Micropropagation of Bael [*Aegle marmelos* (L.) Corr.] – An Indigenous Medicinal Fruit Tree of India

Sandhya Gupta*, Devendra Chauhan and Manju Bala
Tissue Culture and Cryopreservation Unit, National Bureau of Plant Genetic Resources, New Delhi-110012, India

A protocol for micropropagation of bael [*Aegle marmelos* (L.) Corr.] was developed. Bael (family Rutaceae) is an indigenous medicinally important fruit of India. The nodal explants of 30 year old tree were used to initiate cultures. Two cytokinins, *viz*., 6–benzylaminopurine (BAP) and kinetin (Kn) were used in varied concentration (0.1-2 mg/l) for shoot multiplication. BAP (2 mg/l) was found better than Kn, where a 3-fold increase in the number of shoots was recorded in 4 weeks. A synergistic influence of cytokinin and auxin was also observed in the present study. A combination of 0.5 mg/l BAP and 0.1 mg/l IAA induced the formation of maximum number (4.5) of shoots (2.5 cm). For rooting of *in vitro* shoots, different auxins, namely, NAA, IAA and IBA (0.1-2 mg/l) were tested. IAA (0.01 mg/l) was found better than NAA and IBA. It was concluded that elite cultivars of bael can be micropropagated, without undergoing callus phase, using the BAP (0.5 mg/l) plus IAA (0.1 mg/l) for shoot multiplication and IAA (0.1 mg/l) for rooting, to produce true–to–type *in vitro* plants. The *in vitro* raised plantlets were acclimatized with 30% success.

Key Words: *Aegle marmelos*, Bael, *In vitro*, Medicinal fruit tree, Nodal segment, Micropropagation

Introduction

Bael [*Aegle marmelos* (L.) Corr.], a member of family Rutaceae, is an important indigenous fruit of India. Bael fruit is valued for its curative properties, which make this tree one of the most useful medicinal plants of India. The leaves of the tree are traditionally used as sacred offering to 'Lord Shiva' according to Hindu custom. It grows throughout the Indian peninsula as well as in Sri Lanka, Pakistan, Bangladesh, Burma, Thailand and most of the South-East Asian countries.

The ripe fruit is a laxative whereas unripe fruit is prescribed for diarrhea and dysentery. It is in great demand from native systems of medicine such as Ayurveda (Kirtikar and Basu, 1975; Wealth of India, 1985). Various chemicals such as alkaloids, coumarins and steroids have been isolated and identified from different parts of the tree such as leaves, wood, roots and barks (Roy, 1985). The bael fruit is one of the most nutritious fruit. According to Gopalan *et al*. (1971), 100 g of fresh fruit pulp contains 61.5 g water, 1.8 g protein, 0.39 g fat, 1.7 g minerals, 31.8 g carbohydrate, 55 mg carotene, 0.13 mg thiamine, 1.19 mg riboflavin, 1.1 mg niacin, and 8 mg vitamin C. No other fruit has such a high content of riboflavin.

Bael is usually propagated by seeds and can be also propagated by budding on 1 or 2 years old rootstock. Budding in the month of June-July favoured best results (Singh *et al*., 1976). The conventional methods of propagation are time-consuming and due to heterozygocity, the seed progeny is not true-to-type. Earlier studies were taken up to develop micropropagation protocol with the various explants such as axillary bud (Kumar and Seeni, 1998; Mishra *et al*., 2008), cotyledonary nodes (Arumugam and Rao, 1996; Nayak *et al*., 2007) and leaves from *in vitro* seedlings (Islam *et al*., 1993) and met with limited success. There is a strong need to produce true-to-type micropropagated plants from mature trees of *A. marmelos* to ensure the supply of quality planting materials (Islam *et al*., 1993). Therefore, the present study was taken up with objective to develop a micropropagation protocol using nodal explants from the field-grown mature tree.

Materials and Methods

Plant Material

The nodal explants were collected from a 30 year old tree grown in the nursery of NBPGR, New Delhi.

