

An efficient protocol for mass multiplication of *Centella asiatica* (L.) Urban and determination of its phenolic content

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ABSTRACT

The present study was focused on standardizing a protocol for callus induction as well as regeneration in *Centella asiatica* from leaf and stem as explants. Stem and leaf explants have been inoculated in B₅ media supplemented with BAP (0.1-2.5 mg/l), kn (0.1-0.4 mg/l) and NAA (0.1-0.5 mg/l), 2, 4-D (0.2mg/l) for callus induction. The combination of BAP and NAA leads to the formation of green, brown, compact and friable calli while Kn and 2, 4-D induced brown calli. Highest shooting was obtained from BAP (1.5 mg/l) and NAA(0.5 mg/l). When the shoots were inoculated in half strength of B₅ media fortified with 0.1 mg/l BAP and 0.5 mg/l NAA showed cent percent rooting with the highest number of roots per shoot (11.05 cm) and maximum root length (1.86 cm). Stem showed the best explants for callus induction as compared to leaf explants. A low concentration of plant growth regulators was unable to induce callus response in leaf and stem explants. Phenolic content analysis showed that calluses contain more amounts of phenol (0.81 mg/gmdw) as compared to both leaf (0.63 mg/gmdw) and stem (0.59 mg/gmdw) explants.

KEYWORDS: *Centella asiatica*, *In vitro* propagation, Phenol content

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INTRODUCTION

Plants are natural source of medicine and used in curing diseases. The medicinal plants have been used as the herbal remedies. At present use of natural products in treating diseases are increasing. Herbal drugs have achieved much popularity due to increasing awareness towards personal health which leads to maintained health through the natural products [1]. The high public demands of these medicinal plants required its conservation through the micropropagation as well as enhancement of secondary metabolites through the callus culture, suspension cultures and treatment of elicitor's molecule [2]. *Centella asiatica* which is also known as Indian pennywort are small herbaceous annual plant widely distributed in India, Malaysia, Sri Lanka, Indonesia, and other parts of Asia [3,4]. The plant belongs to the family Apiaceae [5]. The extract of *Centella asiatica* is used for wound healing [6] as well as various skin diseases viz: leprosy, lupus, varicose ulcer, eczema, psoriasis and female genital urinary tract infections [7]. Ethanol extract of *Centella* has been well elucidated for antibacterial activity [10]. Apart from antibacterial activity, essential oil of *C. asiatica* is endowed with antimicrobial activity [9,11]. Crude methanolic extract as well as hydroalcoholic activity showed

antioxidant properties [8]. *Centella asiatica* is assumed to be effective on the connective tissues by strengthened weakened viens [12]. Triterpene of *C. asiatica* also showed antidepressant activity [13]. *Centella asiatica* of Asiatic region enhances GABA in cerebral cortex which depicts its conventional anxiolytic and anticonvulsant property [7,14]. The major constituents of these medicinal plants are madecassid acid, asiatic acid, centellasaponins as well as three types of asiaticoside viz: asiaticoside, asiaticoside A and asiaticoside B [15]. Because of great demands of these bioactive molecules, researchers focused to develop plant tissue culture techniques to increase the number of plantlets as well as to enhance the secondary metabolite production through the callus culture [16, 28]. Therefore, the major thrust of present investigation was to standardize a protocol for micropropagation of this valuable medicinal plant in B₅ media. We also determined bioactive molecules production *in vivo* and *in vitro* condition.

MATERIAL AND METHODS

Source of Explant and Surface Sterilization

Germplasm of *Centella asiatica* were collected from different ecological niches of Bihar such as Betiah, Purnia, Motihari

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and Patna, planted in garden of PG Department of Botany, Patna University Patna. One year grown plants were selected as explants for micropropagation on the basis of their morphological features and vigorous growth. The germplasm of Motihari showed maximum growth in leaf as well as size of leaf and stem as compared to other districts of Bihar. Therefore, the germplasm of Motihari District of Bihar for the Micropropagation of *Centella asiatica*. Stem and leaf were cut from the mother plant and washed under the running tap water for 30 minutes. After then washed explants were soaked in 5% (v/v) teepol for 8 min and washed properly with double distilled water, followed by surface sterilization by 0.1% (w/v) freshly prepared mercuric chloride for 5 minute and repeated washing 3-4 times with sterile double distilled water under laminar airhood. Explants were cut in 2-2.5 cm in small pieces.

