Original Research Article

Establishment of *In-vitro* Regeneration Protocol of *Aegle Marmelos* (L.): A Sacred Medicinal Tree.

ABSTRACT

Bael (*Aegle marmelos*L.) is an important medicinal tree and sacred tree species of the Hindu religion. The large-scale multiplication through seed was difficult due to low seed germination.Micropropagation is an alternative technique to produce clonal plants on a largescale.Hence, the present study was conducted to regenerateefficient regeneration protocol. For the regeneration study, the different explants such as the nodal segments and shoot tipswere used for the proliferation of the explant with MS medium,however, only the nodal segment showed positive results. To avoid the browning of the media the explants were kept in distilled water for 60 minutes and then sterilized. The maximum survival of explants (93.33%) and less contamination percentage (6.67%) was observed when explants were washed and treated with 0.1% Tween-20 concentration for 15 minutes + 0.1% bavistin and 0.05% streptocyclinetreatment for 25 minutes andfinally 70% ethanol treatment for 30 seconds.Different hormonal combinations *viz*. BAP alone, BAP + IAA, and Kinetin + IAA were usedtoinitiateshooting. Among all eighteen treatments, the BAP 2.5 mg/l showed a maximum number ofshoots per explant (8.33 ± 2.88), the highest shoot initiation(93.33%), and the earliest initiation (30 ± 5.25 days). For shoot multiplication 2.5 mg/l BAP + 0.5 mg/l NAA showed maximum shoots per explant (28.00 ± 5.08) and minimum days required for shoot proliferation.The regenerated multiple shoots were separated from each other and transferred to half MS media with IBA of different concentrations for rooting. Among the six different treatments only two treatments *viz*. ½ MS media + 1.0 mg/l IBA and ½ MS media + 1.5 mg/l IBA recorded best root length, 2.90 cm ± 1.97 and 2.67 cm ± 1.91, respectively. For primary hardeningthe rooted plants were planted in vermicompost: sand: soil at 1:1:2 proportion and then after one and half months survived plants were again transferred to soil: organic manure (2:1)for secondary hardening.

Keywords:*Aegle marmelos*,regeneration, shooting, rooting, hardening

INTRODUCTION

*Aegle marmelos*is a tropical deciduous and highly heterozygous fruit tree. The plant is very hardy and can adapt to a wide range of habitats, from arid to mesophytic (Arya and Shekawat, 1986; Singh et al. 1976; and Zaman, 1988). It belongs to the Rutaceae family and is an economically important multipurpose social tree of the Indo-Burman subcontinent (Hooker, 1875), that is now being introduced into tropical countries in Western Africa and South America. It has been around since prehistoric times in India and is also known as Bael, Bengal Quince, Golden Apple, Japanese Bitter Orange, Stone Apple or Wood Apple. It is considered auspicious, sacred and, a symbol of prosperity in ancient Sanskrit poems. Hindus worship Lord Shiva with its trifoliate compound leaves. The Bael appears frequently in the Sanskrit scriptures of Vedic times (C 2000BC - C 800BC). Bael is mentioned in the Yajurveda, early Buddhist and Jain literature (C 800BC - C 325BC), the Ramayana, and the *'BrihatSamhita'* (Aiyer, 1956; Om Prakash, 1961; Singh and Roy, 1984). As a sacred tree, the bael tree is usually planted around Hindu temples.

The root is a component of *'dasmula'* (10 roots), an ayurvedic treatment, widely used by practitioners. The roots have a variety of tastes and flavors, including sweet, acidic, bitter, and febrifuge. Bael fruit is significant because of its curative properties, which makes the tree one of India's most useful medicinal plants (Kirtikar and Basu, 1935). The leaves are astringent, laxative, febrifuge, and expectorant, and can be used to treat ophthalmia, deafness, inflammations, catarrh, diabetes, and asthmatic symptoms. The unripe fruits are bitter, acrid, sour, astringent, digestive, and stomachic, and are excellent in diarrhea and dysentery. The ripe fruits are sweet, aromatic, cooling, febrifuge, laxative, and tonic, and are beneficial to the brain and heart in disorders (Warrier et al. 1996).The pulp of the ripe fruit is used in the preparation of sherbet, which is an aromatic summer drink. Bael can also be used forestation in dry areas due to its high drought tolerance capacity (Anonymous, 1985).

