MICROPROPAGATION OF AEGLE MARMELOS AND AGROBACTERIUM RHIZOGENES MEDIATED ROOT INDUCTION IN NODAL EXPLANT

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 4 week old nodal explants were incubated for 5, 10, 15, 20 and 25 minutes with *Agrobacterium rhizogenes* and transferred to MS agar. Shoot induction was observed in 5 minutes, 10 minutes, 15 minutes incubated explants after 3 days of incubation. Shoot growth increased and tumor induction was observed in 5 minutes and 10 minutes incubated explants after 3 weeks.

Keywords: Aegle Marmelos**,** Nodal Explants, Direct Regeneration, Agrobacterium Rhizogenes, Co- Inoculation.

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I. INTRODUCTION

Aegle marmelos (L.) Corr. (Rutaceae) commonly called "Bael"is a spiny medicinal tree, 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 cooling, astringent, carminative, flatulence, febrifuge, restorative, expectorant, stomachic, laxative, used in dysentery, diarrhoea (Shoba and Thomas, 2001) and vomiting. The leaves possess astringent, laxative, antifungal (Rana *et al.,* 1997), antitumour and antimutagenic (Lampronti *et al.*, 2003) properties. It is useful in ophthalmia, deafness, inflammations, diabetes (Kamalakkanan and Prince, 2003; Kar *et al.,* 2003) and asthmatic complaints.

The tender fruits are digestive, bitter, astringent and antilaxative. The ripe fruits are also cooling, febrifuge, astringent, aromatic, laxative and heart and brain tonic (Sivarajan and Balachandran, 1994). Seeds have antiulcer activity. (Goel *et al.,* 1997).

The plant has wide adaptability to varied environmental conditions and is associated to socio-cultural values. Generally the tree is propagated through seeds. Only drawback is that the seeds have short viability and vulnerable to insect attack (Raghu, 2006). Alternatively root suckers can be used, but are slow and difficult to propagate. Indiscriminate collection of roots for medicinal use has resulted in loss of wild source. In india it is reported to be vulnerable in Karnataka, Tamil Nadu and Western Ghats (Ravikumar and Ved, 2000). Micropropagation is another alternative to propagate such tree varieties. However rooting in woody trees cannot be achieved in tissue culture. Hence *Agrobacterium rhizogenes* mediated rooting is a successful method. *Agrobacterium rhizogenes* is a gram-negative soil bacterium that induces adventitious roots 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 regeneration of nodal explants, Regeneration of shoot buds from callus and induction of roots using *Agrobacterium rhizogenes.*

II. 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:** The apical shoot of lower branches with four to five nodes were collected. (Plate-1). The nodal region was excised, washed thoroughly and surface sterilized with 70% ethanol for 2 min. Followed by surface sterilized with 0.1 % (w/v) mercuric chloride for 3 min and inoculated into full strength Murashige and Skoog (MS) basal medium medium with 3% (w/v) sucrose, 0.7% (w/v) agar. pH -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.

Plate 1: Habit of A*egle Marmelos*

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* (MTCC-532) was revived in nutrient broth for 48 hrs.The revived culture was incubated for 5, 10, 15, 20 and 25 minutes with 4 week old nodal explants that were suface sterilized with 70% ethanol and 0.1%mercuric chloride. The explants were blotted dry and co-cultured in MS broth for 48 hrs. The incubated explants were blotted dry and transferred to MS agar with 3% sucrose and maintained at 25 ºC in dark. The explants were observed after 4 weeks.

III. 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. Calli initiation and proliferation does not happen on basal medium alone, but needs a supplementation of exogenous hormones. 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.

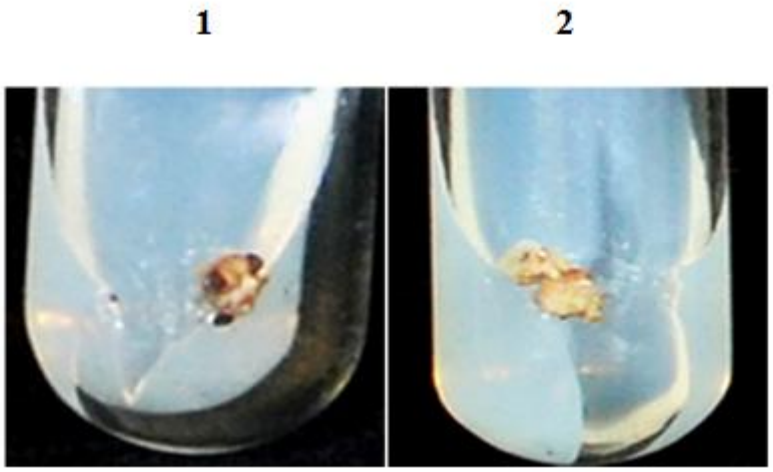

Plate 2: Callus Initiation in Nodal Explants of *Aegle Marmelos*

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) reported 100% calli induction with 2, 4-D and 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.

