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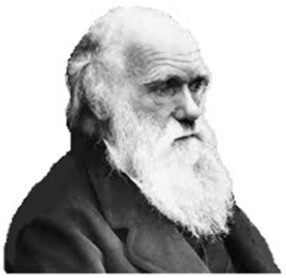

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The effect of additional additives on the axillary shoot micropropagation of medicinal plant *Aegle marmelos* (L.) Corrêa

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ABSTRACT

India is an immense medicinal plant repository, used in traditional medical treatments. The use of herbal medicine is growing day by day due to toxicity and allopathic drug effects. About 90 percent of the phytochemicals marketed come from wild resources and over 120 major phytochemistry come from plants. *Aegle marmelos* leaves contained π-sitosterol, aegelin, lupeol, rutin, marmesinine, flavone, glycoside, Oisopentenyl halfordiol, marmeline, and phenylethyl cinnamides. The additional growth promoters show its effect on tissue culture of plant.

***Keywords*:** Tissue Culture, *Aegle marmelos*, Micropopgation, Axillary Shoot Culture, Amino Acids, PGR, Additives

1. INTRODUCTION

India is an immense medicinal plant repository, used in traditional medical treatments. Different ethnic systems, like Siddhi, ayurveda, Unani, Homeopathy, and Allopathy, use several species of plants to treat different diseases [1]. The use of herbal medicine is growing

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day by day due to toxicity and allopathic drug effects. Because of this, the number of herbal drugs produced suddenly increases.

Herbal medicines are used as the principal remedy in the traditional medicine system. Nowadays they are used extensively because all over the world of their biomedical advantages as well as places in the cultural beliefs. Medicinal plants generate and contain a variety of chemical compounds that work on the body and are used for disease prevention or treatment or health promotion. The plant's medicinal properties are due to these active constituents that are found in them. Secondary metabolites are the active principles of the medicinal plants used for medicinal preparation. About 90 percent of the phytochemicals marketed come from wild resources and over 120 major phytochemicals come from plants [2-4]. A proportion of medicinal plant material used to make plant extract is considered. *Aegle marmelos* (Beal) were found all over India and Southeast Asia. It belongs to the Rutaceae family. *Aegle marmelos* are found in northern, central, and southern India, southern Nepal, Pakistan, Myanmar, Sri Lanka, Bangladesh, Vietnam, Laos, Cambodia, and Thailand in dry forests. It is a medium-sized, slender, aromatic, armed, gum-bearing tree growing to a height of 18 meters. It has three leaflets on it. Its fruits are used all over its range in traditional medicine and as food. The unripe fruit is useful for treating diarrhea, dysentery, and stomachalgia [5]. The stem and root bark aqueous extracts are used to treat malaria, fever, jaundice, and skin diseases like ulcers, hives, and eczema. Both the fruit and root have demonstrated antiamoebial [6] and hypoglycaemic behaviours in pharmacological trials. Specific phytochemical and biological tests have been identified as anti-diabetic [7], antioxidant [8, 9], and antithyroid.

Aegle marmelos leaves contained π-sitosterol, aegelin, lupeol, rutin, marmesinine, β-sitosterol, flavone, glycoside, Oisopentenyl halfordiol, marmeline, and phenylethyl cinnamides [10], a sequence of phenylethyl cinnamides that included new compounds called anhydromarmeline, aegelinosides A, and B were isolated as α-glucosidase inhibitors from the *Aegle marmelos* leaves [11].

Ali *et al.* isolated Marmenol, a new 7-geranyloxycoumarin [7-(2,6-dihydroxy-7-methoxy-7-methyl-3-octaenyloxy)coumarin] from the leaves of methanolic extract of *Aegle marmelos* [12]. α-Phellandrene was found to be the common constituent of the essential oil from leaves, twigs, and fruits. α-Phellandrene (56%) and p-cymene (17%) were reported from leaf oil [13]. Sitosterol was identified from leaves. Stem bark, leaves, fruit, and root investigations revealed α-and β-amyrin and β-sitosterol.

