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Comparison of Different Medium and Establishment of an Efficient Micropropagation Technique of *Clerodendrum Serratum* L. An Endangered Medicinal Plant

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Abstract: *Clerodendrum serratum* L. *is an important medicinal plant, used in many ayurvedic preparations. Medicinal importance of this plant caused it's as much as exploitation become endangered species. In the present study we have developed an efficient protocol for in vitro micropropagation of Clerodendrum serratum to conserve its natural resources. Four plant growth media were used in the study viz. MS, SH, WPM & B5. In all these media MS medium gave best shoot bud induction and shoot multiplication. All the media were supplemented with different concentration of three different cytokinin viz. BAP (6-Benzylaminopurine), KIN (Kinetin), TDZ (Thidiazuron). BAP at lower concentration of 0.5 mg/L gave best shoot bud induction and multiplication. Effects of all cytokinin was similar at higher concentration, where they show declining effect in shoot bud induction and multiplication. For root development three auxin viz. NAA (α-Naphthalene acetic acid), IBA (Indole- 3- butyric acid) & IAA (Indole- 3 acetic acid) alone and in combination with BAP were used. At higher concentration root development declines in all hormones whereas at lower concentration of 0.5 mg/L, NAA gave best root development. After formation of complete plantlets, they were acclimatized in the green house. Their survival rate was 73%.*

I. Introduction

Plants are the basis of life that's why they are being worshiped in all religion from ancient time. Around 1400 plants are presently being used in diverse ayurvedic medicines preparation. *Clerodendrum serratum* L. is one of the important medicinal plants of Lamiaceae family. It is being used in many countries of the world from ancient time. It is a shrub having maximum height of 3-8 ft, with hollow quadrangular stems and not having much branching. It is native of East India and Malaysia but now distributed throughout the forests of Sri Lanka and India. Flowering in *Clerodendrum serratum* can be seen in the months from August-September. The leaf and root of this plant have much medicinal value. According to the traditional knowledge roots of this plant are the good source of drugs for diseases like "asthma, bodyache, bronchitis, cholera, dropsy, eye diseases, fever, inflammations, malaria, ophthalmia, rheumatism, snakebite, tuberculosis, ulcers and wounds" (Keshavamurthy, 1994). It is one of the few medicinal plants that show antagonistic effect on histamine (Chopra et al., 1956). Ethanolic extract of the root is accounted for antinociceptive, anti-inflammatory and antipyretic activities (Narayanan et al., 1999).

Medicinal importance of this plant fascinated the people towards it and causes its unrestricted exploitation, as well as limited cultivation and inadequate attempts for its reforestation, which leads to the depletion of the wild stock of this plant. *Clerodendrum serratum* L. is one of the threatened species according to Chhattisgarh Medicinal Plant Board. Therefore these plants need to be protected and conserved through various approaches. In vitro multiplication/ micropropagation technique is very much inevitable for the multiplication and conservation of these plants. In vitro micropropagation is a technique of growing plant cells, tissues, organs, seeds or other plant parts in a sterile environment on a nutrient medium. It has many advantages over traditional breeding/ cultivation like fast commercial propagation, fast selection for improvement of crop's nutritional value, pest control, production of virus free plants and production of pharmaceutically important secondary metabolites. A protocol was developed for in vitro cloning of *Clerodendrum serratum*on on MS media by using nodal explants by Sharma et al., 2009. In another experiment Vidya et al., 2012 developed an efficient tissue culture and plant regeneration protocol for *Clerodendrum serratum* L. by node and internode explants on LM media. In the present study we have compared different plant growth media and plant growth hormone for standardization of an efficient micropropagation technique for mass production of *Clerodendrum serratum* by using nodal explants. In this technique small portion of shoot including node which contains axillary bud is cultured in vitro for the multiple shoot formation. This method can be used for the production of clones and prevention of somaclonal variations. The nutritional requirements and culture conditions for each developmental

stage are species specific. Meristem and shoot bud culture technique has been extensively studied by Hu and Wang (1983).