The Bael fruit is extremely nutritious and high in vitamins and minerals. Per 100g of edible portion, it contains 1.8 g protein, 0.39 g fat, 1.7 g minerals, 37.8 g carbohydrates, 55 mg carotene, 0.13 mg thiamine, 1.19 mg riboflavin, 1.1 mg niacin, and 8 mg vitamin C (Gopalan et al. 1971). Riboflavin is abundant in baelfruit (Mukherjee and Ahmad, 1957). It also contains marmelosin and has 88 calories of energy per 100g of fruit pulp, making it more nutritious than other fruits (Jauhari and Singh, 1971).

The Bael is typically propagated by seed. However, because it is a cross-pollinated species, seed propagation results in a highly heterozygous population(Ajithkumar and Seeni, 1998).Seeds also have short viability and are vulnerable to insect attack. Plantlets from seed grow slowly and are susceptible to pathogens and pests in the early stages. The rate of vegetative proliferation via root suckers is slow also difficult and time-consuming (Ray and Chattarjee, 1996).

Conventional propagation methods, such as seeds or root cuttings and layers are associated withproblematic issues such as seasonal seed production (which takes a year), pest attacks, and low seed germination. Non-conventional ways, such as *in-vitro*propagation procedures, can helpsolvethese issues. The *in vitro*multiplicationof endangered species could be important for those texa whose reproduction is problematic through conventional methods. Thus, the development of a specific sterilization technique for plant materials obtained from the field is an essential requirement before identifying the growth stage for the collection of plant materials and perfecting the medium composition for the multiplication of plants. Therefore, the present study was conducted to develop an *in vitro* regeneration protocol for the multiplication of bael.

MATERIAL AND METHODS

This study was conducted at the State Level Biotechnology Centre, Mahatma Phule Krishi Vidyapeeth, Rahuri. Plant materials *viz*. nodal segment and shoot tip were collectedin the morning hours from healthy mature plants from the experimental orchard of Medicinal and Aromatic Plants Project, (*Dhanwantary* Garden), Mahatma Phule Krishi Vidyapeeth, Rahuri.

The glassware, and instrumentsused for aseptic manipulations weresterilizedas per standardized procedure.For the sterilization of explants, different sterilants wereused and the best combination was identified. Sterilized explants were inoculated on MS (Murashige and Skoog, 1962) media with full-strength basal medium supplemented with 18combinations of different plant growth hormones for shoot proliferationto identify the best treatment for shoot initiation.They were initially kept in the dark for a week and then incubated at 25°C temperature with a 16 h light period and 8 h dark period. Initiated shoots were then transferred to 8 different media combinations to identify the best treatment for the shoot multiplication.Forthe root initiationstudy,different concentrations of IBA with half MS media were used. As per Das et al.2008, 6 treatments were formulated to carry out rooting

experiments,and the best treatment was identified.Plantlets thus produced were then transferred to pots containing different combinations of sterile soil, sand,vermicompost and coco peat.All the pots were irrigated at regular intervals. They were initially covered with plastic bags for a week and then kept in a polycarbonated polyhouse. All the experiments were conducted in a Completely Randomized Block design with three replicates and 10 explants per replication were used.The data collected were analyzed for mean and standard error as given by Snedecor and Cochran (1967). Calculations of standard error for the number of days required for the multiplication, number of shoots formed per explant, shoot initiation percentage, number of days required for the rooting, length of roots formed and rooting percentage were done. Each replication's value was transformed by use of the square root transformation method.

RESULTS AND DISCUSSION

Explant preparation

The present study describesthe development of a rapid and efficient plantlet regeneration protocol in baelusing nodal segments. For the proliferation of the explants, different explants *viz*. nodal segments and shoot tips were used with a modified MS medium. For shoot initiation, only nodal segments showed positive results (73.33%), while in the case of shoot tip not a single explant developed the shoot (0%), and later on all the shoot tip explants were dried off. Hence, for further experimental study, only nodal segmentswere used as explants. Due to the presence of pre-existing meristem which can easily be developed into shoots, plant regeneration from nodal explant is one of the most promising ways of multiplying a selected species to its true type (Shubhjeetet al. 2021).