2. HISTORY

Based on that, Gottieb Heberlandt, a German physiologist, developed the concept of in-vitro cell culture in 1902. All these attempts at research involving the culture of isolated cells, root tips, and stem tips ended in callus development. When advances in plant tissue culture accelerated rapidly in 1930, due to a significant discovery that vitamin B [14, 15] and auxins [16, 17] are necessary for the growth of meristem-containing tissues. The first plant growth regulator, Indole Acetic Acid (IAA), was discovered by Fritz Went in 1926. IAA is an auxine which occurs naturally [18-21]. In addition to PGRs, scientists have tried to boost the culture media by basically differing in mineral content. In this direction Murashige and Skoog prepared a medium by increasing salt concentration 25 times higher than the solution from Knop. This solution increased tobacco tissue development by fivefold [21, 22].

Kanta and Maheshwari, 1962, first exemplified the role of the plant tissue culture in plant genetic engineering. The developed a test tube fertilization technique that involved growing excised ovules and pollen grains in the same medium were overcoming the barriers to sexual incompatibility. The cultivation of plant tissue is a fast and effective technique used to multiply ex-situ, uses small shoots, and can succeed when other methods fail [23].

A lot of research has been carried out on the micropropagation of medical plants. For the organogenesis of *Aegle marmelos* by nucellar culture, Hossain [24] reports plantlet production of *Aegle marmelos* by nucleus callus culture [25]. In 1994, nodal explants of *Aegle marmelos* have been reported [26]. Nodal explants deriving callus culture have been reported in 2009 [27]. Direct organogenesis from cotyledon of *Aegle marmelos* has been reported [28]. Puhan and Rath show the culture of axillary bud formation of *Aegle marmelos* [29]. Recently Devi and Gopal cultured the direct regeneration from shoot tip [30, 31].

3. METHOD

3. 1. Preparation of culture media

In these experiments the MS medium was chosen. To prepare 1 liter of MS media, 50 ml of A & C 20X stock solution and 5 ml each of B & D's 200X stock solution were added. The medium was supplemented with 0.1 per cent Myo-inositol, 3.0 per cent sucrose, plant growth regulator, amino acids*, antibiotics*, vitamins*, for a final volume of up to 1000 ml. Media were fortified with different concentrations of growth regulators, such as 2,4-D, IAA, IBA, and NAA for callus induction, somatic embryogenesis, and root induction, and different concentrations of cytokinins, such as BAP, Kn, TDZ for shoot induction alone and in combination with both Auxin and Cytokinins. The pH of the medium was adjusted to 5.8 either with 0.1 N HCl or 0.1 N NaOH. Then 0.8% agar was added into the medium and medium was heated to boil to homogenize agar in the microwave oven. Then media were poured in culture bottles (50 ml × 300 ml) or 25X150 Borosil test tubes or Phyta jars. After pouring the medium, the test tube was plugged with non-absorbent cotton plug or bottles were tightly capped and labelled properly.

3. 2. Sterilization of culture media

After plugging the culture tube with a non-absorbent cotton plug wrapped with the muslin cloth, the culture bottle was tightly capped. Media were steam-sterilized at 15 lb/in, 121oc for 20 min in Autoclave. After autoclaving, the tubes were left to cool down and solidify, or placed in slanting stands to prepare the slants for leaves explants. The autoclaved media were stored in a cool and dry place until further use.

3. 3. Collection of plant materials

The nodes, internodes, shoot tip, and leaves were selected as explants. Young leaves or fourth nodes were taken as explants from healthy and disease-free *Aegle marmelos* plants from the University staff colony, Sardar Patel University, and the campus of BRD School of Bioscience, Sardar Patel University in the morning time. For the axillary shoot, proliferation nodes were collected from healthy *Aegle marmelos* buds containing only a single node, were cut and leaves were removed from it before inoculation**.**

3. 4. Establishment of aseptic culture

3. 4. 1. Pretreatment of Explants

The collected explants were washed thoroughly in running tap water for 30 minutes. Then explants were washed with 5-10% liquid soap (Himedia) for 5-10 minutes, followed by 30 minutes washing in running tap water. Then explants were washed with 1% bavistine solution for 15 minutes, followed by washing in running tap water. Then explants were washed with distilled water twice to remove any trace of fungicides.

3. 4. 2. Surface Sterilization

For surface sterilization, sodium hypochlorite was used. The explants were sterilized with 4% sodium hypochlorite solution for 10 minutes. Then explants were rinsed with sterile distilled water for 2-3 times inside the laminar airflow. For the microbe-free explants, they were washed with the antibiotic solution for 15-20 minutes.

