**Micropropagation of *Aegle marmelos* and *Agrobacterium rhizogenes* mediated root induction in nodal explant.**

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**Abstract**

*Aegle marmelos* is a medicinal tress which has less seed viability and poor seed germination. Hence an alternative to propagate such woody tress is through micropropagation. Nodal explants were used for callus induction and direct shoot regeneration. Callus induction was observed in 0.2 and 0.5 mg/l 2, 4 –D after five weeks of inoculation. The callus was subcultured for 3 weeks in MS with 2 mg/L Benzyl Adenine +0.5 mg /l 2,4D. MS medium fortified with 0.5 mg/l IAA and 1-3 mg/l kinetin for adventitious shoot buds. It was observed that in higher concentration of kinetin (3mg/l) alone, the callus turned green with small adventitious shoot buds after 6 weeks. Direct regeneration of shoot was observed in 2 mg /l and 3 mg /l kinetin. However, 3 mg/l kinetin induced better regeneration of the shoot. Direct regeneration was observed after 6 weeks of incubation. Tumor induction was observed after 6 weeks. Rooting was not observed till 60 days, however young shoots were produced from the axillary buds of the nodes after 2 weeks of incubation. The nodal explant of 4 week old plant was co-cultured with *Agrobacterium rhizogenes* after an incubation of 5, 10,15,20 and 25 minutes and transferred to MS agar. After 3 days of incubation shoot induction was observed in 5 minutes, 10 minutes, 15 minutes incubated explants. After 3 weeks of incubation shoot growth increased and tumor induction was observed in 5 minutes and 10 minutes incubated explants.

**Keywords:** *Aegle Marmelos***,** Nodal explants, Direct regeneration, *Agrobacterium rhizogenes*, Co- inoculation

**Introduction:**

*Aegle marmelos* (L.) Corr. (Rutaceae) a spiny medicinal tree commonly called “Bael”is sparsely distributed on the plains and hilly region . This tree originated in Burma and now cultivated in South Asian countries (Zaman, 1988) . *Aegle marmelos* has been cited in ancient Ayurvedic Text such as Charaka Samhita and Sushruta samhita for its medicinal uses. The entire tree is highly medicinal. It is reported to be astringent, febrifuge, expectorant ,cooling, carminative, laxative, restorative and stomachic and is used in dysentery, diarrhoea (Shoba and Thomas, 2001),, flatulence, fever, vomiting and colic. The leaves are astringent, laxative, useful in ophthalmia, deafness, inflammations, diabetes (Kamalakkanan and Prince, 2003; Kar *et al.,* 2003), asthmatic complaints, antifungal activity (Rana *et al.,* 1997), antitumour and antimutagenic activity ( Lampronti *et al.,* 2003) has also been evaluated.

The tender fruit is bitter, astringent, antilaxative, digestive and promotes digestion and strength, overcome vata, colics and diarrhoea. The ripe fruits are astringent, aromatic, cooling, febrifuge, laxative and tonic and are good for the heart and brain (Sivarajan and Balachandran,1994). Seeds are reported for its antiulcer activity. (Goel *et al.,* 1997).

The plant has wide adaptability to varied environmental conditions and is associated to socio-cultural values. This plant is conventionally propagated through seeds that have short viability , low germination and are prone to insect attack(Raghu, 2006). Alternatively root suckers can be used , but are slow and difficult to propagate. Root being the major medicinally useful part, Indiscriminate collection has resulted in loss of wild source and reported to be vulnerable in the Western Ghats of Kerala, Tamil Nadu and Karnataka states of India (Ravikumar and Ved, 2000). Propagation through tissue culture is a viable alternative in this species. Many important medicinal woody plants are often difficult to root, both in conventional and *in vitro* propagation. This issue could be overcome by using *Agrobacterium rhizogenes* mediated rooting *.Agrobacterium rhizogenes* is a soil gram-negative bacterium that induces adventitious root formation at the site of infection in a large number of plants. In the present study an attempt has been made to micropropagate *Aegle marmelos* through Induction of callus from nodal explants ,direct reneration of nodal explants ,Regeneration of shoot buds from callus and induction of roots using *Agrobacterium rhizogenes.*

**MATERIALS AND METHODS**

**1.Sterilization of glassware** :

The glasswares used for micropropagation were washed thoroughly in running tap water and soaked in cleaning solution overnight. The glasswares were washed with detergent and running tap water. These were air dried and used for the study.