The nodal segmentswere collected from newly emerging branches of healthy plants from the DhanwantaryGarden, M.P.K.V, Rahuri. The explants of 0.5 cm to 1.0 cm in length were dissected from the mother plant and immediately the slant cut (near about 45º angle) was given at the bottom of the explants. In preliminary experiments darkening or browning of the culture medium proved toxic to plant growth and hindered the growth, hence the explant was kept in double distilled water for 60 minutes to avoid the browning of media. Most of the phenolic compound was released in water and further, no browning was observed.

The various sterilants were used in different concentrations to standardize the surface sterilization protocol for obtaining the contamination-free culture. The part and partial similar proceduresdescribed by earlier workers were followed for surface sterilization of the nodal

segment. First, the explants were disinfected with Tween-20 solution (0.1%) for 15 minutes and then washed with double distilled water 4 times.Further sterilized by Bavistin and Streptocycline,maximum survival of explant 93.33% andless contamination (6.67%) was observed with 0.1% Bavistin and 0.05% Streptocycline concentration for 25 minutes (Table 1).

Table 1: Effect of sterilization treatments on explants.

Treatment	Time	Survival (%)	Contamination / Dead (%)
Bavistin (0.1%) + Streptocycline (0.05%)	15 min	46.66	53.33
	20 min	60.00	40.00
	25 min	**93.33**	**6.67**
Bavistin (0.2%) + Streptocycline (0.1%)	15 min	33.33	66.67
	20 min	40.00	60.00
	25 min	46.67	53.33
Bavistin (0.1%) + Streptocycline (0.2%)	15 min	53.33	46.67
	20 min	60.00	40.00
	25 min	66.67	33.33
Bavistin (0.2%) + Streptocycline (0.2%)	15 min	46.67	53.33
	20 min	66.67	33.33
	25 min	86.67	13.33

Then the explants were transferred to the inoculation room. In laminar airflow, the explants were treated with 0.1% $HgCl_2$ for 2 minutes and thoroughly washed with double distilled water 4 times to remove the traces of mercuric chloride and again treated with 70% ethanol for 30 seconds and washed with double distilled water 4 to 5 times and finally inoculated on culture media. The duration of treatment is important, long duration of treatment will damage the explants or can cause browning (Singhal et al. 2011).

Shoot initiation

The type and concentration of a growth regulator were found to have a significant impact on morphogenetic responses in bael. The results of different experiments using different hormone concentrations and combinations are presented here. The MS media gave a good response to explant proliferation. The different types of growth regulator concentrations were used for the regeneration study (Fig. 1). The aim was to identify the best media combination for the shoot initiation. For the proliferation of explant BAP alone with different

concentrations, theBAP + IAA combination and Kinetin + IAA combination were used. Among all the treatments, the T_5treatment *i.e.*BAP 2.5 mg/l showed best shoot initiation (93.33%), fastest shoot proliferation (30.00 ± 5.25 days) and higher shoot initiation (8.33± 2.88) followed by T_9 treatment (BAP 1.0 mg/l + IAA 0.3 mg/l) (86.67%, 31.00 ± 5.33, 6.33 ± 2.55) and T_{17} Kinetin 2.0 mg/l + IAA 0.4 mg/l (86.67%, 31.33 ± 5.36, 5.00 ± 2.31). Because of the better responseto shooting as compared to others,BAP (2.5 mg/l)proved to bethe best media combination for the shoot proliferation (Table 2).Similar results were reported by Bindhu (2015) and Pathirana et al. (2020).

Table2 :Effect of different BAP concentrations on shoot proliferation.