3. 4. 3. Inoculation of explants on to medium

All transfer operations were carried out under strict aseptic conditions. The laminar airflow cabinet was sprayed with 70% alcohol before use. The surface of all the vessels and other accessories, such as sprit lamps, lighter, were cleaned with alcohol. Forceps, Scaple, and blade were normally autoclaved before inoculation. Hands and arms were wiped with alcohol or sprit or sanitizers before inoculation. Sterilized explants were excised from both ends, using a fine sterile forceps and Scaple, and the rims of the sugar tubes or mouth of bottles were flame sterilized, and then the explants were inoculated into respective media in culture tubes. The culture tubes were closed with cotton plugs. The necessary care was taken to avoid any contamination.

3. 5. Maintainance of culture

All culture was maintained at 25 ºC. The source of illumination consisted of 2.5 feet wide 40 watt fluorescent tubes. The intensity of illumination was 2500-3500 LUX at the level of cultures and a 14-18-hour light regime was followed by 6-10-hour darkness. The cultures are maintained at 60% relative humidity.

3. 5. 1. Subculture

Subcultures are carried out regularly at an interval of 3-4 weeks depending upon growth stages. Subculture is required for further shoot multiplication and elongation. In the case of callus culture, to maintain it, it has to be subcultured after 2-3 weeks. For callus culture subculture is required for organogenesis and somatic embryogenesis.

3. 5. 2. Shoot induction and multiplication

Nodal segments were placed on MS media provided with various concentrations of cytokinin, it was also provided in combination with auxins in lower concentrations. Sub-culturing was done every 3 weeks on the same medium. The number of shoots per explants, shoot length, and bud breakage was recorded after 6 to 12 weeks of culture. A minimum of 12 replicates was tested and the experiments were repeated twice.

3. 6. In-vitro rooting

Healthy and elongated shoots of 3 to 4-cm were cut from the culture and transferred to rooting media composed of ½ MS media supplemented with different concentrations of auxins. Data were recorded after 3 to 4 weeks of transfer.

3. 7. Observation and data analysis

The data were recorded after 15-20 days of inoculation. The experiments were performed with 10 replicates per treatment. The data were on bud breakage, number of shoots, shoot length; the number of roots, and percentage response were calculated. The percentage response for bud break was quantified using the following formula:

$$Response = \frac{\text{Total number of shoots responded in bud break}}{\text{Total number of replicates}} \times 100\%$$

The rate of multiplication represents the number of shoots produced per explant on a medium after number of days of its inoculation is mentioned in results.

4. RESULTS

The present studies focus primarily on high-frequency axillary proliferation and callus mediated micropropagation from *Aegle marmelos* for the development of phytochemical substances of medicinal importance. Previous studies on *Aegle marmelos* micropropagation for clonal spread exist [25, 27, 28, 32-35]. 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.

4. 1. Effect of Various Cytokinins in the Nodal Explant of *Aegle marmelos*

Nodal explants were placed into vessels containing sterilized MS media [36], after treating with 4% NaOCl, 40 mg/l citric acid, and a combination of antibiotics. The duration of treatment is important, long duration of treatment will damage the explants or can cause browning [37]. The media are augmented by different cytokinin concentrations; exogenous cytokinins facilitate the multiplication of axillary fired. After 7-8 days of inoculation, a split of the nodal bud was observed. The percentage response and induction of multiple shooting are high when explants are inoculated in such a way that the node contacts the media.

4. 1. 1. Effect of BAP in the nodal explant of *Aegle marmelos*

Different concentrations of BAP were used, i.e. 1-5 mg/l to study the effect of shoot multiplication in *Aegle marmelos* nodal explants, and 1, 2 and 3 mg/l BAP were observed to be effective [38]. Among these three, 2.0 mg/l BAP more effective with 98.5% response and 2.1 average numbers of shoots were observed (**Table 1**). Bud breakage was observed after 6-7 days of inoculation.