II. Materials and Methods

2.1. Chemicals:

All the chemicals used were of analytical grade and purchased from Hi- Media, S.D. Fine chemicals, Merck, Sigma chemicals and Qualigens.

2.2. Collection of plant material:

Plants were collected from the nursery of Energy Park Raipur (C.G.) India, identified and planted in the botanical garden of Pt. Ravishankar Shukla University Raipur (C.G.) for further use. For micropropagation, young shoots were collected and each shoots were cut into small pieces (about 1.5cm long) having a nodal portion in between with axillary bud. These shoot pieces were used as explants.

2.3. Surface sterilization of explants:

Explants were washed under running tap water to remove dust particles then dipped in aqueous Laboline detergent (1%) for 10 min and rinsed three times in sterile water. For sterilization explants were dipped in the 0.1% mercuric chloride solution for 10 min then washed 3 times with sterile distilled water. Excess water on the explant was removed by using sterile tissue paper before culturing.

2.4. Shoot bud induction and multiplication:

Sterilized explants were inoculated into four different basal media viz. MS medium (Murashige and Skoog 1962), Schenk and Hildebrandt medium (SH) **(**Schenk and Hildebrandt, 1972), Woody Plant Medium (WPM) (Lloyd and McCown, 1980) and B_5 medium (Gamborg et al. 1968). All the media were supplemented with different concentration (0.5mg/L, 1.0mg/L and 2.0mg/L) of cytokinin (BAP, KIN, TDZ). Media containing no hormone were used as a control. Cultures were kept for five weeks at 25 ± 2^{o}C, under 16h/8 h light and dark period. Light intensity was 100µE/m^2 provided by cool- white fluorescent light. Experiments were repeated three times each with ten replicates. Percent shoot bud induction, number of shoots each explants and shoot length (cm) was recorded after five weeks of inoculation.

2.5. Rooting of in vitro shoots:

To optimize the rooting medium half strength of MS medium supplemented with different concentration (0.5mg/L, 1.0mg/L and 2.0mg/L) of auxin (NAA, IAA and IBA) and cytokinin (BAP) alone and their combinations were used. Half strength MS medium without plant growth regulators were used as control. Twenty in vitro shoots were transferred to each type of medium combination for induction and development of roots. Cultures were kept for 16h/8 h light and dark period at 25 ± 2^{o}C. Percent root induction, number of roots and root length was measured after 6 weeks of culture. Plantlets with well- developed roots were later kept for acclimatization.

2.6. Hardening and acclimatization:

Healthy plants with well developed roots were taken out of the culture room and kept for hardening at 25-32^{o}C temperature, light (2000 lux) and humidity (70- 80%) in green house under diffused sunlight. The plants were carefully removed from culture vessels without damaging the roots. It was washed in sterile double distilled water to insure removal of traces of agar. Then plants were transferred to the plastic cups containing sterilized vermiculite and placed in the green house.

2.7. Statistical analysis:

Data were analyzed by the analytical software SPSS (version 16). Mean difference of all the treatments were compared by one way analysis of variance (ANOVA) and significant difference between the treatments were analyzed using Duncan's multiple range test at 5% significance level. Shoot number and root number was non parametric data, so they were subjected to log transformation before analysis, to make the data parametric.

III. Results

3.1. In vitro shoot induction:

The effects of different media MS, WPM, B5 and SH supplemented with different types and concentration of cytokinin on shoot bud induction and shoot parameters are summarized in fig.1 and table 1 respectively. All the data are represented as mean ± SE. Maximum percentage of shoot induction (90%) was achieved in MS medium supplemented with 0.5mg/L BAP (fig 3a & 3b). Shoot bud induction was observed in 5 – 8 days of culture. Higher concentration of BAP lowers the percent shoot bud induction. This phenomenon was observed in KIN and TDZ also. At higher concentration of cytokinin shoot bud induction declines.