Treatment No.	**Growthregulator (mg/l) addedto MS media**	**Shoot establishment %**	**No. of days required for shootproliferation (Mean ± SE)**	**No. of shoots per explant (Mean ± SE)**
T_1	0.5 BAP	60.00	33.00 ± 5.50	2.33 ± 1.72
T_2	1.0 BAP	53.33	31.33 ± 5.36	3.33 ± 1.96
T_3	1.5 BAP	86.67	31.00 ± 5.33	7.00 ± 2.67
T_4	2.0 BAP	66.67	32.67 ± 5.47	6.00 ± 2.49
T_5	**2.5 BAP**	**93.33**	**30.00 ± 5.25**	**8.33 ± 2.88**
T_6	3 BAP	66.67	34.67 ± 5.63	4.33 ± 2.18
T7	1.0 BAP + 0.1 IAA	73.33	31.83 ± 5.40	3.67 ± 2.04
T8	1.0 BAP + 0.2 IAA	80.00	32.27 ± 5.44	3.67 ± 2.04
T9	**1.0 BAP + 0.3 IAA**	**86.67**	**31.00 ± 5.33**	**6.33 ± 2.55**
T10	1.0 BAP + 0.5 IAA	66.67	33.33 ± 5.52	5.00 ± 2.31
T11	2.0 BAP + 0.1 IAA	53.33	32.00 ± 5.42	3.33 ± 1.89
T12	2.0 BAP + 0.2 IAA	60.00	31.20 ± 5.35	3.00 ± 1.89
T13	1.0 Kinetin + 0.05 IAA	53.33	31.50 ± 5.37	3.00 ± 1.89
T14	2.0 Kinetin + 0.1 IAA	73.33	32.00 ± 5.42	5.33 ± 2.37
T15	2.0 Kinetin + 0.2 IAA	66.67	31.00 ± 5.33	3.33 ± 1.96

T16	2.0 Kinetin + 0.3 IAA	80.00	31.67 ± 5.39	4.00 ± 2.11
T17	**2.0 Kinetin + 0.4 IAA**	**86.67**	**31.33 ± 5.36**	**5.00 ± 2.31**
T18	2.0 Kinetin + 0.5 IAA	53.00	33.33 ± 5.52	3.00 ± 1.89

Shoot multiplication

The initiated, well-developed, 45 to 60 days-old explants were transferred to the multiplication media. But, initially, all explants were dried off and could not survive. Then some of the bottom portion of initiated explants was removed and directly transferred to the multiplication media so the survival percentage was increased.All explants were cultured on multiplication media containing BAP + NAA growth regulator with different concentrations (Fig. 2).Among all the treatments M_8*i.e.* BAP 2.5 mg/l + NAA 0.5 mg/l showed maximum shoots per explant (28.00 ± 5.08). The shoot establishment was 93.33% and the number of days required for shoot proliferation was 60.00 (± 7.82). Hence, it was accepted as the best treatment for shoot multiplication (Table 3). Das, et al. (2008)reported that the highest percentage of multiple shoots (91.23%) within 40 days and the maximum number of multiple shoots per explant (22.7) was found in BAP 2.0 mg/l with NAA 0.2 mg/l. Hossain et al.(1993) reported the maximum frequency of adventurous bud proliferation was observed in MS medium with BAP and NAA.

Table 3: Effect of different concentrations of BAP + NAA on multiplication of shoots.

Treatment No.	Growth Regulator BAP +NAA (mg/l) added to MS media	Shoot establishment %	No. of days required for shoot proliferation (Mean ± SE)	No. of shoots per explant (Mean ± SE)
M_1	2.0 BAP + 0.1 NAA	46.67	60.43 ± 7.85	10.33 ± 3.17
M_2	2.0 BAP + 0.2 NAA	80.00	60.70 ± 7.86	18.00 ± 4.11
M_3	2.0 BAP + 0.3 NAA	73.33	60.55 ± 7.85	18.67 ± 4.18
M_4	2.0 BAP + 0.5 NAA	66.67	60.45 ± 7.85	10.00 ± 3.13
M_5	2.5 BAP + 0.1 NAA	86.67	60.30 ± 7.84	21.67 ± 4.49
M_6	2.5 BAP + 0.2 NAA	60.00	61.67 ± 7.93	10.00 ± 3.13