Table 1. Effect of various cytokinins on nodal multiplication of *Aegle marmelos* (L.) Corrêa

Concentration of BAP, mg/l	No. of Replicates	Response (%)	Average No. of Shoots	S.D.
1	24	87.5	1.54	0.93
2	24	96	2.1	0.9
3	24	91.6	2.1	1
4	24	67	0.8	0.7
5	24	58	0.9	0.9
Concentration of Kn, mg/l				
1	24	71	0.9	0.8
2	24	62	0.83	0.76
3	24	67	1	0.8
4	24	70	1	0.8
5	24	58	0.8	0.8
Concentration of TDZ, mg/l				
1	24	63	0.8	0.7
2	24	70	1.2	1
3	24	75	1.1	1
4	24	87	1.2	0.7
5	24	75	1	0.7
Concentration of 2IP, mg/l				
1	24	75	1.2	0.9
2	24	63	0.9	0.8
3	24	70	0.9	0.7
4	24	79	1.2	0.9
5	24	62	0.9	0.9
Concentration of Zeatin, mg/l				
1	24	71	0.9	0.8
2	24	87.2	1	0.6
3	24	87	1	0.7
4	24	75	0.8	0.6

5	24	71	0.9	0.7
Concentration of metatropolin, mg/l				
1	24	70.8	0.98	0.75
2	24	75	1.04	0.8
3	24	79	1.92	0.88
4	24	83	2.01	0.73
5	24	71	1	0.07

4. 1. 2. Effect of Kinetin in the nodal explant of *Aegle marmelos*

Different concentrations of kinetin (Kn) were used for induction of shoot multiplication in nodal explant of *Aegle marmelos*. It was observed that among the five conc. of Kn, 1, 3 and 4 mg/l are effective (Ajithkumar and Seeni 1998), in which 4.0 mg/l gave the best result with 70% response (**Table 2**). Bud breaking was observed after 10-12 days of inoculation.

Table 2. Effect of additives in axillary shoot multiplication in *Aegle marmelos* (L.) Corrêa

	Replicates	**Response (%)**	**Average No. of Shoots**	**S.D.**
1.0 mg/l BAP with AdSO$_4$				
10	18	61	1.11	1.02
20	18	83	0.94	0.53
30	18	56	0.83	0.85
40	18	61	0.94	0.93
2.0 mg/l BAP with AdSO$_4$				
10	18	56	0.78	0.80
20	18	95	2.67	1.41
30	18	89	1.44	1.24
40	18	89	1.05	0.53
1.0 mg/l BAP with Tryptophane				
10	18	78	1.11	0.75
20	18	100	1.22	0.42

30	18	89	1.22	0.64
40	18	83	1.38	1.03
2.0 mg/l BAP with Tryptophane				
10	18	89	1.27	0.75
20	18	94	1.05	0.41
30	18	89	1.05	0.63
40	18	100	1.16	0.38
1.0 mg/l BAP with Glutamine				
10	18	78	0.77	0.42
20	18	94	1	0.34
30	18	95	1.61	1.24
40	18	100	1.38	0.60
2.0 mg/l BAP with Glutamine				
10	18	100	1.11	0.32
20	18	94	1.22	0.64
30	18	94	1.16	0.51
40	18	95	1.27	0.66
1.0 mg/l BAP with Proline				
10	18	95	1.47	0.71
20	18	100	1.33	0.59
30	18	100	1.72	0.66
40	18	100	1	0
2.0 mg/l BAP with Proline				
10	18	94	1.38	0.69
20	18	100	1.16	0.38
30	18	100	1	0
40	18	100	1.55	0.51
1.0 mg/l BAP with Arginine				
10	18	100	1.61	0.84

20	18	100	2.05	1.10
30	18	100	1.11	0.32
40	18	100	1.22	0.42
2.0 mg/l BAP with Arginine				
10	18	100	2.16	1.15
20	18	100	2.52	1.32
30	18	100	2.33	1.28
40	18	100	1.33	0.59
2.0 mg/l BAP with combination of Tryptophane, Glycine, Proline, Glutamine, Lycine and Metheonine, Glutamic acid				
10	18	95	1.72	0.82
20	18	100	2.22	0.64
30	18	95	1.89	1.02
40	18	95	1.11	0.47

4. 1. 3. Effect of TDZ in the nodal explant of *Aegle marmelos*

Concentrations ranging from 1-5 mg/l of TDZ were used to study the multiple shoot induction in *Aegle marmelos* [39]. Out of these five concentrations 2, 3 and 4 mg/l were effective (**Table 1**). Among these three, 4.0 mg/l is more effective with 87% response and 1.2 average no. of shoots. Bud breaking was observed after 13-14 days of inoculation.

4. 1. 4. Effect of 2IP in the nodal explant of *Aegle marmelos*

1-5 mg/l ranges of 2IP were used for the multiple shoot induction in *Aegle marmelos* [40]. It was observed that 1 and 4 mg/l are effective. Out of these, 4.0 mg/l is more effective with 79% response and 1.2 average no. of shoot (**Table 1**). Bud breaking was observed after 13-14 days of inoculation.

