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Tissue culture of endangered Bael tree (*Aegle marmelos*): A Review

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ABSTRACT

Tissue culture is a proven means of producing millions of identical plants under a controlled and aseptic condition, independent of seasonal constraints. It not only provides economy of time and space but also gives greater output and allows further augmentation of elite disease free propagules.

Most of these crops have medicinal value and are suitable for growing under marginal situations. The commercial production of these crops is restricted due to the shortage of desirable planting material. Tissue culture can play an important role in rapidly increasing new cultivars of these fruit crops. This review paper outlines the work done on *Aegle marmelos (Linn)* family *Rutaceae*.

Aegle marmelos (Linn) family *Rutaceae* is highly reputed ayurvedic medicinal tree commonly known as the bale fruit tree, is medium sized tress growing throughout the deciduas forest of India of altitude 1200 meter. The collection and evaluation of over 1200 bael (*Aegle marmelos* Correa) trees in West Bengal was initiated in 1994. Six types have been identified as superior clones and are being conserved at the Faculty of Horticulture Research Station located at 23.5 North latitude and 89 East longitudes. Wide variability in yield (20–437 fruits/tree), fruit weight (130–1825 g), fruit shape, rind thickness, pulp colour, number of seeds, total soluble solids (23–42 °Brix), fruit acidity (0.23–0.44%) and ascorbic acid (22–30 mg/100 g) content of fruit were observed among the genotypes. The clones T1, T5, T8, T10, T15 and T16 were selected for cultivation. The method standardised could be used for large scale planting material production and conservation of this important endangered medicinal plant. The hardened plants are being evaluated under field condition. The micropropagated plants were tested for its genetic fidelity using 12 RAPD, 2 micro-satellite and 2 mini-satellite primers. Profile obtained by all the three single primer amplification reaction (SPAR) technique obtained from mother tree and micropropagated plants were found identical which suggests the genetic uniformity of micropropagated plants with that of mother tree.

Keywords: Bael, Clonal multiplication, Micropropagation, Organogenesis, Rutaceae, Nodal explants

INTRODUCTION

Tissue Culture

Plant tissue culture is the science and art of growing plant cells, tissue, or organs isolated from the mother plant on artificial media. Tissue culture industry in the world has potential to grab a sizable chunk of the growing international market for production of secondary metabolite of medicinal important, flowers and other plants and earn valuable foreign exchange. Netherlands stand first in the world market of tissue culture plant business. A. V. Thomas and Co (AVT) is first to commercialize plant tissue culture in Kerala. They created a superior variety of cardamom through tissue culture and exported it. The most viable technology in plant tissue culture today is the clonal/micropropagation of elites plants. Several industries and nurseries have standardized protocols for the multiplication of ornamentals like orchid's genera, carnation, roses, chrysanthemum and plantation crop like banana and cardamom. There are more than 20 private industries engaged in commercial production of horticulture plants, forests, fruit crops such as apples, peaches, pears, strawberries, plums and forest trees, radiate pine. The major benefits of this method include the following. (1)Rapid multiplication of superior clones and maintenance of uniformity, (2) multiplication of disease free plants and (3)multiplication of sexually derived sterile hybrids.

Tissue culture is a proven means of producing millions of identical plants under a controlled and aseptic condition, independent of seasonal constraints. It not only provides economy of time and space but also gives greater output and allows further augmentation of elite disease free propagules. Most of these crops have medicinal value and are suitable for growing under marginal situations. The commercial production of these crops is restricted due to the shortage of desirable planting material. Tissue culture can play an important role in rapidly increasing new cultivars of these fruit crops. This review paper outlines the work done on *Aegle marmelos* (Linn) family *Rutaceae.*

Bael tree

***Habitat*:** Bael is indigenous to dry forests on hills and plains of central and southern India, southern Nepal, Sri Lanka, Myanmar, Pakistan, Bangladesh, Nepal, Vietnam, Laos, Cambodia and Thailand. It is cultivated throughout India, as well as in Sri Lanka, northern Malay Peninsula, Java in the Philippines and Fiji Islands.[1,2]

Alternative names*:* It is also popularly known as Vilva maram, Bilwa, Bel, Kuvalam, Koovalam (in Malayalam), Madtoum, or Beli fruit, Bengal quince, stone apple, Maredu (in Telugu),and wood apple. The tree is the only species in the genus[1-2]. In Javanese language it is called **Maja** which lent its name to Majapahit Empire, since its capital was built on former betel forest[1].

Yield: The average yield is 300-400 fruits per tree[3]. The quality of fruits is greatly associated with the weight and size of the seed-sacs. The larger and heavier the seed sacs, the greater is the amount of mucilage and poorer the quality.

PHARMACOGNOSY OF PLANT

Macroscopic characters [2, 4, 5]

A small to medium-sized aromatic tree, deciduous; stem and branches, light brown to green; strong axillary spines present on the branches; the average height of tree, 8.5 metres.