M_7	2.5 BAP + 0.3 NAA	80.00	61.33 ± 7.90	25.00 ± 4.81
M_8	**2.5 BAP + 0.5 NAA**	**93.33**	**60.00 ± 7.82**	**28.00 ± 5.08**

***In vitro* rooting**

The regenerated multiple shoots were separated from each other and transferred to half MS media with IBA of different concentrations. The observations were recorded and depicted in. Among the six different treatments only two treatments R_3(½ MS media + 1.0 mg/l IBA) and R_4(½ MS media + 1.5 mg/l IBA) showed root proliferation (Table 4 and Fig. 3). The rooting treatment R_3 recorded 2.90 cm ± 1.97 and R_4 recorded root length of 2.67 cm ± 1.91. The percent root initiation was higher in R_3 (33.33%). The number of days required for root proliferation was approximately similar (R_3: 90.67 ± 9.03; R_4: 91.00 ± 9.04) in both treatments. As a hard woody plant species, the root formation was slow, and more duration was required for root induction. Similar results were reported by Das, et al. 2008. and Akter, et al. 2013.

Table 4: Effect of different concentrations of IBA on root formation.

Treatment No.	Growth Regulator IBA (mg/l) added to ½ MS media	% of root initiation	No. of days required for root proliferation (Mean ± SE)	Average length of roots (cm) (Mean ± SE)
R_1	½ MS media + 0.1 IBA	-	-	-
R_2	½ MS media + 0.5 IBA	-	-	-
R_3	**½ MS media + 1.0 IBA**	**33.33**	**90.67 ± 9.03**	**2.90 ± 1.97**
R_4	½ MS media + 1.5 IBA	20.00	91.00 ± 9.04	2.67 ± 1.91
R_5	½ MS media + 2.0 IBA	-	-	-
R_6	½ MS media + 2.5 IBA	-	-	-

Hardening for plant acclimatization

The regenerated *in vitro* plants were taken out after weeks from the inoculated bottle and washed with double distilled water to remove the media traces. The rooted portion was treated with 0.1% Bavistin solution to prevent further fungal infection. For primary hardening, the rooted plants were transferred to the small pots containing two different

potting hardening mixtures of sterile Soil: Coco peat: Sand in the proportion of 1:1:1 and Vermicompost: Sand: Soil of 1:1:2 respective. To maintain the proper moisture level pots were drilled to make proper holes and kept under controlled environment conditions in the greenhouse for one month. The plant survival rate for sterile Soil: Coco peat: Sand (1:1:1) was 60% and Vermicompost: Sand: Soil (1:1:2) was 80%. After one monthwhen new leaves emerged the plants were shifted to a new potting mixture containing Soil: Organic manure (2:1) for secondary hardening. After one month old plant survival rate was 80%. Results obtained in hardening are presentedin Table 5 and Fig 4. Similar results were reported by Das, et al. 2008. After sufficient acclimatization, the plants were transferred to natural conditions and showed 80% plantlet survival.

Table 5: Hardening of the regenerated plants.

Sr. No.	Type of hardening	Hardening composition	Plant survival percentage
1.	Primary hardening	Sterile soil: Coco peat: Sand (1:1:1)	60%
		Vermicompost: Sand: Soil (1:1:2)	**80%**
2.	Secondary hardening	Soil + Organic manure (2:1)	80%

CONCLUSION

The nodal segment 0.5cm to 1.0cm in length showed better response for shoot initiation. To avoid the browning of the media, the explants were kept in distilled water for 60 minutes immediately after removal from the mother plant. The explant was kept free from contamination by using surface sterilization such as Tween 20 (0.1%) for 15 minutes, Bavistin (0.1%) + Streptocycline (0.05%) for 25 minutes, Mercuric chloride 0.1% treated for 2 minutes and 70% ethanol for 30 seconds. The M.S. Medium supplemented with BAP 2.5 mg/lwasthebest media combination for shoot proliferation.For shoot multiplication, M.S. Medium supplemented with 2.5 mg/l BAP + 0.5 mg/l NAAproved to be the best media combination. Likewise, ½ MSmedium with 1.0 mg/l IBAgave the best resultfor root development.For primary hardening Vermicompost: sand: soil at1:1:2 proportion and for secondary hardening soil: organic manure (2:1)gave the better response for plant survival.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

ETHICAL ISSUES: None.

