as high as 38 ± 0.58 shootlets per explant with an average length of 4.2 ± 0.03 cm followed by 1 mg/l BA + 0.2 mg/l IAA. Also when BA alone was used there was a maximum of 7.3 ± 0.33 shoots per explant produced. The least response was observed in the medium supplemented with 2 mg/l KN. In such cultures there was

Table 1. Effect of various growth regulator on shoot regeneration from cotyledon explants of *A. marmelos*.

MS medium with hormones (mg/l)	% Culture responded	Number of shoots	Length of shoot
Control	NR	NR	NR
Kn0.5	20.3±0.33f	3±0.58k,l	2±0.03h
Kn1	41±0.58d	3±0k,l	2.2±0.02f
Kn1.5	30.7±0.33e	2.3±0.33l	2.1±0.07g
Kn2	20.7±0.33f	NC	NC
BA0.5	50±0.58c	6.7±0.33h,i	2.8±0.03e
BA1	60±0.58b	7.3±0.33h	3.2±0.03c
BA1.5	60.3±0.33b	5.7±0.33i,j	3.1±0.04c,d
BA2	40.3±0.33d	4.3±0.33j,k	2.5±0.03f
BA 0.5 + 0.2 IAA	50.3±0.33c	15±0.58e	3.6±0.03b
BA 1.0 + 0.2 IAA	60.7±0.67b	25±0.58b	3.6±0.01b
BA 1.5 + 0.2 IAA	81±0.58a	38±0.58a	4.2±0.03a
BA 2.0 + 0.2 IAA	61±0.58b	21±0.33c	3±0.03d
BA 0.5 + 0.5 IAA	51±0.58c	9.3±0.33g	3.1±0.05c,d
BA 1.0 + 0.2 IAA	60±0.58b	18±0.88d	3.5±0.06b
BA 1.5 + 0.5 IAA	50±0.58c	13±0.88f	3.1±0.03c,d
BA 2.0 + 0.5 IAA	40.3±0.88d	9±0.58g	2.5±0.03f

NR: None responded.
Data scored after 7 weeks of culture are mean ± SE of 3 experiments each with 10 replicates. Shootlets measuring <2 cm is not taken into account.
Means followed by the same letter within the column are not significantly different ($P<0.05$) as tested by the multiple range test of Duncan (1955).

Table 2. Rooting response of excised shoots of cotyledon explants of *Aegle marmelos* on MS medium supplemented with various growth regulators

½ MS medium with	Shoots rooted (%)	Days to rooting	Number of roots	Mean root length	Rooting culture produce callus at the base (%)
IBA 2.0 mg/l + 0.5%A.C.	25±1.732d	45-60	1-2Roots	1.9±0.057b	10±0.577a
IBA 2.5 mg/l + 0.5%A.C.	39±1.732c	40- 55	1-2Roots	1.1±0.115c	5±0.577c
IBA 4.0 mg/l + 0.5%A.C	89±0.577a	25-30	1-2Roots	2.4±0.057a	3±0.577d
IBA 5.0 mg/l + 0.5%A.C.	85±1.732a	35-40	1-2Roots	2.0±0.057b	3±0.577d
IBA 6.0 mg/l + 0.5%A.C	54±1.154b	60-80	1-2Roots	1.3±0.057c	8±0.577b

A.C.: Activated charcoal.
Data (mean ± SE) of three 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).

only 3-4 shootlets having shoot length less than 1 cm were observed and did not developed any more elongation of shootlets length even after another prolonged observation period of more than 4 weeks. So it is not taken into consideration for calculation (Table 1).

The *in vitro* shootlets were carefully excised and transferred individually to the rooting medium containing half-strength MS basal medium supplemented with either of IAA or IBA. Depending upon the media concentration the roots developed at the base of the shoots with or without intervening callus. Root initiated after 3 weeks of culture irrespective of media composition. However, addition of IBA at 4 mg/l showed maximum response (89 ± 0.577%) with an average number of 2.4 ± 0.057 roots per shootlet were recorded. Higher concentration of IBA (6.0 mg/l) and also lower concentration resulted in reduction in percentage of rooting as well as root length (Table 2).

After 5 weeks of maintenance in the rooting media (Figure 3) the plantlets were carefully removed from the culture tubes, washed thoroughly to remove any remains of medium and planted in the polybags containing sand + soil (1:1) and maintained in a temperature controlled green house. Temperature was set to 28 ± 2°C and the

Figure 3. *In vitro* propagation of Aegle marmelos through direct shoot regeneration from cotyledon explants. A single shootlet rooted on half-strength MS basal media supplemented with 4 mg/l IBA (5 weeks old).

Figure 4. *In vitro* propagation of Aegle marmelos through direct shoot regeneration from cotyledon explants. Hardened plantlets in the polybags containing sand + soil (1:1) (5 weeks old).

potted plantlets were allowed to grow in the same condition for 5 weeks. Within 3 - 5 weeks new leaves began to form (Figure 4). Then they were transferred to the field.

DISCUSSION

The characteristics of cell culture to undergo complex developmental sequences lie in controlled manipulation of auxin and cytokinin. Organogenic or embryogeneic differentiation depends on a delicate balance between the concentration of auxin and cytokinin in the nutrient medium. In the present study, MS medium without any growth regulator failed to elicit regeneration responsive. But addition of BA proved to be significantly useful in shootlets production. Another interesting point to be noted is that, BA along with auxin IAA gave better response. Shoot growth in such combinations were better than the shoot growth observed in the medium with BA alone. This is in corroboration with results obtained in cotyledon (Hossain et al., 1994) and hypocotyls (Hossain et al., 1995) explants of *A. marmelos*. The results of the present study indicated that auxin along with cytokinin is essential for enhanced sprouting response. A review of literatures indicates that addition of either of IAA or NAA in the culture medium improved the response in a number of species in terms of shoot growth. It has been reported that nodal cultures of *Acacia senegal* responded better when BAP (4.0 mg/l) and NAA (0.5 mg/l) were added to the culture medium (Kaur et al., 1998). A similar response was observed in *Prosopis juliflora* cultures (Nandwani and Ramawat, 1991). This was also true in case of *Acacia seyal* shoot tip cultures, in which multiple shoot formation was better when the culture medium was supplemented with BA and NAA, but not when either of them was used separately (Al-Wasel, 2000). BA in combination with auxin was also found to be essential for multiple shoot induction in some other trees (Arya and Shekhawat, 1986; Tabone et al., 1986; Shekhawat et al., 1993).

Successful regeneration of plantlets using mature nodal explants (Varghese et al., 1993), nucellar tissue (Hossain et al., 1993), cotyledon (Hossain et al., 1994), hypocotyl (Hossain et al., 1995), embryonic tissue (Islam et al., 1995) and cotyledonary node (Arumugam and Rao, 1996) of *A. marmelos* has been reported. The present results represent a three step protocol for efficient plant regeneration system through direct organogenesis from cotyledon explants.

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