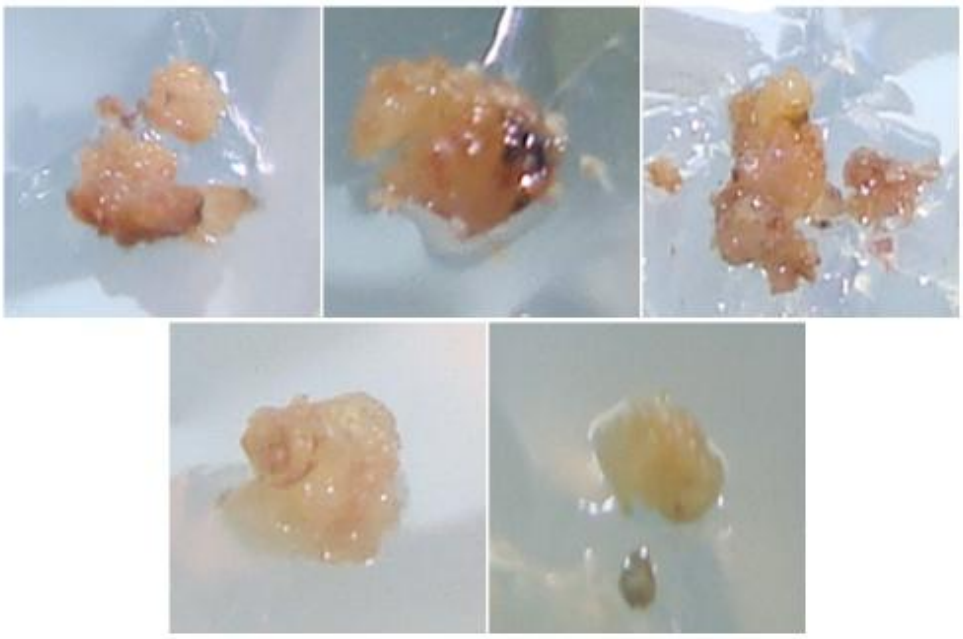

Plate 3: Culturing of Callus
(Growth of calli in 2mg/l Benzyl adenine with 0.5mg/l 2,4D after 3weeks)

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).

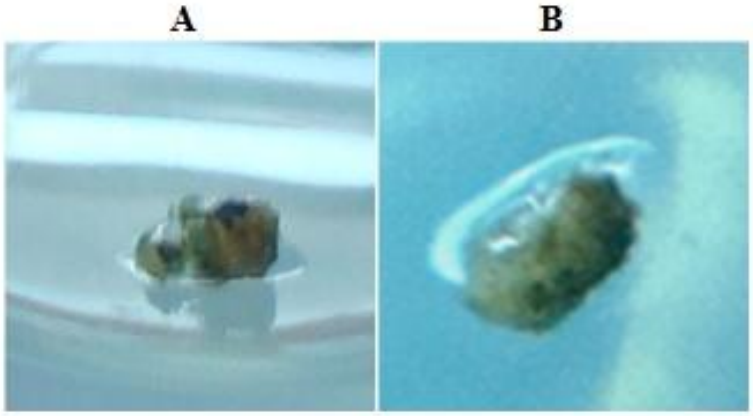

Plate 4: Shoot Bud Induction in Nodal Explant Derived from Callus

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

Adventitious shoot buds were produced by 42 day old calli that were supplemented with auxin and cytokinin (Islam, 2007). Successful micro propagation using nodal explants of *A.vasica* has been reported (Jaiswal *et al*., 1989). In the case of a

woody plant such as *Flacorita jangomas*,all explants do not have the potential to regenerate shoot buds and only nodal explant derived callus produced shoot buds (Chandra and Bhanja ,2002).

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.

