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In vitro Plant Regeneration from Leaf Primordia of Gum-bearing Tree *Aegle marmelos*

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Abstract: A protocol has been developed for regeneration of plantlets from leaf primordial of *Aegle marmelos*. *Aegle marmelos* is a middle sized slender aromatic armed tree native to India and distributed South-east Asia. The *in vitro* plant regeneration studies indicated that the following combinations of hormones with MS media containing Kn (1.5 mg L^{-1}) + 0.5 mg L^{-1} 2, 4-D with NAA (1.0 mg L^{-1}) is most suitable for organogenic calli formation and shoot induction. These calli later developed shoots when transferred to MS medium containing NAA (1.5 mg L^{-1}) with (0.5 mg L^{-1}) IBA for root initiation. Rooted plants survived well under acclimatization.

Key words: *Aegle marmelos*, organogenic callus, shoot regeneration, leaf primordia, napthalene acetic acid

INTRODUCTION

Aegle marmelos, commonly known as bael, is a spiny tree belonging to the family Rutaceae. The leaves, roots, bark, seeds and fruits of *A. marmelos* are edible. The medicinal properties of this plant have been described in the Ayurveda. It is indigenous to Indian subcontinent and mostly found in Tropical and subtropical region (Purohit and Vyas, 2005). It is medium sized tree having profuse dimorphic branched, alternate, trifoliate, deep green leaves; membranous leaflets, large, sweet scented, greenish white flowers; large, oblong or globose fruits (Purohit and Vyas, 2005). Almost all parts of the tree are used in preparing herbal medicine (Kala, 2006). The roots and bark are used in the treatment of diarrhea, fever (Mazumder *et al*., 2006) and to control pain in the abdomen (Kirtikar and Basu, 1935). The leaves are used in the treatment of diabetes (Narendhirakannan *et al*., 2005), snakebites (Purohit and Vyas, 2005). Fresh aqueous and alcoholic leaf extracts of *A. marmelos* are reported to have a cardio tonic effect, like digitalis and to decrease the requirement for circulatory stimulants (Nadkarni, 1976). Conventionally, grafting and layering are carried out to achieve this, but for large-scale propagation, they are not feasible methods. There is wide genetic variability in terms of quality, form and size of the fruit (Bhati *et al*., 1992). Also, seeds have short viability and are prone to insect and fungal attack (Purohit and Vyas, 2005). Although, vegetative propagation through root suckers is possible, the number of propagules produced through this technique is very limited. Alternatively, *in vitro* micropropagation techniques offer opportunities for multiplying disease-free planting material in a larger quantity within a short span of time. Nucleolus culture of *Aegle* has been

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attempted to develop plants with seedless fruits (Hossain *et al*., 1993) and *in vitro* axillary shoot proliferation is reported for rapid clonal propagation (Ajithkumar and Seeni, 1998; Arumugam and Rao, 1996). A tissue-to-plant regeneration system is thus an important pre-requisite for application of this technology for *Aegle marmelos*. Micro propagation using tissue culture is an alternative method used to multiply mainly vegetatively propagated crops. In this circumstance, the objective of this study was to develop an efficient method of plant regeneration system using leaf primodia explants of *A. marmelos*.

MATERIALS AND METHODS

Plant Material and Surface Sterilization

Aegle marmelos were freshly collected during (April 2010) from the southeast coast of Tamil Nadu, India and kept under shade net (50%) house environment. The specimen was certified by Botanical Survey of India (BSI) Coimbatore and by the Herbaria of CAS in Marine Biology, Annamalai University, Parangipettai, Tamil Nadu and India. The leaf primordia were used as explants material for initiating the organogenic calli. The explants were surface sterilized with 70% (v/v) ethyl alcohol for 1-5 min followed by 0.1% $HgCl_2$ for 3 min. The explants were then washed 4 times with sterile distilled water to remove traces of $HgCl_2$.