4. 1. 5. Effect of Zeatin in the nodal explant of *Aegle marmelos*

Different concentrations of zeatin were used for the induction of shoot multiplication in nodal explants of *Aegle marmelos* [41]. It was observed that 2 and 3 mg/l are more effective (**Table 1**). Among these two, 2.0 mg/l is more effective, having 87.2% response and 1.2 average no. of shoots. Bud break was observed after 15 days of inoculation.

4. 1. 6. Effect of Metatropolin in the nodal explant of *Aegle marmelos*

1-5 mg/l ranges of metatropolin were used for the multiple shoot induction in *Aegle marmelos*. It was observed that media having concentrations. of 2, 3 and 4 mg/l of metatropolin are effective (**Table 1**) in which 4.0 mg is more effective with 83% response and 2.01 average shoot no. Bud breakage was observed after 9-10 days of inoculation.

As shown above, BAP and metatropolin are most effective in the nodal explants of *Aegle marmelos*. BAP having concentration of 1.0 mg/l, 2.0 mg/l and 3.0 mg/l are effective. Metatropolin having concentration of 3.0 mg/l and 4.0 are effective.

4. 2. Effect of various additives in the axillary shoot proliferation of *Aegle marmelos*

There are different additives supplemented along with the cytokinins. Additives such as $AdSO_4$ and amino acids, i.e. Proline, Glutamine, Tryptophane and Arginine were used [42, 43]. The additive media showed rapid growth in plant and multiple shoot development. Bud time to break is reduced to 5-6 days of inoculation. The results of various additives, are as follows:

4. 2. 1. Effect of $AdSO_4$ with BAP

BAP was used with the additive because it gave good results in previous experiments. 1.0 mg/l and 2.0 mg/l of BAP were used with 10, 20, 30, and 40 mg/l $AdSO_4$. The media having 2.0 mg/l BAP and 20 mg/l and 30 mg/l $AdSO_4$ were effective. Among them, 2.0 mg/l BAP with 20 mg/l $AdSO_4$ gave the best results with 95% response and 2.67 average no. of shoots per explant (**Table 2**).

The time of bud breakage was greatly reduced to 6-7 days of inoculation. $AdSO_4$ was used in cultures from which plant regeneration is required. It seems sometimes to improve growth or to bring about or reinforce responses normally attributed to cytokinin action.

4. 2. 2. Effect of Glutamine with BAP

Glutamine is an amino acid that is supplemented with BAP having a range of 10-40 mg/l. The concentration of BAP, 1.0, and 2.0 mg/l was used. It was observed that the combination of 1.0 mg/l BAP with 30 mg/l and 40 mg/l Glutamine was effective on *Aegle marmelos*. Among them, 1.0 mg/l BAP with 30 mg/l Glutamine is the best for Axillary shoot proliferation. It shows 95% response and 1.65 average shoot numbers per explant (**Table 2**).

Glutamine is important because of its structural role in protein synthesis. It also has a significant role in NO_3- uptake and is found in Xylem and Phloem inconsiderable amount.

4. 2. 3. Effect of Tryptophane with BAP

10-40 mg/l Tryptophane were supplemented with 1.0 mg/l and 2.0 mg/l BAP. It was observed that the composition of 1.0 mg/l BAP with 40 mg/l Trypytophane and 2.0 mg/l BAP with 10 mg/l Tryptophane were effective. Among them, 1.0 mg/l BAP with 40 mg/l Tryptophne are more effective, with 83% response and 1.3 average shoot no., other combinations also gave great response (Table 2).

Tryptophane is an aromatic amino acid and it is the precursor of the IAA biosynthesis pathway. Tryptophane provides a basic indole ring in the Auxin synthesis. Tryptophan is also important for the pollen fertility of the plant. Tryptophan plays a structural role in protein synthesis.

4. 2. 4. Effect of Proline with BAP

1.0 mg/l and 2.0 mg/l of BAP were selected with 10-40 mg/l Proline and supplemented it with basal media. It was observed that the combination of 1.0 mg/l BAP with 30 mg/l Proline is effective with 100% response and 1.7 average shoots no. (**Table 2**). Proline is needed for pollen fertility and protein synthesis.