**2.Collection and Inoculation of explants for callus initiation:**

Stem cuttings with four to five nodes were collected from the tip of the lower branch of *Aegle marmelos* tree (Plate-1). (After leaf excision, the stem was cut into single node pieces, thoroughly washed under running tap water for 10 mins. The explants were surface sterilized with 70% ethanol for 2 min followed by three washing in distilled water. The explants were then taken to the laminar airflow chamber and surface sterilized with 0.1 % (w/v) mercuric chloride for 3 min followed by three washing in sterile distilled water.

**Plate-1 Habit of A*egle marmelos***



Under aseptic conditions the nodal explants were inoculated vertically into the culture medium consisting of full strength Murashige and Skoog (MS) basal medium medium with 3% (w/v)sucrose, 0.7% (w/v) agar., The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 20 min. The cultures were incubated at 24±2°C in dark for a week. The calli were sub cultured on to MS medium 3% sucrose, 0.5 mg / l 2,4,-D and BA (1-5 mg/l) maintained under dark.

**3.Shoot induction from calli** :

The callus thus obtained was inoculated into MS with 3% sucrose 0.5 mg/l IAA and 1-3 mg/l kinetin with 0.7% agar. The pH of the medium was adjusted to 5.7 prior to autoclaving. About 10 ml of the medium was evenly dispersed into test tubes and plugged with non-absorbant cotton plug and autoclaved to maintain sterility. The callus was maintained under 16 h photoperiod with 3000 Lux with cool white fluorescent tubes at 25º C till the callus turned green . Once the callus turned green the calli were transferred to MS medium .

**4.Direct regeneration of shoots and adventitious rooting**  :

Approximately 4 weeks old young plants of *A.marmelos* were used as explant for direct regeneration of shoots. Nodal explants were utilized for this purpose. Nodal explants were surface sterilized and inoculated into MS media with 3% sucrose 0.5 mg/l IAA and 1-3 mg/l kinetin under aseptic condition shooting and IBA (1-5mg/l) for rooting separately. The explants were incubated under alternative light condition at 25 ºC.

***5.Agrobacterium rhizogenes* mediated root induction** :

*Agrobacterium rhizogenes* was obtained from Microbial Type Culture Collection, Chandigarh (MTCC-532). *A.rhizogenes* culture was revived in Nutrient broth for 48 hrs. The nodal explants collected from 4 weeks old plants were surface sterilized with 70% ethanol and 0.1%mercuric chloride and incubated with *A.rhizogenes* for different time intervals such as 5, 10, 15,20 and 25 min. The explants were blotted dry and co-cultured in MS broth for 48 hrs. After incubation the explants were again blotted dry and transferred to MS agar with 3% sucrose without any exogenous auxin and incubated at 25 ºC in dark . The explants were observed for the formation of adventitious rooting for a period of 4 weeks.

**Results and Discussion**

**1.Callus intitiation**

The nodal explants of *Aegle marmelos* was inoculated in MS medium with 2,4,-D at a concentration of 0.1-0.5 mg/l. Callus induction was observed in 0.2 and 0.5 mg/l 2, 4 –D after five weeks of inoculation. The callus was solid brown in color in lower concentration of 2, 4-D (0.2).However at 0.5mg/l the callus was white friable in nature (Plate-2 ).Islam *et al* .,1992;1993 reported that the color of the induced callus was brownish and hard in nature and however semi-friable callus was also found which was similar to the observation made in the present study. It was evident from the study that lesser concentrations of 2,4 D (0.1-0.5mg/l) were efficient in initiating callus from nodal explants. A number of reports indicate that callus initiation and proliferation from nodal explants strictly depended on exogeneous growth regulator supplementation and no callus was induced on basal MS medium only. Islam *et al.,*1992 reported the induction of calli from young leaves of *A.marmelos* using 2, 4-D at higher concentration of 1-5 mg/l.