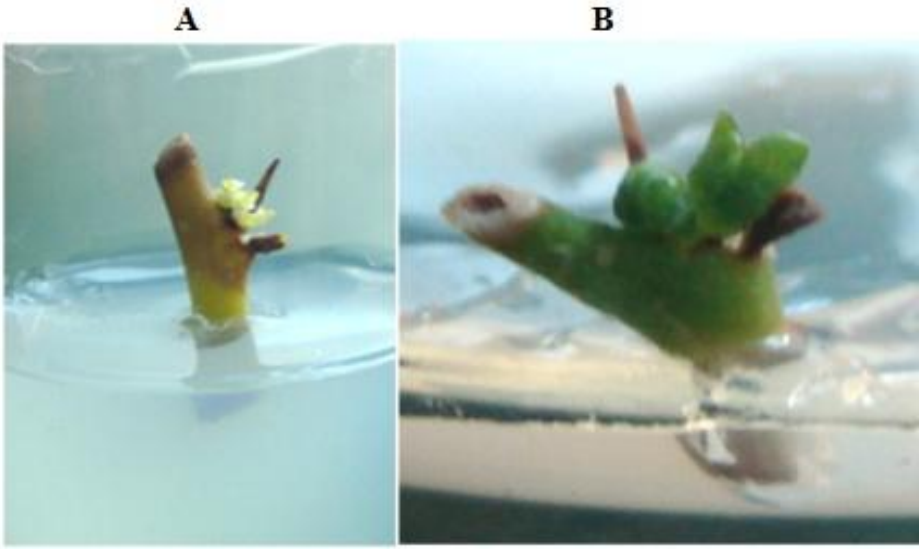

Plate 5: Direct Shoot Regeneration from Nodal Explants

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.

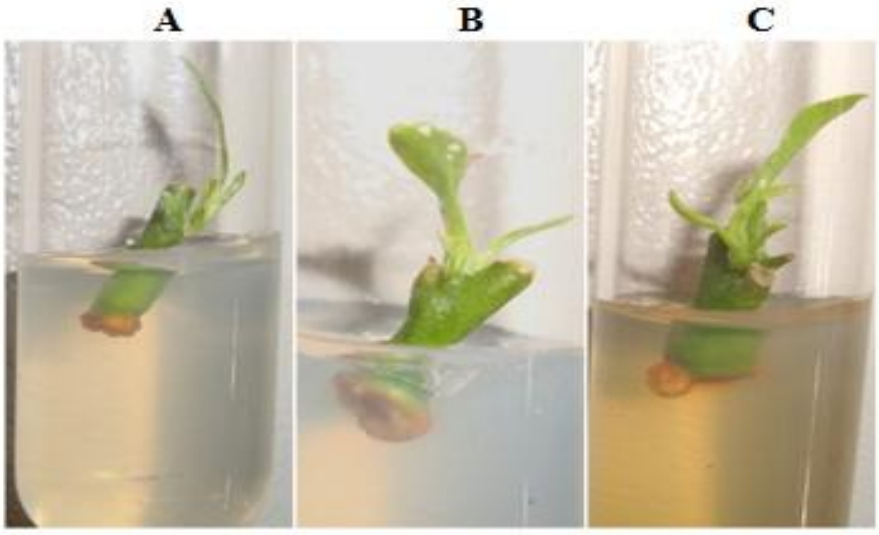

Plate 6: Adventitious Rooting in Nodal Explants

A- Flattened shoot base at 1mg/l IBA after 5weeks
B- Flattened shoot base at 4mg/l IBA after 5 weeks
C- Tumor induction in 5mg/l IBA after 6 weeks

Highest percentage of root induction was observed in *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) in MS media augmented with 1 mg/L of IBA.

5. **Agrobacterium Mediated Rooting:** After 3 days of incubation shoot induction was observed in co cultured explants for 5 minutes, 10 minutes and 15 minutes (Plate-7).

