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# A study on effects of antioxidants in micropropagation of Bael (*Aegle marmelos* L.)

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**Abstract**
Bael (*Aegle marmelos* L.) is a rare endangered medicinal plant, requires rapid mass multiplication and germplasm conservation as it has slow growth and expensive cultivation in nature. Multiplication through *in vitro* technique has problems of culture media browning which delimits its pace of micropropagation. Present investigation was carried out to study effect of different antioxidants on shoot proliferation and callus induction. Antioxidants *viz:* activated charcoal, ascorbic acid, citric acid and polyvinylpyrollidone were used to control the accumulation of phenolic compounds in the MS medium and enhance the rate of micropropagation were worked out at most responsive level of plant growth regulators. Supplementation of activated charcoal at concentration of 200 mg/l in the MS medium appeared most optimum level for maximum shoot bud proliferation in nodal segment explants followed by 150 mg/l level of activated charcoal. Maximum callus proliferation in leaf explant was observed using polyvinylpyrrolidone at the rate of 6.0 mg/l followed by at 8.0 mg/l polyvinylpyrrolidone with low intensity of browning in the medium. Further, these optimum levels of antioxidants may be utilized in culture media to enhance growth for mass multiplication of Bael.

**Keywords:** Activated charcoal, *Aegle marmelos*, antioxidant, bael, micropropagation, polyvinylpyrrolidone

## Introduction
Bael [*Aegle marmelos* (L.) Corr.] is a cross pollinated tree having chromosome number 2n=18 [8]. It is originated in India [19] and commonly known as Bengal quince, Bilva, Indian quince, Golden apple, Holy fruit, Bel, Belwa, Sriphal, Stone apple and Maredo [4]. Bael is a subtropical plant and grows up to an altitude of 1,200 m altitude from sea level. It grows well in the dry forests on hilly and plain areas. It is now growing well throughout South East Asia, especially in India, Bangladesh, Pakistan, Sri Lanka, Burma and Thailand either wild or under cultivation [13 & 18]. Bael is a slow-growing, medium sized tree, up to 12-15 m tall. It takes 10-12 months from flowering to fruit ripening. Ripe bael fruit is sweet, aromatic and nutritive, whereas fresh fruit is astringent and has laxative properties. The testa is woody and the seeds are oblong, compressed, the embryo has a thick fleshy cotyledon [3]. Bael is one of the most important tree species used in various indigenous systems of medicine in India [6]. It is used in all Tridosa-vata (air), pitta (phlegm) and kapha (cough). Out of more than 66 ethno-botanical uses of bael, 48 are exclusively for medicinal purposes. Bael possesses antiviral, anti-helminthic anti-inflammatory, anti-bilious, anti-parasitical, antipyretic, anti-scorbutic, aphrodisiac, aromatic, astringent, digestive, febrifuge, haemostatic, anti-diarrhoeal, laxative and nutritive properties. Bael fruit powder exhibits anti-cancerous and anti-proliferative activities. Bael fruits are edible, contain high protein and are used in making tasty aromatic cold drinks and jam. Its fresh juice is bitter and pungent.

In nature bael is propagated by seeds and root suckers though many problems are associated with these methods. Progeny raised from this tree seeds are usually not uniform besides remaining viable for short duration and prone for insect attacks. Vegetative propagation by root suckers and by conventional methods of budding and soft wood grafting are very slow, seasonal, difficult and labour intensive. Due to all these drawbacks, micropropagation techniques have been successfully employed for rapid multiplication of the superior bael plants. One of the major problems associated with plant tissue culture is browning of the culture medium and the explants, which invariably leads to death of plants. In order to control browning of medium and explants *in vitro*, many workers have tried to incorporating non specific absorbents like activated charcoal, ascorbic acid and polyvinylpyr-rolidone (PVP) in to the culture medium but only met to limited success [1]. The majority of woody plants and some herbaceous species under *in vitro* culture show browning of medium. If this browning

was so extreme, the explants turn its colour brown to black and become necrotic and finally lead to die [5]. The browning of the medium is due to releasing phenol by the explants which get oxidized, and this oxidation product could be phytotoxic. Thus the present investigation has been undertaken to establish to study the effects of antioxidant on shoot proliferation and callus induction.

## Materials and Methods

The present investigation was carried out at Tissue Culture Laboratory of Department of Plant Breeding and Genetics, S.K.N. Agriculture University, Jobner, Rajasthan, India during 2018-19. Murashige and Skoog Medium (1962)[9] used as basal medium for shoot proliferation and callus induction. Nodal segment with 2-3 nodes and leaves were used as explants which were collected from healthy tree planted in department of horticulture. Mercuric chloride (0.1 per cent) was used for surface sterilization. BAP (6-Benzyl-aminopurine) was used at 2 mg/l concentration to see the effect of antioxidants on *in vitro* degree of browning and culture establishment for shoot proliferation taking nodal segment explant in MS medium. To observe effects of antioxidants on callus induction the most responsive level of 2,4-D (2 mg/l) were incorporated in MS medium using leaf explant. Antioxidants *viz:* activated charcoal, ascorbic acid, citric acid and polyvinylpyrollidone were used to control the accumulation of phenolic compounds in the culture medium and enhance the rate of micropropagation were worked out at most responsive level of plant growth regulators.