4. 2. 5. Effect of Arginine with BAP

Arginine with a range of 10-40 mg/l was supplemented with 1.0 mg/l and 2.0 mg/l BAP in Media. It was observed that 20 mg/l arginine is effective with both 1.0 and 2.0 mg/l BAP. Among them, 2.0 mg/l BAP with 20 mg/l Arginine is best for axillary shoot proliferation. It showed 100% response and 2.5 average no. of shoots per explant (**Table 2**).

Arginine is essential for protein synthesis. It also has a significant role in NO_3- uptake.

4. 2. 6. Effect of combinations of amino acid with 2.0 mg/l BAP

Different concentrations of amino acids, i.e. Methionine, Glutamic acid, Glycine, Tryptophane, Proline, Lycine, Arginine, Glutamine were used in the range of 10-40 mg/l with 2.0 mg/l BAP. It was observed that the combination of all amino acid in 20 mg/l with 2.0 mg/l BAP are effective, having 100% response and 2.22 average shoot length per explant (Table 2). Glycine helps in the formation of vegetative tissues, synthesis of phytochrome, photosynthesis, pollen fertility, and it also acts as a chelating agent.

Methionine helps in protein synthesis, synthesis of growth substance, pollen fertility, and activator of phytochromes.

Glutamic is important for the Opening of Guard cells of stomata, pollen fertility, and formation of vegetative tissues. Lysine helps in pollen fertility. According to the combination of the above results of Arginine with BAP and BAP with a mixture of amino acid is best for the axillary shoot proliferation of *Aegle marmelos* (**Table 2**).

4. 3. Subculture of *Aegle marmelos*

In the fresh media, the callus and shoots are subcultured daily. The callus is subcultured for organogenesis in BAP media of 0.5 mg/l. Shoots are also subcultured for the development of multiple shoots and shoot elongation. The shoots are moved to new media within a 15-20 day period (**Figure 1**).

The multiple shoots are produced in the different concentrations of cytokinins and Auxins, and then the individual shoots are transferred to the rooting media.

Shoots elongation Nodal explants were transformed into the medium elongation (MS + various concentrations of BA, KN, and GA3 either or in combinations) to restrict defoliation and callusing with healthy shoot growth. At fortnightly intervals, the cultures were subcultured three times by moving to fresh media (**Figure 2**).

The assessments were made in terms of the number of shoots per explant inducing healthy growth, shooting duration after 4 and 6 weeks of culture, and the number of nodes per shoot.