Leaves are alternate, pale green, trifoliate; terminal leaflet, 5.7 cm long, 2.8 cm broad, having a long petiole; the two lateral leaflets, almost sessile, 4.1 cm long, 2.2 cm wide, ovate to lanceolate having reticulate pinnate venation; petiole, 3.2 cm long.

Leaflets are ovate or ovate-lanceolate, margins crenate, apex acuminate, glabrous and densely minutely glandular-punctuate on both surfaces; lateral leaflets to 7 cm long and 4.2 cm wide, petiolules 0-3mm long.

Flowers greenish white, sweetly scented, bisexual, actinomorpbic, ebracteate. hypogynous, stalked; stalk, 8 mm long; diameter of a fully open flower, 1.8 cal; flowers, borne in lateral panicles of about 10 flowers, arising from the leaf axil; calyx, gamosepalous, five-lobed, pubescent, light green, very small in comparison with petals; corolla polypetalous, with 5 petals, imbricate, leathery, pale yellow from above and green from beneath, length 4 mm; androecium, polyandrous, numerous, basifixed, 4 mm long, dehiscing longitudinally; gynoecium, light green, 7 mm long, having capitate stigma and terminal style.

Stamens numerous; another elongate, apiculate; filaments free or fascicled, inserted round an inconspicuous disk. Ovary ovoid, cells 10-20; style terminal, short, deciduous; stigma capitate; ovules numerous, 2-seriate.

Fruits yellowish green, with small dots on the outer surface, oblong to globose, 5.3 cm to 7 2 cm in diameter; weight, 77.2 g; volume, 73.7 ml; pulp, yellow and mucilaginous, the pulp of dried fruits retains its yellow, and also remains intact; rind woody, 4 to 5 mm thick.

Seeds numerous, embedded in the pulp, oblong, compressed, white, having cotton-like hairs on their outer surface. seeds numerous, oblong, compressed, embedded in sacs covered with thick, orange coloured sweet pulp root bark is 3 to 5 cm thick covered, with creamy yellowish surface.

It has a firm leathery texture, a sweet taste and fracture is fibrous. Stream bark is extremely gray and internally cream in colour. The outer surface is rough warty due to a number of lenticels, ridges and furrows. It is 4-8 mm thick, film in texture and occurs as flat or channeled pieces[6]. The fracture is tough and gritty in outer region and fibrous in the inner.[5]The taste is sweet and there is no characteristic odour[5].

Chemical constituents [5, 6, 7, 8]

Various chemical constituents were found in bael like alkaloids, coumarins, steroids, polysaccharides, tannins, carotenoids etc.

***Alkaloids*:** Agelin, aegelenine, marmeline, dictamine, fragrine, O-methylhalfordinine, O-isopentanylhalford iniol, N-4-methoxy styryl cinnamide.

Coumarins: Marmelosin, marmesin, imperatorin, marmin, alloimperatorin, methylether, xanthotoxol, scoparone, scopoletin, umbelliferone, psoralen and marmelide.

***Polysaccharides*:** Galactose, arabinose, uronic acid and L-rhamnose was obtained on hydrolysis.

***Tannin**:* Tannin was also present in leaves and fruit as skimmianine.

Carotenoids were also reported, which import pale colour to fruit.

Seed oil: composed of palmitic, stearic, oleic, linoleic and linolenic acid.

The fruit pulp contains 60.7 per cent moisture. The pulp contains 0.46 per cent acidity, 8.36 per cent total sugars, 6.21 per cent reducing sugars, 2.04 per cent non-reducing sugars and 0.21 per cent tannins. The pectin content is 2.52 per cent, which is quite high. The fruit pulp, however, is not a good source of vitamin C which is only 920 mg per 100 g of pulp[4].

This fruit is a very good source of protein which is 5.12 per cent of the edible portion. The total mineral content of the edible portion, as represented by ash, is 2.663 per cent. The percentage content of some of the minerals, viz. phosphorus, potassium, calcium, magnesium and iron is 0.137, 0.746, 0.188, 0.127 and 0.007 respectively[4].

Medicinal properties

The roots are useful for treating diarrhoea, dysentery, and dyspepsia[3, 9]. The leaf is used for opthalmia, diabetes, and asthmatic complaints. Unripe fruit is useful for treating diarrhoea, dysentery and stomachalgia. The aqueous extracts of the stem and root bark are used to treat malaria, fever, jaundice, and skin diseases such as ulcers, urticaria, and eczema[9, 10.] In pharmacological trials, both the fruit and root showed antiamoebic and hypoglycaemic activities[9, 11, 12]. Aqueous leaf extract and methanolic extract of the root bark of *A. marmelos* showed preventive effects on myocardial diseases[3, 10, 11]. The leaves are used to cure sinusitis, dyspepsia and anorexia[6]. This fruit was used to cure tuberculosis, loss of appetite, emaciation etc. There are several such pharmacopoeias in Siddha medicine. *Aegle marmelos (Linn) family Rutaceae* was highly reputed ayurvedic medicinal tree[1]. Various phytochemical and biological evaluations have been reported as anti-diabetic[13, 14], antioxidant, antithyroid[14]. 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 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. Antidiabetic property, antidiarrhoeal activity, antiulcer activity of seeds, antifungal activity of leaves and antitumour and antimutagenic activity of this plant are clinically evaluated, [1, 15].