Shoot number and shoot length was observed after five weeks of culture (fig. 3c & 3d). Number of shoots was highest in MS medium supplemented with 0.5mg/L BAP (3.3 ± 0.04) followed by MS medium supplemented with 0.5mg/L KIN (2.0 ± 0.04). Number of shoots is very less in WPM medium supplemented with 2.0mg/L BAP. Effects of all three medium viz. WAP, SH and B5 is almost similar when supplemented with lower concentration of hormones but varies greatly at higher concentration. Shoot length was also highest (4.52 ± 0.32 cm) in MS + 0.5mg/L medium and least in WPM + 2.0mg/L BAP medium. Effect of all three hormones shows similar trend at lower and higher concentration. BAP shows significantly higher effect at lower concentration on shoot bud induction, number of shoots and shoot length. There was no response of shoot bud in the control for all four types of media.

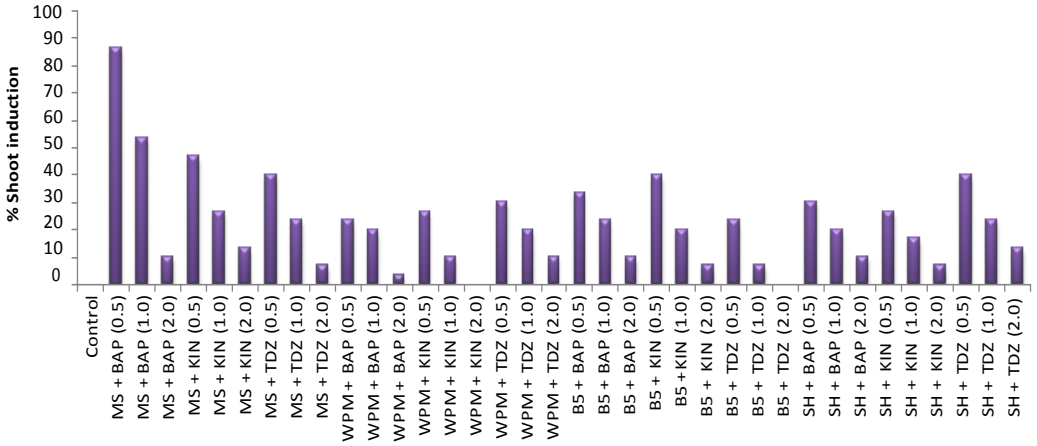


Fig. 1: Effects of different plant growth media and growth hormones on % shoot bud induction

Table 1: Effects of different plant growth media and growth hormones on shoot number per explants and shoot length (cm)

S.N.	Treatments	Shoot number per explants (Mean ± SE)	Shoot length (cm) (Mean ± SE)
1	MS + BAP (0.5)	3.34 ± 0.04^{a}	5.22 ± 0.34^{a}
2	MS + BAP (1.0)	2 ± 0.04^{b}	3.83 ± 0.33^{bc}
3	MS + BAP (2.0)	1.33 ± 0.02^{ghij}	2.8 ± 0.16^{hij}
4	MS + KIN (0.5)	2 ± 0.04^{bc}	5.27 ± 0.44^{b}
5	MS + KIN (1.0)	1.62 ± 0.03^{defghi}	3.62 ± 0.30^{defghi}
6	MS + KIN (2.0)	1.25 ± 0.02^{ghij}	2.8 ± 0.18^{ghij}
7	MS + TDZ (0.5)	1.75 ± 0.04^{bcde}	3.16 ± 0.30^{cdef}
8	MS + TDZ (1.0)	1.28 ± 0.03^{efghij}	3.13 ± 0.25^{efghij}
9	MS + TDZ (2.0)	1 ± 0.01^{hijk}	0.6 ± 0.12^{hij}
10	WPM + BAP (0.5)	1.28 ± 0.03^{efghij}	2.93 ± 0.24^{efghij}
11	WPM + BAP(1.0)	1.5 ± 0.03^{efghij}	2.78 ± 0.21^{efghij}
12	WPM + BAP (2.0)	1 ± 0.01^{ij}	2.3 ± 0.08^{i}
13	WPM + KIN (0.5)	1.62 ± 0.03^{defghi}	3.26 ± 0.27^{defghij}
14	WPM + KIN (1.0)	1.33 ± 0.02^{ghij}	2.57 ± 0.14^{hij}
15	WPM + (2.0)	NR	NR