Culture Medium

The nutrient medium used for the study was MS (Murashige and Skoog, 1962), with 0.8% bacto-agar and 3% sucrose (Qualigens Fine Chemicals). The pH of the media was adjusted to 5.8 using 0.1 N NaOH or 0.1 N HCl. The media was poured into culture tubes (20 ml/ tube). The culture tubes (25 mm x 150 mm, Borosil, India) were closed with polypropylene caps and autoclaved for 15 minutes at 121°C at 1.06 kg cm^{-2} pressure.

**Author for Correspondence: Email: sandhya_gupta87@yahoo.com; sandhya@nbpgr.ernet.in*

Culture Initiation

The nodal explants were cut to the size of 2-3 cm, all leaves were removed and washed under running tap water for 30 minutes. The explants were then washed with Tween 20 for 5 minutes, followed by a washing with distilled water. Explants were surface sterilized with 0.1% mercuric chloride ($HgCl_2$) and washed thoroughly with sterile distilled water. Nodal segments were trimmed to the size of 1.5-2.0 cm, bearing single axillary bud, and inoculated on to the MS basal medium (with no growth hormone). The established cultures were further sub-cultured on the same medium to raise sufficient stock material for experiments.

Shoot Multiplication

For shoot multiplication experiments, shoots with single node were cultured on MS media (AEG0-AEG8) supplemented with different growth regulator alone, *i.e.*, BAP (0.1, 0.5, 1, 2 mg/l), kinetin (0.1, 0.5, 1 and 2 mg/l) and a combination of BAP (0.5 mg/l) and IAA (0.1 mg/l).

Rooting

Four-week-old cultures of bael were used for rooting experiments. *In vitro* shoots of about 0.5-1 cm size were transferred (1 shoot/ tube) on rooting medium (AEGR0-AEGR12) containing different concentrations of NAA (0.1, 0.5, 1, 2 mg/ l), IAA (0.1, 0.5, 1, 2 mg/ l), and IBA (0.1, 0.5, 1, 2 mg/ l). Medium without auxin was used as control.

Acclimatization

Rooted plantlets of 8-week-age were taken out of the culture tubes, washed thoroughly and planted in the plastic pots containing sterile soilrite. The pots were covered with polythene.

Culture Conditions

The cultures were kept at 25±1°C and 16 h photoperiod under 40 m mol/m–2s–1 light, which was provided by 40 Watt cool day light fluorescent lamps adjusted 30 cm high from the culture tubes.

Observation and Statistical Analysis

Observations were taken every week. Data were recorded on shoot parameters, namely, number of shoot, shoot length, number of nodes, presence or absence of callus. The observations on root parameters include root initiation, number of roots, root length and presence and absence of callus. A total of 12 tubes (single node per tube) were inoculated for each treatment (n=12). Data are presented as percentage and mean. Mean values of treatments were compared using the Least–Significant–Difference Test. Significance is stated at $P \leq 0.05$.

Results and Discussion

Culture Initiation

The contamination-free cultures showed axillary buds swelling as the first response. Bud break occurred in 50% explants in 4 weeks.

Shoot Multiplication

The cultures showed single shoot in almost all media including the basal (AEG0), used as control. The average shoot number increased from 1 to 2.9 in AEG4 followed by AEG3 (2.4) and AEG2 (2) (Table 1). The BAP was better than kn in terms of mean number of shoot per culture. The highest number of shoots with kn was 1.7 (AEG7) followed by 1.5 (AEG8) after 4 weeks of subculture. The cultures showed about 3-fold shoot multiplication on BAP (2 mg/l).