Culturing Tissues and Shoots Regeneration

The leaf primordial explants were cultured for plantlet regeneration in 250 mL conical flask and 25 mL Petri plates containing Murashige and Skoog (1962) medium (MS) supplemented with 30 g L^{-1} sucrose, 8 g L^{-1} agar and various concentration of cytokinin and auxin. Four growth regulator combinations, denoted as T1-T4 were tested [T1: 2,4-D (0.5 mg L^{-1}); T2: 2,4-D (0.5 mg L^{-1}) with NAA (0.5 mg L^{-1}); T3: Zeatin (1.5 mg L^{-1})+ 0.5 mg L^{-1} 2,4-D with NAA (1.0 mg L^{-1}); T4: Kn (1.5 mg L^{-1}) + 0.5 mg L^{-1} 2,4-D with NAA (1.0 mg L^{-1})]. The pH was adjusted to 5.8 prior to the addition of agar. Media were autoclaved at 15 lbs for 15 min. Culture was incubated at 25±2°C and relative humidity of 60±10%. An 8 h photoperiod (16 h dark) with light intensity of 20-30 μmol/m^2/sec was provided by cool day light fluorescent tubes. The experimental chemicals were purchased from (Hi-Media, Mumbai).

Each treatment consisted of 10 replicates. Cultures Once calli were initiated, globular-shaped yellowish green organogenic calli were selected and sub cultured in a fresh medium and incubated under dark conditions to promote further calli proliferation. Proliferated calli that were sub-cultured in fresh medium were transferred to light conditions and when the calli turned green in colour, The shoots (1-2 cm) were separated and cultured in various concentration of MS medium (above mentioned T1, T2, T3 and T4) to enhance further shoot growth.

Root Formation

Shoots that were 3-4 cm were separated and cultured in MS-based basal medium [MS medium, sucrose (3% w/v) and solidified with agar (0.8% w/v)] added with several growth regulator combinations to promote root initiation and growth. Four media compositions, denoted as R1 – R4, were tested [R1: IBA (0.5 mg L^{-1}); R2: IBA (0.5 mg L^{-1}) with BAP (0.2 mg L^{-1}) +IAA (0.5 mg L^{-1}); R3: IBA (1.0 mg L^{-1}) + NAA (2.0 mg L^{-1}) with 0.5 mg L^{-1}; R4: NAA (1.5 mg L^{-1}) with 0.5 mg L^{-1} IBA]. Each treatment consisted of 10 replicates.

Acclimatization of Plants

The rooted plantlets were transferred to a potting mixture of compost: sand (1:1) in 5 cm diameter pots. They were kept inside a poly chamber for 3 weeks and the poly cover was gradually removed. The plants were allowed to grow in a plant house with application of a liquid fertilizer once a week.

RESULTS AND DISCUSSION

Calli formation was observed in leaf primordia explants after 7-8 weeks. This response was highly observed in medium T3: Zeatin (1.5 mg L^{-1}) + 0.5 mg L^{-1} 2, 4-D with NAA (1.0 mg L^{-1}); T4: Kn (1.5 mg L^{-1}) + 0.5 mg L^{-1} 2, 4-D with NAA (1.0 mg L^{-1})]. They were compact yellowish green in color with an embryogenic nature. These calli proliferated rapidly when separated and sub cultured in a fresh T4 medium. Hence this system was selected for further studies. The results showed (Table 1) that, out of the several types of cytokinins tested, zeatin is suitable for callusing of *Aegle* at lower concentrations (2.0 mg L^{-1}) but at higher levels (4.0 mg L^{-1}), the response was very low. It was also shown that NAA and lower concentration of 2, 4-D was the suitable auxin here to initiate calli in the presence of zeatin (T3) as well as of Kn (T4). Creamish friable competent callus was achieved from nodal segments on MS medium augmented with 4.0 mg L^{-1}, 2, 4-D within two weeks of inoculation. The callus produced large number of shoots when cultured on MS medium fortified with 2.0 mg L^{-1} BAP + 0.1 mg L^{-1} NAA within ten days of culture. *In vitro* raised shoots were rooted on half strength MS medium enriched with 1.0 mg L^{-1} IBA within fifteen days of culture (Ranjoy *et al*., 2009). Similar results were reported in several plants including, *Ceropegia candelabrum* (Beena and Martin, 2003), *Ocimum sanctum* L. (Singh and Sehgal, 1999), *Gentiana* sp. (Fiuk and Rybczynski, 2008), *Azadirachta indica* (Quraishi *et al*., 2004). Since the growth regulator 2, 4-D is promoting abnormal cell divisions that can induce mutations 2, 4-D was in this study for callus initiation at lower concentration, although Islam *et al*. (1992) reported 2, 4-D at 1-5 mg L^{-1} induced calli from young leaves of *A.marmelos*. Organogenic calli formation on a MS-based medium fortified with Zeatin or 6-furfurylamine (KN) (2.0 mg L^{-1}) and NAA (0.5 mg L^{-1}) under dark conditions. Such type of plant regeneration was also reported in several medicinal plant species including, Carica papaya (Islam *et al*., 2000), *Phellodendron amurense* Rupr (Azad *et al*., 2005), *Amorphophallus albus* (Hu and Li, 2008), *Gentiana* sp. (Fiuk and Rybczynski, 2008). Separated shoots continued to grow in liquid medium, free of hormones and produced roots at 30% efficiency in the presence of NAA (1.0 mg L^{-1}). This protocol is suitable to produce number of plants from cotyledon, hypocotyls and immature leaves of *A. marmelos*