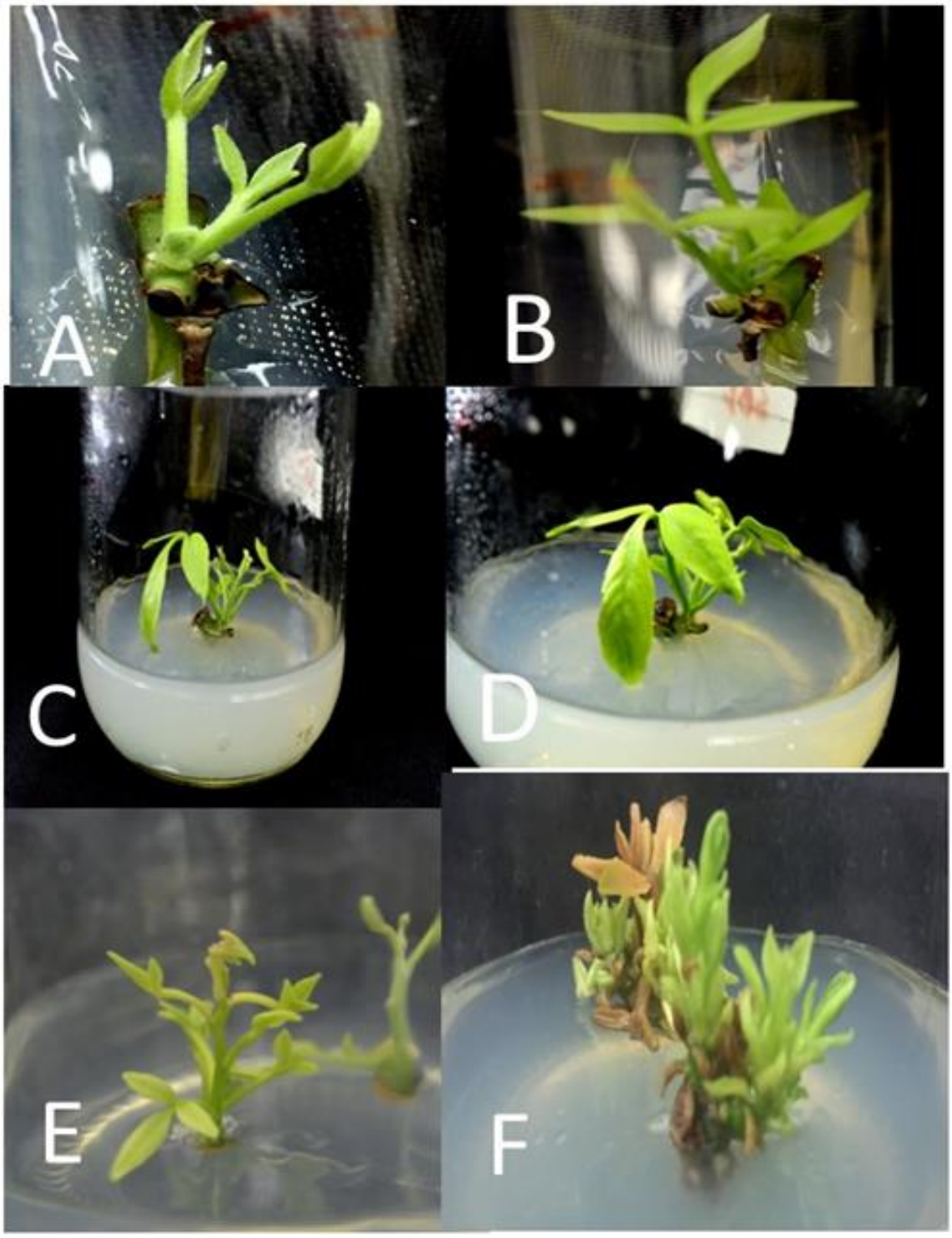

Figure 1. In vitro culture of nodal explant of *Aegle marmelos.*
A: Bud breaking, **B-D:** Growth of axillary shoots on MS media, **E:** Subculture in fresh madia, **F:** Initiation of multiple shoots.