1. Activated charcoal (50, 100, 150, 200, 250 and 300 mg/l).
2. Ascorbic acid (50,100,150, 200, 250 and 300 mg/l).
3. Citric acid (10, 20, 30, 40, 50 and 60 mg/l).
4. Polyvinylpyrollidone (2, 4, 6, 8 and 10 mg/l).

All the cultures were maintained at temperature of 25 ± $2^0$C under fluorescent light in a 14:10 hour's photoperiod. MS basal medium without supplementation of any antioxidant was used as a control in all the experiments. The observations was recorded at 45 days of culture on days taken in shoot induction, number of shoots per explant, browning intensity, days taken for callus initiation, callus growth, callus color, callus texture and percent of morphogenetic response. The experiment was conducted in completely randomized design (CRD) comprising ten replications. The data were analyzed for mean and standard error accordingly as described by Snedecor and Cochran (1972) [14]. Test of significance was done according to Duncan's Multiple Range Test (DMRT) for different traits [2].

## Results and Discussions

Effect of antioxidants on degree of browning and culture establishment of nodal segment explants and leaf explants supplemented with BAP and 2,4-D @2.0 mg/l respectively in MS medium are presented in table 1 and table 2. When different antioxidants incorporated singly in MS medium supplemented with responsive level of plant growth regulators for shoot proliferation and callus induction elicited different responses because it controls the accumulation of inhibitory substances (phenolic compounds) in the growth medium. Among the studied antioxidants, maximum number of shoot bud (3.9) with low browning intensity was observed using activated charcoal @ 200 mg/l with nodal segment explant in the MS medium (Table 1 & Fig. 1). 50 mg/l activated charcoal was insufficient to control the accumulation of phenolic compound as medium showed intense browning. Maximum callus response was also observed at 200 mg/l activated charcoal followed by at 250 mg/l activated charcoal with low intensity of browning in the medium using leaf explant (Table 2). Ascorbic acid concentration of 150 mg/l found best for shoots proliferation (3.7) with lowest browning in nodal segment explants while maximum callus induction frequency (70 per cent) was observed at 200 mg/l ascorbic acid with lowest intensity of browning using leaf explant in the medium. Among different concentrations of citric acid, highest bud shoot induction (3.8) and callus induction (70 per cent) with low browning was observed at 40 mg/l concentration in both nodal segment and leaf explants. Addition of polyvinylpyrrolidone (PVP) in culture vessels both higher and lower level showed medium browning in culture vessels. PVP at 6.0 mg/l was found best among the all antioxidants with respect to callus proliferation response (80 per cent) coupled with profuse callus growth in the medium (Fig: 2).

The results of present investigation to use of activated charcoal for efficient reduction of browning of medium and explant were in close agreement with Madhusudhanan and Rahiman (2000) [7] in piper species. Activated charcoal can alleviate toxic metabolites, phenolic exudation, their accumulation and promotes regeneration by absorbing inhibitory compounds. Activated charcoal could promote growth by releasing substances that are naturally present in the charcoal. It also immediately adsorbed PGRs and vitamins from medium and gradually releases them again in the medium [15]. Parmar and Kant (2012) [10] and Sharma *et al.*, (2012) [12] also reported positive response of activated charcoal (0.2 per cent) for shoot bud break in nodal segment of *Commiphora wightii*. PVP was also found effective in browning control when supplemented in medium [11] & [16]. The effectiveness of different antioxidants in control of browning was varying among plants and species. This could be due to the specificity of these chemicals to certain plant and species [17].

## Conclusion

Browning of culture media during *in vitro* mass multiplication of Bael (*Aegle marmelos* L.) is one of the major problems hinders the pace of propagation. The present investigation was given an efficient protocol to minimize the problem of media browning. The addition of activated charcoal (200 mg/l) in the MS medium with 2.0 mg/l BAP found best for maximum shoot bud proliferation in nodal segment explants, while addition of polyvinylpyrrolidone (6.0 mg/l) with responsive level of plant growth regulators (2.0 mg/l 2,4-D) show more callus induction in leaf explant. Further, these levels of activated charcoal and polyvinylpyrrolidone may be utilized in mass propagation of Bael.