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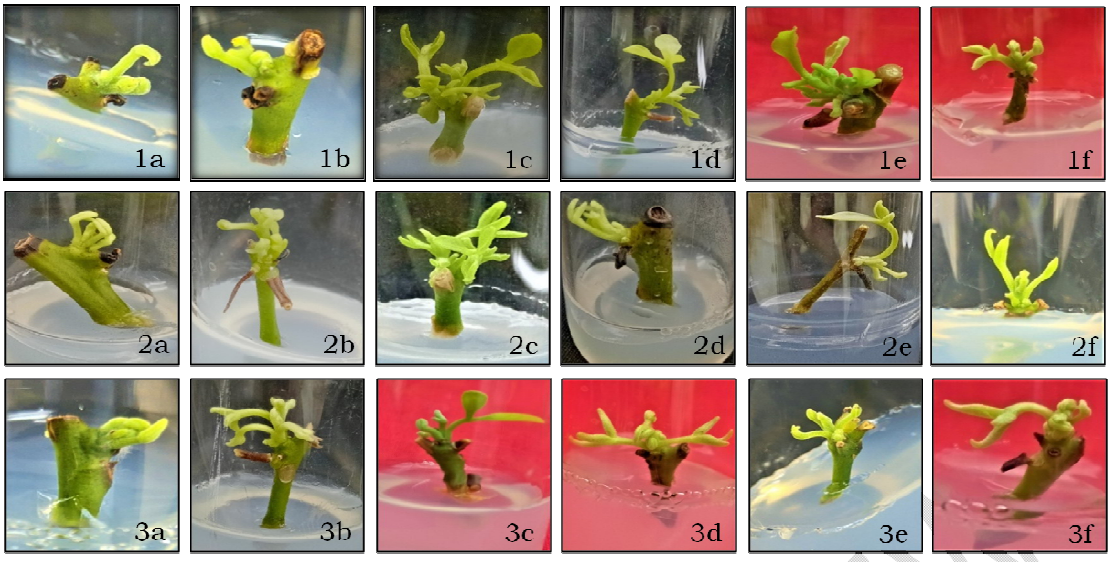

Fig. 1: Initiation of nodal segment of *Aegle marmelos* L. on MS medium with different concentration od BAP mg/l, BAP mg/l + IAA mg/l and Kinetin mg/l + IAA mg/l.

1a. MS +BAP 0.5 mg/l
1b. MS + BAP 1.0 mg/l
1c. MS + BAP 1.5 mg/l
1d. MS + BAP 2.0 mg/l
1e. MS + BAP 2.5 mg/l
1f. MS + BAP 3 mg/l
2a. MS + BAP 1.0 mg/l + IAA 0.1 mg/l
2b. MS + BAP 1.0 mg/l + IAA 0.2 mg/l
2c. MS + BAP 1.0 mg/l + IAA 0.3 mg/l
2d. MS + BAP 1.0 mg/l + IAA 0.5 mg/l
2e. MS + BAP 2.0 mg/l + IAA 0.1 mg/l
2f. MS + BAP 2.0 mg/l + IAA 0.2 mg/l
3a. MS + Kinetin 1.0 mg/l + IAA 0.05 mg/l
3b. MS + Kinetin 2.0 mg/l + IAA 0.1 mg/l
3c. MS + Kinetin 2.0 mg/l + IAA 0.2 mg/l
3d. MS + Kinetin 2.0 mg/l + IAA 0.3 mg/l
3e. MS + Kinetin 2.0 mg/l + IAA 0.4 mg/l
3f. MS + Kinetin 2.0 mg/l + IAA 0.5 mg/l