16	WPM + TDZ (0.5)	1.77 ± 0.04^{cdefg}	3.21 ± 0.28^{defghi}
17	WPM + TDZ (1.0)	1.16 ± 0.02^{fghij}	2.85 ± 0.21^{efghij}
18	WPM + TDZ (2.0)	1 ± 0.02^{ghij}	2.33 ± 0.13^{hij}
19	B5 + BAP (0.5)	1.9 ± 0.04^{bcdef}	4 ± 0.36^{cde}
20	W5 + BAP (1.0)	1.43 ± 0.03^{defghij}	3.13 ± 0.25^{efghij}
21	W5 + BAP (2.0)	1.66 ± 0.02^{ghij}	2.57 ± 0.14^{hij}
22	W5 + KIN (0.5)	1.75 ± 0.04^{bcde}	4.08 ± 0.38^{bcd}
23	W5 + KIN (1.0)	1.33 ± 0.03^{efghij}	3.03 ± 0.23^{efghij}
24	W5 + KIN (2.0)	1 ± 0.01^{hijk}	2.3 ± 0.10^{ij}
25	W5 + TDZ (0.5)	1.71 ± 0.03^{defghij}	3.56 ± 0.28^{defghij}
26	W5 + TDZ (1.0)	1 ± 0.01^{hijk}	2.85 ± 0.13^{hij}
27	W5 + TDZ (2.0)	NR	NR
28	SH + BAP (0.5)	1.55 ± 0.03^{defgh}	3.92 ± 0.33^{defgh}
29	SH + BAP (1.0)	1.16 ± 0.02^{fghij}	3.16 ± 0.24^{efghij}
30	SH + BAP (2.0)	1.33 ± 0.02^{ghij}	2.53 ± 0.14^{hij}
31	SH + KIN (0.5)	1.5 ± 0.03^{defghi}	4.46 ± 0.37^{cdefg}
32	SH + KIN (1.0)	1.4 ± 0.03^{fghij}	3.1 ± 0.20^{fghij}
33	SH + KIN (2.0)	1 ± 0.01^{hijk}	2.35 ± 0.11^{ij}
34	SH + TDZ (0.5)	1.69 ± 0.04^{bcd}	4.32 ± 0.41^{bc}
35	SH + TDZ (1.0)	1.57 ± 0.03^{defghij}	3.17 ± 0.26^{efghij}
36	SH + TDZ (2.0)	1.25 ± 0.005^{ghij}	2.78 ± 0.18^{ghij}

Data shown are mean ± SE of three experiments, each experiment consist of 10 replicates. Means followed by same letter within each column are not significantly different at $p<0.05$ according to Duncan's multiple range test. NR: No response

3.2. In vitro rooting:

For all the shoot parameters MS medium gave best result so we used MS medium for rooting experiments. For in vitro rooting auxins (NAA, IAA, and IBA) and cytokinin (BAP) alone and in combination of different concentrations (0.5mg/L, 1.0mg/L and 2.0mg/L) were supplemented with half strength of MS medium. The results of rooting experiments have been summarized in fig. 2 and table 2. Highest number of root induction (85%) was observed in NAA 0.5mg/L supplemented with half strength of MS medium (fig. 3e). Root induction was observed in 14 to 17 days after inoculation. Root induction is better at lower concentration of all four hormones alone and in combination, and declining of root induction at higher concentration is also similar for all four hormones alone and in combination. IAA, IBA and BAP show similar effects on root induction (50%) at 0.5mg/L concentration. Very less root induction (15%) was observed in hormone free medium.