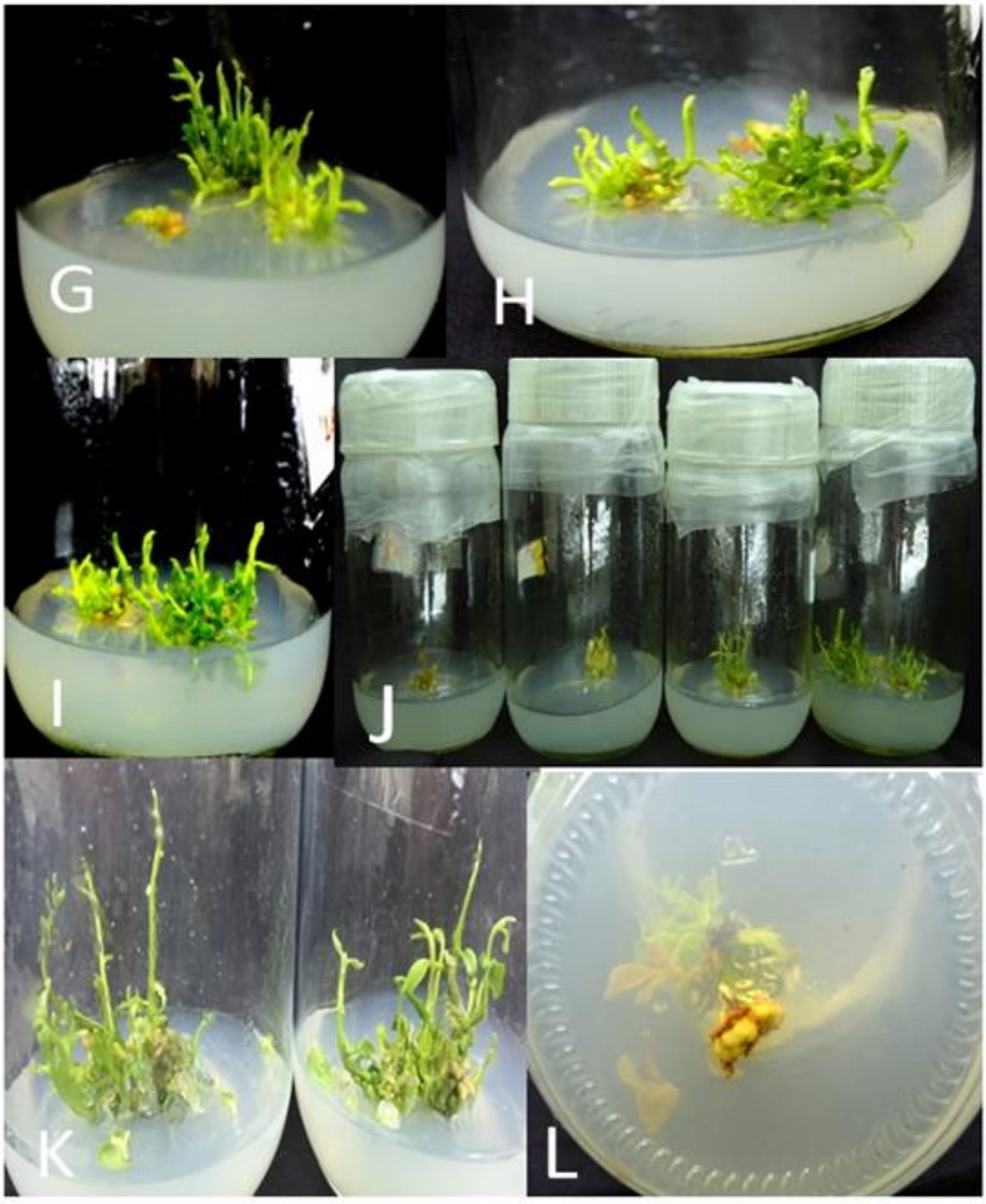

Figure 2. Stages of multiple shoot generation.
G-I: Multiple shoots genetarion, **J:** Different stages of multiple shoot generation, **K:** Developed multiple shoot, **L:** Wound in the end of the shoot.

4. 4. Rooting of *Aegle marmelos*

After the multiple shoot generation (**Figure 2**), individual shoots were placed in ½ MS media containing a high concentration of auxins for in-vitro rooting [44]. Shoots were excised (2 cm or longer) and moved into rooting media. The rooting medium was replaced by the basal medium MS (1962) with different NAA concentrations. The roots have been established with 0.4 mg/l NAA with a ½ MS media from the shoot base. The base of the shoot first develops a wound, develops from this wound roots. Roots were established after 40 days of transferring into rooting media.

Hardening after 4 weeks of cultivation, the rooted plantlets were the approximately washed with tap water and dipped for 5 minutes in an anti-fungal solution 0.1 per cent (w/v) of Bavistin, then washed and planted in pots (5 cm diameter) containing autoclaved artificial soil and provided 4 weeks of sterile distilled water. Established plants were repotted in polythene bags (20 cm or 10 cm) filled with autoclaved sand, soil/red earth and farmyard manure in the proportions of 1:1:1 and then in larger pots (25 cm diameter) containing non-sterile sand:soil:compost mixture (1:3:1) and kept under shade in the garden for another 3 weeks and watering as and when required.

5. CONCLUSIONS

An efficient protocol was introduced for the fire multiplication and also for callus induction. *Aegle marmelos* is an important medicinal herb. It has many properties such as antibacterial, antifungal, antidiabetic, and antioxidant. Nodal explants were taken to multiply shoots, and leaves were taken to induce the callus. The segment of nodal stems was found to be the strongest explant. The explanation for the nodal stem segment's suitability was the existence of covered axillary buds that do not get affected during surface sterilization. The segment of nodal stems proved to be a stronger explant (*Aegle marmelos*) as suggested by Ajithkumar and Seeni (1998). Because the significant seasonal fluctuations influence the explant viability in culture, the establishment of aseptic bael cultures was not possible all year round. The season for collecting plants is an important factor in the establishment and growth of *in vitro* cultures [45, 46]. The effect of the explant size on crop growth and cultural differentiation was documented in *Aegle marmelos* and *Tacomela undulate*. Various cytokinins and auxins were used. BAP 2.0 mg/l which had 92 per cent response and produced around 1.6 average shoots was best used among various cytokinins. Cytokinin BAP was was the most effective in inducing multiple shootings. However, a mixture of an auxin IAA with BAP augmented the multiplication of shoots. The synergetic effect of auxin along with cytokinins on shoot multiplication and shoot bud induction has been reported by several workers [33, 47-49]. For callus formation, numerous mixtures of auxins and cytokinins were used.

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