The mean shoot length was not significantly different but ranged from 0.7 cm in AEG3, AEG4 medium to 0.9 cm in AEG0, AEG1 and AEG2 (BAP) medium after 4 week and 0.8 cm AEG5, AEG6 medium to 1 cm in AEG8 medium after 4 week. The shoots started growing from the 1 week and gradually reached to their maximum by 4th week. Kn and BAP were more or less equally effective as seen by the mean of shoot length after 4 weeks. However, a significantly high number of shoots (4.5) with 2.5 cm shoot length were produced on AEG9 medium

Table 1. Effect of various cytokinins in MS media on shoot multiplication of *A. marmelos* after 4weeks of subculture

Medium code	Cytokinin conc. (mg/l)	4 week growth	
		Mean shoot no	Mean shoot length (cm)
AEG0	BAP 0	1.9^{bc}	0.9^{b}
AEG1	BAP 0.1	1.4^{c}	0.9^{b}
AEG2	BAP 0.5	2.0^{bc}	0.9^{b}
AEG3	BAP 1	2.4^{b}	0.7^{b}
AEG4	BAP 2	2.9^{b}	0.7^{b}
AEG5	Kn 0.1	1.4^{c}	0.8^{b}
AEG6	Kn 0.5	1.4^{c}	0.8^{b}
AEG7	Kn 1	1.7^{bc}	0.9^{b}
AEG8	Kn 2	1.5^{c}	1^{b}
AEG9	BAP 0.5+ IAA 0.1	4.5^{a}	2.5^{a}

The values are mean (n=12); Mean in a category followed by different letters are significantly different at P<0.05

with a combination of BAP (0.5 mg/l) and IAA (0.1 mg/l) (Table 1).

The shoot initiation was observed without the formation of callus in nodal segments, ensuring direct shoot formation in all the treatments. The results of the present findings show low rate of shoot multiplication on cytokinin medium when used alone. The low rate of shoot production was reported earlier in the callus induced shoot cultures of *A. marmelos* (Varghese *et al*., 1993). Repeated sub-culturing of nodes from shoot cultures may help to achieve continuous production of callus-free, healthy shoots through further subculture cycles. A synergistic influence of cytokinin and auxin was observed when a combination of them was tested in the present study. Combination of 0.5 mg/l BAP and 0.1 mg/l IAA induced the formation of maximum number (4.5) of stout and long (2.5 cm) shoots.

Rooting

Shoots formed grew uniformly and could be harvested in four weeks for rooting. The cultures showed no rooting in media used for shoot induction or multiplication, which shows the requirement of auxins to root *in vitro* shoots. Different concentration of NAA (0.5 mg/l), IAA (0.1 mg/l) and IBA (1 mg/l) were required to induce formation of one to three roots in 3 weeks at a frequency (number of shoots rooted) of 10%, 50% and 10%, respectively (Table 2). Roots were normal, *i.e*., straight, white, thick and healthy in all the cultures (Fig.1).

Table 2. Effect of various auxins in MS media on root parameters of *A. marmelos*

Medium code	Auxins conc. (mg/1)	Root frequency (%)	Root no per shoot	Mean root length per shoot (cm)	Root condition
AEGR0	–	0^{c}	0	0	–
AEGR1	NAA 0.1	0^{c}	–	–	–
AEGR2	NAA 0.5	10^{b}	1	0.5	normal
AEGR3	NAA 1	0^{c}	–	–	–
AEGR4	NAA 2	0^{c}	–	–	–
AEGR5	IAA 0.1	50^{a}	3	1.5	normal
AEGR6	IAA 0.5	0^{c}	–	–	–
AEGR7	IAA 1	0^{c}	–	–	–
AEGR8	IAA 2	0^{c}	–	–	–
AEGR9	IBA 0.1	0^{c}	–	–	–
AEGR10	IBA 0.5	0^{c}	–	–	–
AEGR11	IBA 1	10^{b}	1	0.9	normal
AEGR12	IBA 2	0^{c}	0	0	–

The values are mean (n=10); Mean in a category followed by different letters are significantly different at P<0.05