**1 2**



**Plate-2 Callus initiation in nodal explants of**

***Aegle marmelos***

**1. Callus initiation at 0. 2mg/l 2,4 D**

**2. Callus initiation at 0. 5 mg/l 2,4 D after 5weeks**

The callus was subcultured for 3 weeks in MS with 2 mg/L Benzyl Adenine +0.5 mg /l 2,4D (Plate-3). It was observed in the present study that BA with less quantity of 2,4 D was not suitable for mass multiplication of callus. Although Rao and Chopra (1987) found 100% callus induction by using 2,4-D along with BA. The maximum % of callusing was found in media having 0.3 mg L-1 BA +2.0 mgL- 2,4-D. Usage of such higher concentrations of growth regulator 2,4,-D may have adversity since they are known to promote abnormal cell divisions that can induce mutations.





(Growth of calli in 2mg/l Benzyl adenine with 0.5mg/l 2,4D after 3weeks)

**Plate-3 Culturing of callus**

**2.Adventitious Shoot buds from callus :**

The callus initiated at 0.5 mg/l strength of 2,4 D was inoculated for shoot buds induction. on MS medium fortified with 0.5 mg/l IAA and 1-3 mg/l kinetin. It was observed that in higher concentration of kinetin (3mg/l) alone, the callus turned green with small adventitious shoot buds after 6 weeks (Plate-4).

**A B**



**Plate-4 Shoot Bud induction in nodal explant derived from callus**

A - Shoot bud at 3mg/l kinetin after 6weeks. Note the green pigmentation of the calli

B - Shoot buds appearing on the surface of the calli after 7 weeks

Islam, 2007 reported that under appropriate condition of auxin and cytokinin 42 day old calli showed formation of number of adventitious shoot buds. Successful micro propagation using nodal explants of *A.vasica* has been reported by Jaiswal *et al*.,1989. Chandra and Bhanja (2002) proved in *Flacorita jangomas* (Lour.), a woody medicinal plant, that all explants do not have the equal potential to regenerate shoot buds and only nodal segment derived callus produced shoot buds.

**3.Direct regeneration of shoot:**

For direct regeneration of the nodal explants from 4 weeks old *A. marmelos* plant was used. Direct regeneration of shoot was observed in 2 mg /l and 3 mg /l kinetin. However, 3 mg/l kinetin induced better regeneration of the shoot. Direct regeneration was observed after 6 weeks of incubation (Plate-5).The proliferation of multiple shoots from cotyledons and shoot tips within 14 days of incubation is reported . However on the other hand, multiple shoot induction from cotyledons was much lower in frequency.



**Plate-5 direct shoot regeneration from Nodal explants**

**A- Shoot induction in 2mg/l Kinetin after 4 weeks**

**B- Shoot induction at 3mg/l Kinetin after 4 weeks**

High percentage of shoot induction was observed in shoot tips which were supplemented with 2 mg of BAP+ 0.3 mg of NAA. According to Islam *et al*.,(1994) presence of low concentration of NAA and IAA in the media markedly promoted shoots regeneration from cotyledon. Among different combinations BA-NAA was found to be the best. The highest shoot regeneration (92.86%) was achieved in the medium supplemented with 2.0 mg/l BA+0.2 mg/l NAA. The maximum mean number of shoot per culture was also observed in that media composition (Islam *et al*.,1994).

**4. Adventitious rooting:**

When 4 weeks old nodal explants were inoculated into MS media with 1-5 mg/l IBA and incubated. After 35 days of incubation the shoot bases showed flattening (Plate-6) which is reported to be the initial step in the initiation of rootlets. Enlargement of shoot base occurred prior to root initiation. Islam also reported the flattening of shoot bases in *A.marmelos.* .Tumor induction was observed after 6 weeks. Rooting was not observed till 60 days, however young shoots were produced from the axillary buds of the nodes after 2 weeks of incubation. The induction of shoots in the explants suggests that the exogenous auxin was lesser than the endogenous cytokinin concentration which was not sufficient for root differentiation, but rapid cell division and cell growth took place resulting in shoot base enlargement and tumors . The higher endogenous cytokinin was responsible for shoot induction from the auxillary buds on the nodes.

**A B C**



**Plate-6. Adventitious rooting in nodal explants**

1. **Flattened shoot base at 1mg/l IBA after 5weeks**
2. **Flattened shoot base at 4mg/l IBA after 5 weeks**
3. **Tumor induction in 5mg/l IBA after 6 weeks**

Highest percentage of root induction was observed in MS media augmented with 1 mg/L of IBA. Similar results were reported in several other medicinal fruit plants such as *Gymnema sylvestre* (Komalavalli and Rao, 2000), *Carica papaya* (Islam *et al*.,2000),*Centella asiatica* (Mohapatra *et al*., 2008), and *Ocimum basilicum* (Sahoo *et al*., 1997).