TECHNIQUES OF TISSUE CULTURE ON BAEL TREE

Tissue culture can play an important role in rapidly increasing new cultivars of these fruit crops. These includes following techniques

Micropropagation

Micropropagation by enhanced axillary shoot proliferation from mature single node[16] Raghu A.V. et al had reported 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[16].

Micropropagation of Bael via The nodal explants[17]

Gupta Sandhya et al had reported a protocol for micropropagation of bael [*Aegle marmelos* (L.)Corr.]. 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.

In-vitro culture

in-vitro culture via clonal multiplication.[18] Ajit kumar D. and Seeni S. had reported Rapid clonal multiplication of *Aegle marmelos* (L.) Corr. (*Rutaceae*), a medicinal tree, was achieved by enhanced axillary bud proliferation in young single-node segments of a 25-year-old tree cultured in Murashige and Skoog (MS) nutrient medium. Bud break was dependent on cytokinin supply, but the synergistic combination of 2.5 mg l^{-1} 6-benzylaminopurine (BAP) and 1.0 mg l^{-1} indole-3-acetic acid (IAA) induced the formation of 12.1 shoots of up to 5.2 cm length in 48% of the explants after 7 weeks of culture. Explants of in-vitro-grown shoots – node, whole leaf, shoot tip and internode were subcultured in the presence of 0.05–2.5 mg l^{-1} BAP to produce 11.3, 18.4, 5.3 and 3.2 shoots and shoot buds at a 100%, 70%, 95% and 40% rate respectively, in 7 weeks. Different shoot nodes and leaves were equally regenerative and adventitious organogenesis in the latter was confined to cut petiolar ends. Nodal explants responded most favourably at low BAP (0.05–0.1 mg l^{-1}) and produced uniform (3.8–5.3 cm) shoots facilitating their simultaneous harvest for rooting. Repeated subculturing through five cycles of nodes and leaves of shoot cultures enabled continuous production of healthy callus-free shoots without any sign of decline. Shoot cuttings (3.0–5.2 cm) were best rooted in half-strength MS medium with 0.5 mg l^{-1} IAA (70%) or 10.0 mg l^{-1} indole-3-butyric acid (90%). Eighty-eight percent of the rooted plants were established in polybags after hardening.

In Vitro plant Regeneration via Organogenic Callus Culture[19]

Prematilake et al had reported a tissue to plant regeneration system for *Aegle marmelos* (L.) Corr. using cotyledon tissues. This system was applicable to immature leaf and root tissues at lower efficiency. This procedure involved organogenic calli formation on a MS–based medium fortified with Zeatin or 6-furfurylamine (KN) (2.0mg/l) and NAA (0.5mg/l) under dark conditions. These calli later developed shoots when transferred to hormone-free medium and under illumination. Separated shoots continued to grow in liquid medium, free of hormones and produced roots at 30% efficiency in the presence of NAA (1.0mg/l). Rooted plants survived well under acclimatization. This protocol is suitable to produce number of plants from cotyledon, hypocotyls and immature leaves of *A. marmelos*.

DISCUSSION AND CONCLUSION

In-vitro multiplication protocols were developed for a number of threatened medicinal and aromatic species, for example Valeriana wallichi, Picrorhiza kurroa, Dioscorea deltoidea, Centella asiatica. Plant tissue culture played important role in the conservation of medicinal plants in the rapid multiplication and reintroduction to nature of endangered species. In the assessment and monitoring of biodiversity, as a source of new tools for conservation and in the search for new gene product of therapeutic use, tissue culture is a proven means of producing millions of identical plants under a controlled and aseptic condition, independent of seasonal constraints. It not only provides economy of time and space but also gives greater output and allows further augmentation of elite disease free propagules.

Most of these crops have medicinal value and are suitable for growing under marginal situations. The commercial production of these crops is restricted due to the shortage of desirable planting material. Tissue culture can play an important role in rapidly increasing new cultivars of these fruit crops.

FUTURE PROSPECTS

Plant tissue culture played important role in the conservation of medicinal plants in the rapid multiplication and reintroduction to nature of endangered species in the assessment and monitoring of biodiversity, as a source of new tools for conservation and in the search for new gene product of therapeutic use. Species of medicinal and aromatic plants at risk need to be multiplied with minimum loss of time and reintroduced for establishment in their natural habits. *In vitro* protocol for multiplication of endangered species could be very useful for those texa whose propogation through conventional means was difficult.

Researchers aim to obtain increased production of secondary metabolites, increased production, higher nutritional value and greater plant resistance to adverse weather, pathogenic agent and pests.

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