Highest mean root number (6.3 ± 0.10) was observed in 0.5mg/L NAA + half strength MS medium. Mean root length was also highest in the same medium viz. 5.37 ± 0.71 cm. All the hormones alone and in combination exhibit declining effects on root number and root length at higher concentration, but at lower concentration their effects varies greatly.

3.3. Hardening and acclimatization:

All the well developed rooted plants were shifted to the plastic pots containing vermiculite and placed in the green house. Their survival rate was 73%. The in vitro regenerated plants grew well in the green house without any phenotypic aberrations. After acclimatization in the green house they were shifted to field.

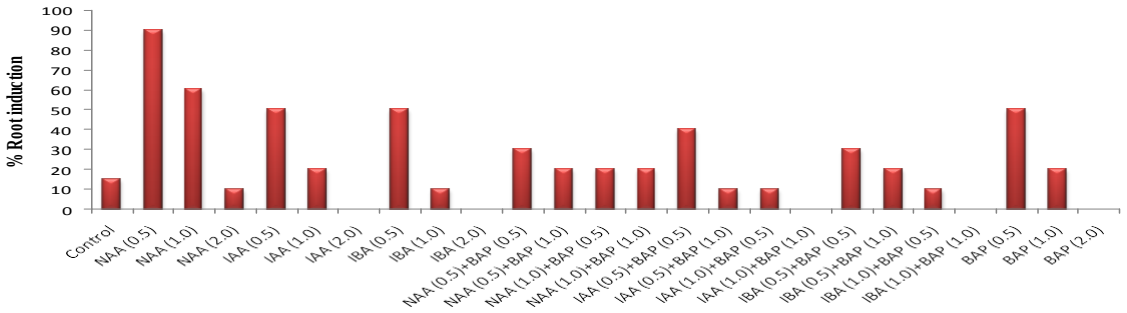


Fig.2: Effects of different plant growth media and growth hormones on % root induction

Table 2: Effects of different plant growth media and growth hormones on Root number per explants and Root length (cm)

S.N.	Treatments	Root number per explants (Mean ± SE)	Root length (cm) (Mean ± SE)
1	NAA (0.5)	7 ± 0.11 [a]	5.97 ± 0.72 [a]
2	NAA (1.0)	3.16 ± 0.11 [bc]	4.00 ± 0.76 [b]
3	NAA (2.0)	3.00 ± 0.06 [ef]	3.40 ± 0.34 [ef]
4	IAA (0.5)	3.6 ± 0.11 [bcde]	4.04 ± 0.72 [bcd]
5	IAA (1.0)	3.00 ±0.08 [cdef]	3.1 ± 0.42 [cdef]
6	IAA (2.0)	NR	NR
7	IBA (0.5)	4.00 ± 0.12 [bcd]	3.64 ± 0.65 [bcde]
8	IBA (1.0)	4.00 ± 0.07 [def]	2.7 ± 0.27 [ef]
9	IBA (2.0)	NR	NR
10	NAA (0.5)+BAP (0.5)	4.33 ± 0.11 [bcdef]	2.80 ± 0.48 [cdef]
11	NAA (0.5)+BAP (1.0)	3.5 ± 0.09 [cdef]	1.85 ± 0.25 [ef]
12	NAA (1.0)+BAP (0.5)	4.5 ± 0.10 [cdef]	2.45 ± 0.36 [def]
13	NAA (1.0)+BAP (1.0)	3.00 ± 0.08 [cdef]	3.40 ± 0.47 [cdef]
14	IAA (0.5)+BAP (0.5)	3.25 ± 0.10 [bcdef]	3.55 ± 0.60 [bcdef]
15	IAA (0.5)+BAP (1.0)	3.00 ± 0.06 [ef]	2.3 ± 0.23 [ef]
16	IAA (1.0)+BAP (0.5)	3.00 ± 0.06 [ef]	1.3 ± 0.13 [f]
17	IAA (1.0)+BAP (1.0)	NR	NR
18	IBA (0.5)+BAP (0.5)	3.67 ± 0.10 [bcdef]	4.47 ± 0.70 [bcdef]
19	IBA (0.5)+BAP (1.0)	2.50 ± 0.07 [cdef]	4.00 ± 0.53 [cdef]
20	IBA (1.0)+BAP (0.5)	2.00 ± 0.05 [ef]	3.8 ± 0.38 [ef]
21	IBA (1.0)+BAP (1.0)	NR	NR
22	BAP (0.5)	6.4 ± 0.14 [b]	4.32± 0.81 [bc]
23	BAP (1.0)	4.5 ± 0.10 [cdef]	4.15 ± 0.56 [cdef]
24	BAP (2.0)	NR	NR
25	Control	4.05 ± 0.09 [cdef]	4.20 ± 0.56 [cdef]