**5.Agrobacterium mediated rooting:**

The nodal explant of 4 week old plant was co-cultured with *Agrobacterium rhizogenes* after an incubation of 5, 10,15,20 and 25 minutes and transferred to MS agar. After 3 days of incubation shoot induction was observed in 5 minutes, 10 minutes, 15 minutes incubated explants (Plate-7).

**1 2**



**3 4 5**



**6 7**



**Plate-7 *Agrobacterium rhizogenes* mediated shooting in nodal explants**

**1 - Initial stage of shooting from axillary buds of the nodal explants (after 3 days ).**

**incubated with *A.rhizogenes* for 5 min**

**2 - shooting from axillary buds of the nodal explants (after 3 weeks) incubated with**

***A.rhizogenes*. for 5 min**

**3 - Initial stage of shooting in nodal explants (after 3 days) incubated with**

**A*.rhizogenes*  for 10 min**

**4 and 5 - shooting in nodal explants (after 3week ) incubated with *A.rhizogenes* for 10**

**min**

**6- Initial stage of shooting (after 3days) in nodal explants incubated with *A.rhizogenes***

**for 15 min**

**7- shooting in nodal explants (after 3 week) incubated with *A.rhizogenes* for 15 min**

After 3 weeks of incubation shoot growth increased and tumor induction was observed in 5 minutes and 10 minutes incubated explants (Plate-8).

1 2

 

**Plate-8 Tumor induction in A*grobacterium rhizogenes* treated nodal explants**

1. **Tumor induction (after 3 weeks) in nodal explant incubation with *Agrobacterium rhizogenes* for 5 min**
2. **Tumor induction (after 3 weeks) in nodal explant incubated with *Agrobacterium rhizogenes* for 10min**

*Agrobacterium rhizogenes* is a soil bacterium responsible for the development of hairy root disease on a range of dicotyledonous plants (Tepfer,1990). In the present study when *A.marmelos* nodal explants were inoculated with *A.rhizogenes* prominent tumor induction was observed .A similar observation was made, where *A.rhizogenes* after 42 days of inoculation induced tumors in the epicotyl of *Cicer arietinum*. The absence of rooting in *A.marmelos* explants with tumor showed different response because most of the species do not appear to be affected by infection and hormonal supply may be required for good rooting. Georgina-Navarrete (2006) reported the formation of a globular tumor in 5-8 days after infection. The efficiency to induce hairy root in different *A.rhizogenes* strains may vary from one species to another. In terms of hairy root induction different populations exhibit varying degree of response. It is reported that a few strains of *A.rhizogenes* did not induce hairy root in any of the population. These results show that genotype is an important factor for hairy root induction via *A.rhizogenes*. A similar observation was reported by Khatodia and Biswas (2014).

Successful initiation of shoots in the explants suggest that cytokinin synthesis dominated the production of auxins. The synthesis of cytokinins in the explants also indicated successful incorporation of Ri plasmid from *A.rhizogenes* into the host*.* The Ri plasmid has region for both auxin and cytokinin production. The expression of these region depended on the strain of *A.rhizogenes*. In the present study it was observed that the absence of rooting, along with tumorous growth indicated that certain strain of *A.rhizogenes* need an exogenous auxin supply for successful rooting.

*A.Marmelos* is one among the many important medicinal plants which have therapeutic values and over exploitation of this medicinal plant has resulted in loss of it population .Today the status of this trees is listed as endangered and there is a need to conserve them. Micropropagation is the best tool to propagate *A.marmelos* in a short duration. Micropropagation of *A.Marmelos* was successfully achieved from embryos & cotyledons in the past. In the present study callus initiation , shoot bud intiation and direct shooting was observed from nodal explants of *A.marmelos*. *A.rhizogenes* strain induced tumor formation in the nodal explants of *A.marmelos* was evident,however extensive shooting was observed while rooting was delayed.

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**Conflict of interest:**  "The authors declare that there is no conflict of interest”

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