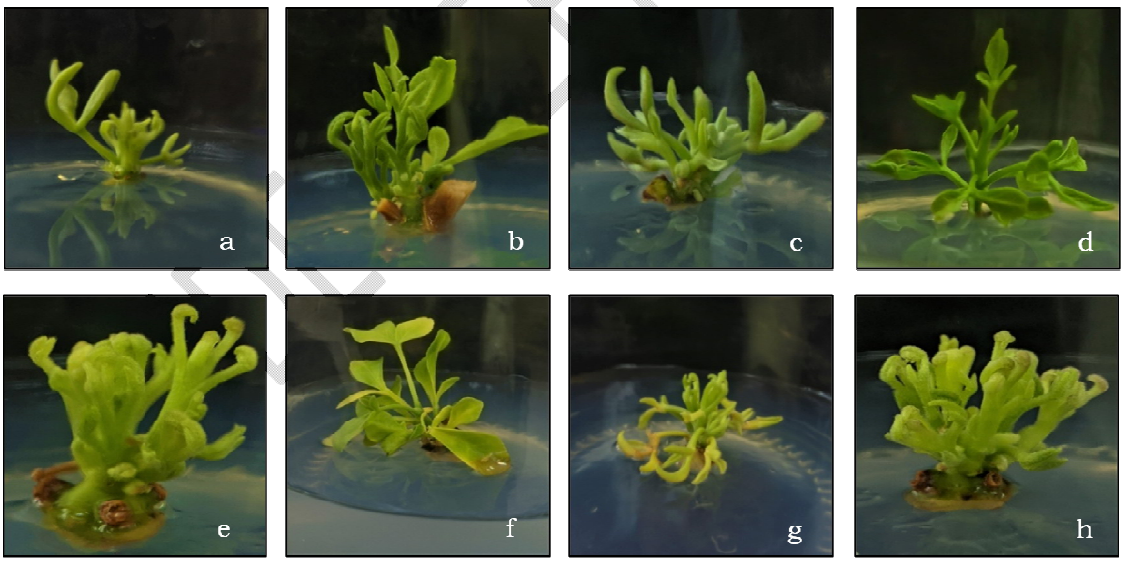

Fig. 2: Multiplication of *Aegle marmelos*L. on MS medium and BAP mg/l + NAA mg/l combination with different concentrations.

a. MS + BAP 2.0 mg/l + NAA 0.1 mg/l
b. MS + BAP 2.0 mg/l + NAA 0.2 mg/l
c. MS + BAP 2.0 mg/l + NAA 0.3 mg/l
d. MS + BAP 2.0 mg/l + NAA 0.5 mg/l
e. MS + BAP 2.5 mg/l + NAA 0.1 mg/l
f. MS + BAP 2.5 mg/l + NAA 0.2 mg/l
g. MS + BAP 2.5 mg/l + NAA 0.3 mg/l
h. MS + BAP 2.5 mg/l + NAA 0.5 mg/l

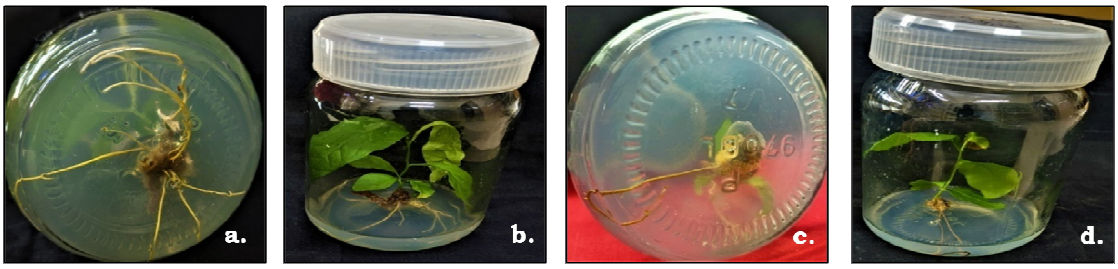

Fig. 3: Rooting of *Aegle marmelos*L. on ½ MS medium and IBA mg/L with different concentrations.

a & b. ½ MS + IBA 1.0 mg/L c & d. ½ MS + IBA 1.5 mg/L mg/L

Fig. 4: Hardening of regenerated tissue culture plantlets of *Aegle marmelos*L.

a. Primary hardening (Vermicompost : Sand : Soil at1:1:2)
b. b. Secondary hardening (Soil : Organic manure at 2:1)