Table 1: Effect of different concentration of auxin and cytokinin for callus induction and plantlet regeneration from leaf primordia explants of *Aegle marmelos*

Hormonal concentrations (mg L^{-1})	No. of explants	Sucrose concentration (g L^{-1})	Agar concentration (g L^{-1})	Percentage of callus+Shoot induction	Percentage of root induction from shooting
0.5 2,4-D (T1)	10	30	8	40.0	-
0.5 2,4-D+0.5 NAA (T2)	10	30	8	52.0	-
1.5 Zn+1.0 NAA+0.5 2,4-D (T3)	10	30	8	75.0	-
1.5 Kn+1.0 NAA+0.5 2,4-D (T4)	10	30	8	87.5	-
0.5 IBA (R1)	-	30	8	-	30.0
0.5 IBA+0.2 BAP+0.5 IAA (R2)	-	30	8	-	55.0
2.0 IBA+2.0 NAA+0.5 IAA (R3)	-	30	8	-	72.0
1.5 NAA+0.5 IBA (R4)	-	30	8	-	80.0

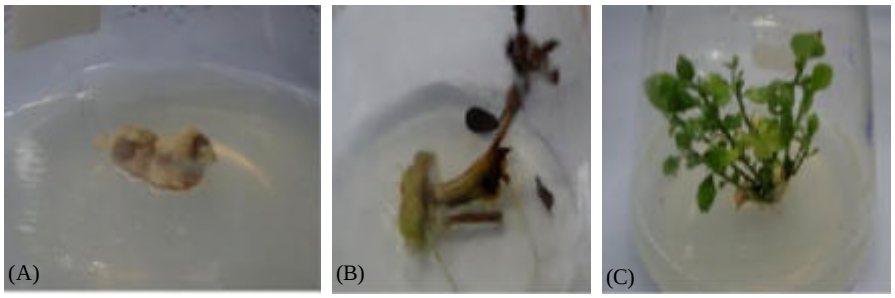

Fig. 1: *In vitro* plant regeneration of leaf primordial explants of *Aegle marmelos* (A) Callus induction, (B) Root and shoot induction on MS medium containing K. (1.5 mg L^{-1})+0.5 mg L^{-1} 2,4-D with NAA (1.0 mg L^{-1}) and (C) Plnatlet regeneration

(Prematilak *et al*., 2006). When the calli that were formed in the T3 and T4 media were allowed to be in the same medium without sub-culture for 6-8 weeks, they turned green and when transferred to light, produced shoot buds. Enlargement of shoot base occurred prior to root initiation. Root initiation efficiency was around and was observed in the presence of NAA (1.5 mg L^{-1}) with 0.5 mg L^{-1} IBA after 3-4 weeks of culture. Many other workers reported similar results for root induction in various types of plants, namely Carica papaya (Islam *et al*., 2000), *Gymnema sylvestre* (Komalavalli and Rao, 2000), *Tylophora indica* (Faisal *et al*., 2007). Plants were successfully acclimatized under the given conditions and are now growing well in the plant house. It is a well known fact that the plants regenerated via a callus phase may carry features that are different to the mother plant (somaclonal variations). After another 3-5 weeks the plants were suitable to be transplanted in soil (Fig. 1A-C). The potted plantlets were covered by transparent polythene sheet to maintain high humidity and within 20 days new leaves were emerged from the plantlets that resumed new growth. After 40-45 days, the plants were transplanted in the field condition, where 80% plants were survived and grown satisfactory.

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