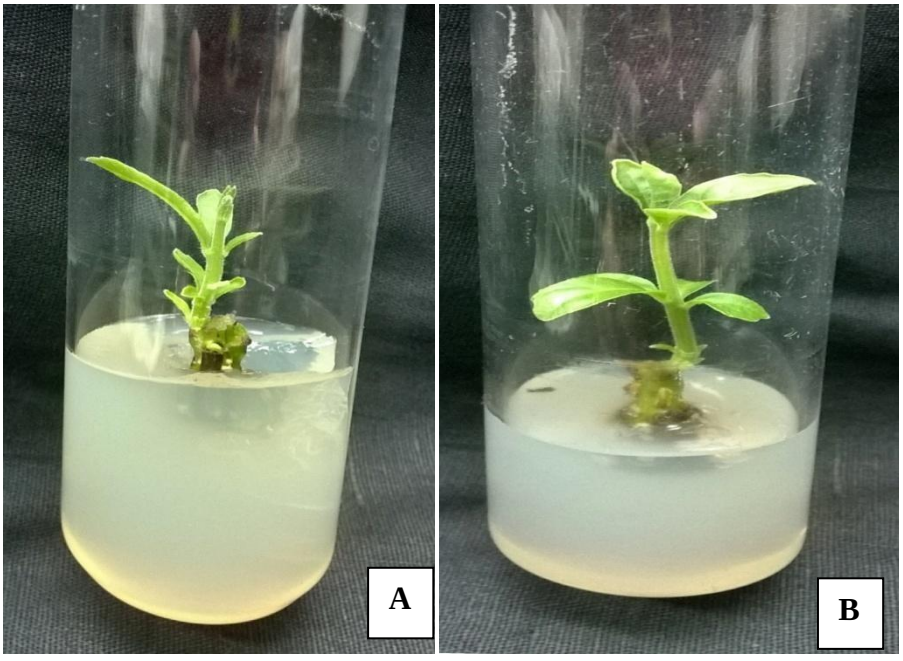
Data shown are mean ± SE of 20 replicates. Means followed by same letter within each column are not significantly different at $p<0.05$ according to Duncan's multiple range test. NR: No response**.**

IV. Discussion

Aim of the present experiment was to develop an efficient micropropagation technique of *Clerodendrum serratum* L. from shoot bud culture. This is a very important medicinal herbs mentioned in ayurvedic medicine and used to cure many diseases. Being an important medicinal plant it was exploited as much as it became endangered species according to Chhattisgarh medicinal plant board. So for conservation of endangered plant species in vitro micropropagation is an important biotechnological technique. As shoot bud

culture produces clonal plants same as parental plants, so we used shoot bud culture technique in this study. Results of this experiment show that MS medium is best in all four media for in vitro mass production of *Clerodendrum serratum* L. Same result was also given in *Saussurea involucrata* by Kuo et al., 2015. In all the three types of cytokinin used in this experiment BAP is best hormone then after KIN also gave better results. BAP at concentration of 0.5mg/ L is showing maximum shoot bud induction, shoot number and shoot length as also reported by Vidya et al., 2012. Effeciency of BAP over other cytokinin was also shown by Zaerr & Maps (1982) and Thomas & Blakesley (1987). Many other authors have also reported that BAP is a good shoot bud inducing hormone (Nadgauda et al., 1990; Chambers et al., 1991).