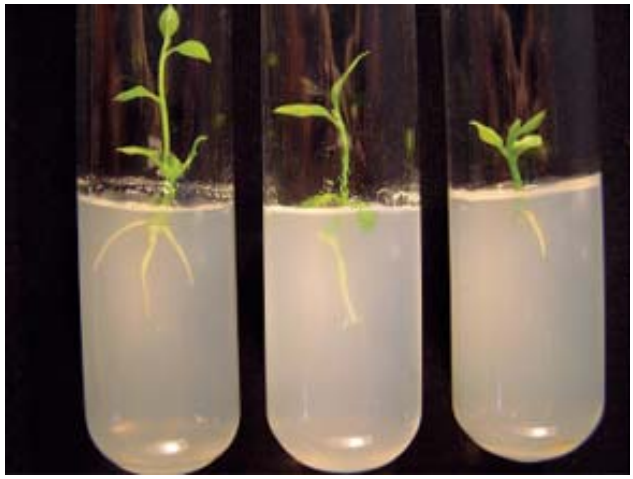

Fig. 1: Four weeks old cultures of *Aegle marmelos*: Plantlets are on medium MS + IAA, MS + IBA, MS + NAA (from left to right)

Acclimatization

Well rooted shoots were transferred into the pot. Only 30% plants could be successfully established in the soil in the present study. However, in the other study, a high rate of establishment was recorded among the rooted plants raised in the presence of IAA (70%) and IBA (90%) compared to NAA (40%) after hardening (Islam *et al*., 1993).

Successful micropropagation has been easily achieved with seed derived explants of *A. marmelos* (Islam *et al*., 1993) and other explants. In the earlier report, regarding stem segments of mature trees (Varghese *et al*., 1993), the formation of a limited number of shoots was preceded by extensive callus formation. The present study showed that the shoot multiplication and rooting without the callus formation is possible in bael and that ensures true-to-type micropropagated plants. Thus, it is concluded that elite cultivars of bael can be micropropagated using a combination of BAP and IAA for shoot multiplication and IAA for rooting.

Acknowledgements

The facilities provided by the former and present Directors of NBPGR, New Delhi, are gratefully acknowledged.

References

Arumugam S and MV Rao (1996) *In vitro* production of plantlet from cotyledonary node cultures of *Aegle marmelos* (L.) Corr. *Advances in Plant Sci*. **9:** 181-186.

Gopalan CBV, Ramasastri and Balasubramanian (1971) *Nutritive Value of Indian Food*. National Institute of Nutrition, ICMR, Hyderabad, India.

Islam R, M Hossian, OI Joarder and MR Karim (1993) Adventitious shoot formation on excised leaf explants of *in vitro* growth seedling of *Aegle marmelos* Corr. *J. Hort. Sci.* **68:** 495-498.

Kirtikar KR and BD Basu (1975) *Indian Medicinal Plants*, 2nd ed. Vol. 1., Bishen Singh Mahendra Pal Singh, Dehra Dun, Uttarakhand, India.

Kumar A and S Seeni (1998) Rapid clonal multiplication through *in vitro* axillary shoot proliferation of *Aegle marmelos* (l.) Corr., a medicinal tree. *Plant Cell Rep.* **17:** 422-426.

Mishra M, R Chandra and R Pati (2008) *In vitro* regeneration and genetic fidelity testing of *Aegle marmelos* (L.) Corr. plants. *Indian J. Hort.* **65:** 6-11.

Murashige T and F Skoog (1962) A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol Plantarum* **15:** 473-497.

Nayak P, P Behera and T Manikkannan (2007) High frequency plantlet regeneration from cotyledonary node cultures of *Aegle marmelos* (L.) Corr. *In Vitro Cell Dev. Biol-Plant* **43:** 231-236.

Roy SK (1985) Bael. In: TK Bose (ed.) *Fruits of India: Tropical and Subtropical.* B. Mitra Naya Prakash Calcutta, India, pp 498-504.

Singh UR, IC Panday and RS Prasad (1976) Propagation of bael (*Aegle marmelos* Corr.). *Punjab Hort. J.* **16:** 57-59.

Varghese SK, JA Inamdar, K Kalia, RB Subramaniam and M Nataraj (1993) Micropropagation of *Aegle marmelos* (L.). *Phytomorphology* **43:** 87-92.

Wealth of India (1985) *Raw Materials.* Vol IA (rev). CSIR, New Delhi, pp 85-91.