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**Table 1:** Effect of antioxidants on *in vitro* degree of browning and culture establishment of nodal segment explants in MS medium supplemented with BAP @2.0 mg/l.

| Antioxidant | Concentration (mg) | Days taken in shoot induction | Number of *de novo* shoot bud induction | Browning intensity |
|---|---|---|---|---|
| Activated Charcoal | 50 | 15.5 | 3.6 ± 0.163 a | +++ |
| | 100 | 14.2 | 3.7 ± 0.153 a | ++ |
| | 150 | 14.1 | 3.8 ± 0.133 a | ++ |
| | 200 | 13.5 | 3.9 ± 0.180 a | + |
| | 250 | 13.6 | 3.6 ± 0.221 a | + |
| | 300 | 13.9 | 3.7 ± 0.153 a | ++ |
| Ascorbic acid | 50 | 15.4 | 3.7 ± 0.153 a | ++ |
| | 100 | 14.2 | 3.6 ± 0.163 a | ++ |
| | 150 | 13.6 | 3.7 ± 0.213 a | + |
| | 200 | 13.9 | 3.6 ± 0.163 a | ++ |
| | 250 | 14.3 | 3.7 ± 0.153 a | ++ |
| | 300 | 14.5 | 3.6 ± 0.153 a | ++ |
| Citric acid | 10 | 15.6 | 3.6 ± 0.221 a | +++ |
| | 20 | 15.1 | 3.6 ± 0.153 a | +++ |
| | 30 | 14.8 | 3.7± 0.153 a | ++ |
| | 40 | 13.9 | 3.8 ± 0.133 a | + |
| | 50 | 14.8 | 3.6 ± 0.163 a | ++ |
| | 60 | 14.6 | 3.6 ± 0.163 a | ++ |
| Polyvinylpyrrolidone | 2 | 14.7 | 3.6 ± 0.163 a | ++ |
| | 4 | 14.1 | 3.6 ± 0.221 a | ++ |
| | 6 | 15.4 | 3.5 ± 0.167 b | ++ |
| | 8 | 13.9 | 3.4 ± 0.163 b | +++ |
| | 10 | 14.8 | 3.4 ± 0.163 b | +++ |

+++= Intense browning, ++= Medium browning, += Low browning
Values followed by same letters in each column are not significantly different ($p<0.05$) using DMRT

**Table 2:** Effect of antioxidants on *in vitro* degree of browning and culture establishment of leaf explants in MS medium supplemented with 2,4-D @2.0 mg/l.

| Antioxidants | Concentration (mg/l) | Days taken for callus initiation | Visual growth | Colour | Texture | Response (%) | Browning intensity |
|---|---|---|---|---|---|---|---|
| Activated Charcoal | 50 | 16.5 | ++ | Light brown | Loose | 50 | +++ |
| | 100 | 17.2 | + | Brownish yellow | Friable | 50 | ++ |
| | 150 | 14.1 | ++ | Brownish green | Friable | 50 | ++ |
| | 200 | 17.5 | ++ | Light green | Semi compact | 70 | + |
| | 250 | 16.6 | ++ | Light green | Semi compact | 60 | ++ |
| | 300 | 15.9 | ++ | Light green | Friable | 50 | ++ |
| Ascorbic acid | 50 | 16.4 | + | Brownish green | Friable | 50 | +++ |
| | 100 | 16.2 | + | Creamish | Semi compact | 50 | +++ |
| | 150 | 17.6 | + | Brownish green | Loose | 60 | ++ |
| | 200 | 16.9 | ++ | Creamish | Semi compact | 70 | + |
| | 250 | 17.3 | ++ | Brownish yellow | Semi compact | 60 | ++ |
| | 300 | 16.5 | + | Brownish green | Semi compact | 60 | +++ |
| Citric acid | 10 | 16.6 | + | Light green | Semi compact | 60 | +++ |
| | 20 | 17.1 | ++ | Brownish yellow | Loose | 70 | +++ |
| | 30 | 16.8 | ++ | Creamish | Semi compact | 60 | ++ |
| | 40 | 16.9 | ++ | Light green | Semi compact | 70 | + |
| | 50 | 15.8 | ++ | Brownish green | Loose | 50 | +++ |
| | 60 | 15.6 | + | Light green | Semi compact | 60 | +++ |
| Polyvinylpyrrolidone | 2 | 16.7 | ++ | Creamish | Loose | 60 | ++ |
| | 4 | 17.1 | ++ | Brownish yellow | Semi compact | 70 | ++ |
| | 6 | 15.4 | +++ | Brownish green | Compact | 80 | + |
| | 8 | 16.9 | ++ | Light green | Semi compact | 70 | + |
| | 10 | 17.1 | + | Creamish | Semi compact | 60 | ++ |

+++= Intense browning, ++= Medium browning, += Low browning
+++ = Profuse callus, ++=Medium callus, +=Slight callus, (-) = No response

**Fig 1:** Effect of activated charcoal (200 mg/l) on shoot bud induction in nodal segment explant of bael, supplemented with BAP @2.0 mg/l.

**Fig 2:** Effect of polyvinylpyrrolidone (6.0 mg/l) on callus induction in leaf explant on MS medium supplemented with 2, 4-D @2.0 mg/l.

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