Results of root induction and other root parameters also support the result of Vidya et al., 2012. In all three types of auxin used in this experiment NAA proves better results at 0.5mg/L. In this experiment we have observed that BAP alone can also induce root initiation at lower concentration when apical meristem is present on the inoculated shoot. The ability of plant tissues to form adventitious roots depends on the interaction of many endogenous and exogenous factors. The role of auxins in root developments is well established and was reviewed by Torrey (1976). Efficient rooting of in vitro grown shoots is a prerequisite for the success of micropropagation. It is a common practice nowadays to employ lower salt concentration in rooting medium for rooting of in vitro shoots. Higher salt levels in the medium have often been found inhibitory to root initiation (George et. al., 2008). Many authors have reported that NAA play an important role in the rooting of in vitro shoots (Wei et. al., 2015). Findings of present study is contrary from the report of Dode et al., 2003 in which presence of NAA inhibited root formation whereas higher concentration of BAP induces higher number of shoots in *Ocimum basilicum* L. Present study provides an efficient protocol for mass production of *Clerodendrum serratum* L. so its natural resources can be preserved.

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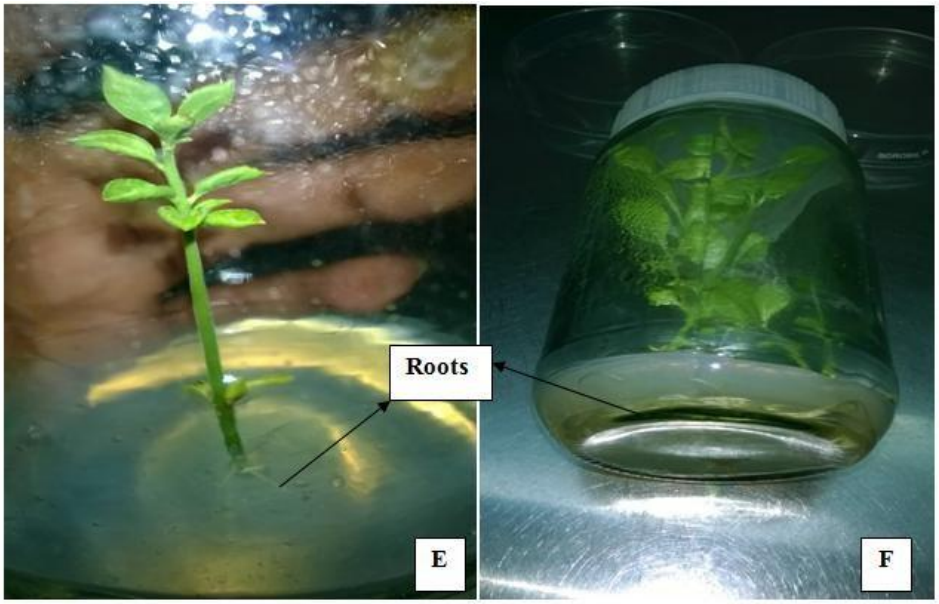
Roots
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F

Fig. 3 In vitro micropropagation of *Clerodendrum serratum* L. from nodal culture. (A & B) Induction of shoot bud. (C & D) shoot multiplication. (E) Root induction. (F) Well developed roots. (G) In vitro generated plantlets. (H) Hardening of in vitro generated plantlets.

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