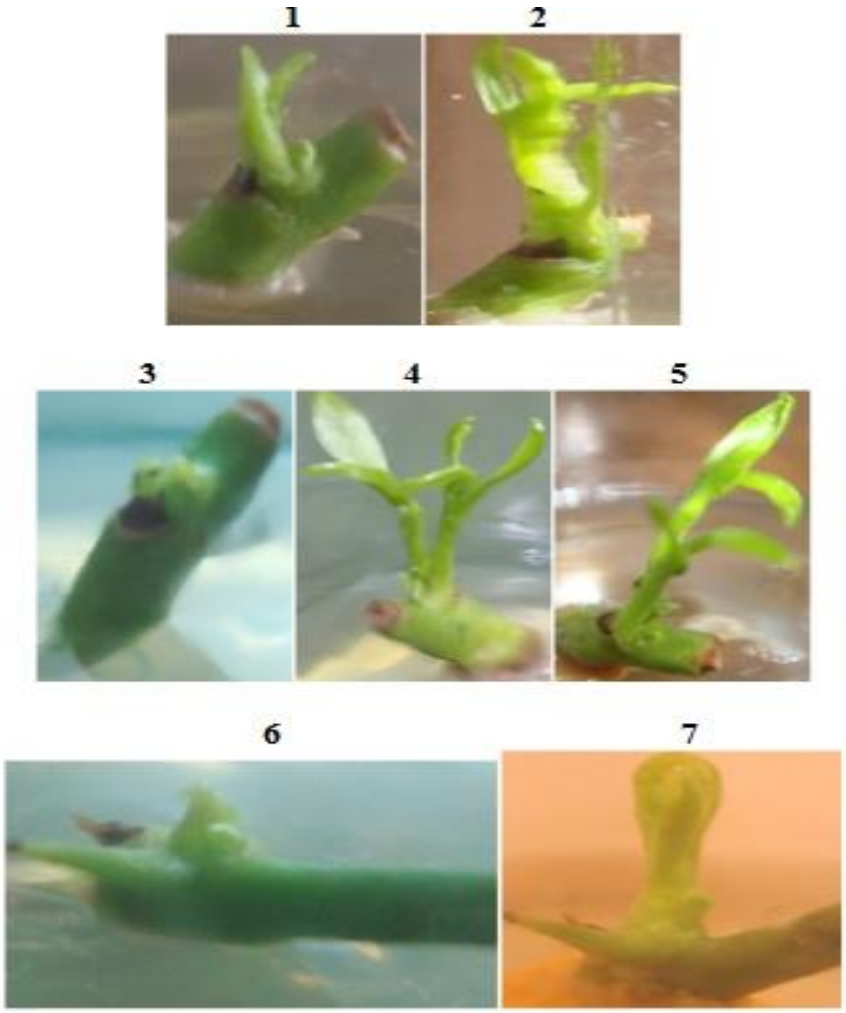

Plate 7: *Agrobacterium Rhizogenes* Mediated Shooting in Nodal Explants

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).

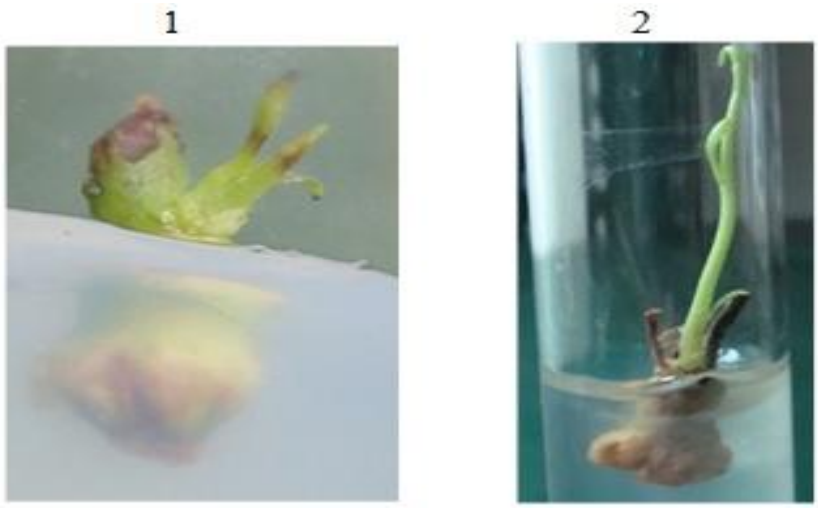

Plate 8: Tumor Induction in A*grobacterium Rhizogenes* Treated Nodal Explants

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 produces hairy root on a wide range of dicotyledonous plants (Tepfer,1990). But as far as woody tress are concerned they may not be able to induce hairy roots. This was evident in the study, where A.rhizogenes instead of inducing hairy roots, tumor induction was observed. A similar observation seen in 42 day old epicotyl of *Cicer arietinum.* There seems to be different response because most of the species do not appear to be affected by infection and external 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 ability to induce hairy root is strain specific and differes in different *A.rhizogenes* strains. A varied response is observed in different populations. Few strains of *A.rhizogenes* do not induce hairy root. Gene regulation is a key factor in hairy root induction through A.rhizogenes (Khatodia and Biswas (2014).

However, successful shoots induction in the explants suggest that cytokinin synthesis and regulation was dominant over auxin production. Synthesis of cytokinin is only possible when the Ri plasmid incorporates in the host. This was evident in the study. The Expression of auxin or cytokinin depends on the strain *of Agrobacterium rhizogenes*. An exogenous supply auxin can trigger the formation of hairy roots in *A. marmelos*.

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.

IV. ACKNOWLEDGMENTS

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V. CONFLICT OF INTEREST

"The authors declare that there is no conflict of interest"

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