Inoculation of Explants in B5 Media

The surface sterilized explants were dried out on autoclaved filter paper and further inoculated in plant tissue culture tubes (150×25 mm) under laminar air hood containing 20-25 ml of B5 media [17] augmented with different combination and composition of auxins such as 2, 4-D, NAA and cytokinins BAP and kinetin. pH of media was adjusted with the help of NaOH and HCl prior to autoclave at 1.05 kg/cm² pressure and 121 °C temperature for 15 min. The uniform culture condition was maintained in plant tissue culture laboratory of department at temperature 25 ± 2 °C under a photoperiod of 16/8 hr with relative humidity (RH) 60-70 % and light intensity of 35-40 μmolm⁻²S⁻¹ (white fluorescent light). Twenty replicates raised per treatment were repeated three times. Values represent means ± SE of 10 explants per culture vessels. The cultures were maintained by subculturing at regular interval of 4 weeks. Data were collected after inoculation of 4 week. Randomly selected *in vitro* grown calluses were further transferred in freshly prepared shooting B5 media supplemented with BAP and NAA for proliferation of callus into shoots. The number of shoots per callus and length of shoots were recorded after 4 week. When the shoots achieved the length of 2-2.5 cm, they were further transferred in rooting media fortified with BAP and NAA in tissue culture jar (PW1300, Himedia). Data were collected after 4 week of inoculation in shooting and rooting media. Percentage of shooting, number of roots and length of root (in cm) were also recorded. Moisture contents of randomly selected calluses were determined by the drying it in incubator at 30 °C. Firstly we measured the weight of fresh calluses then kept the material in Petri plates and placed it in incubator for 72 hours. The moisture of calluses was determined using the formula, % of moisture content = $(FC - P) - (FD - P) \times 100 / (FC - P)$, where, P is weight of empty Petri plates, FC is weight of Petri plates with fresh calluses and FD is weight of Petri plates with dry calluses.

The total phenolic content of *In vitro* grown callus and *In vivo* explants was studied by the method of Govarthan et al. with slightly modification [4]. The folin-ciocalteu's (FC) reagent was used for determination of total phenolic. One ml of methanolic extract and nine ml of double distilled water was added in 25 ml of conical flask. One ml of folin - ciocalteu reagent was added in the mixture with constant stirring. After 5 min 10 ml

of 7 % (Na₂CO₃) was added in the mixture and volume was made up to 25 ml with sterile distilled water. The reaction was incubated for 90 min on room temperature and absorbance was determined at 550 nm with the help of an ultraviolet (UV) visible spectrophotometer. Total phenolics content was expressed as mg gallic acid equivalents (GAE)/gm of *C. asiatica* extract. Meanwhile after induction of roots plantlets were removed from the media and cleaned with double distilled luke warm water for removal of agar and transferred into sterile plastic pots containing sand, soil, vermiculite (1:1:1) covered with sterile polythene bags. In order to acclimatize plants into field, after 2 week polythene bags were opened and transferred in earthen pots filled with 3:1 mixture of soil and vermicompost. *In vitro* grown plantlets were maintained in green house in normal day-night condition.

Statistical Analysis

A complete random block designed was design for all investigations such as callus culture, shoot induction, rooting and plant regeneration. Data were calculated on the basis of number of explants used. Twenty replicates per treatment were repeated three times and values represented by mean ± SE ten explants per culture vessels.

RESULTS AND DISCUSSION

The stem and leaf explants were inoculated in B5 media augmented with different combination and composition of BAP, kn NAA and 2, 4-D. The results have been presented in (Table 1) and Figure 1). After one week of inoculation calluses turned up reddish due to secretions of secondary metabolites. It was observed that B5 media augmented with BAP concentration ranges from 0.1 to 2.5 mg/l and NAA ranges 0.1-0.5 mg/l lead to formation of callus from leaf and stem explants (Figure 1). The calli obtained from the leaf were green, brown, compact and friable in nature. The same type of calli was also obtained from stem explants in B5 media fortified with BAP 0.1-2.5 mg/l and NAA 0.1-0.5 mg/l. Higher concentration of plant growth regulators leads to formation of brown and friable calli (Tables 1 and Figure 1-g,i) while lower concentration of plant growth regulators induced compact and green calluses (Tables 1 and Figure j). Lower concentration of BAP and NAA induced green and compact calli in both stem and leaf whereas higher concentration was responsible for brown and friable callus. Leaf and stem explants showed callus induction after one week of inoculation. BAP 0.1 mg/l and NAA 0.1 mg/l induced callus formation after 12 days in leaf explants and 13 days in stem explants after inoculation. BAP 2.5 mg/l and NAA 0.5 mg/l lead to formation of callus after 07 days in leaf and 09 days in stem explants. B5 media supplemented with Kn and 2, 4-D formed brown, compact and friable calluses (Tables 1). Keeping the concentration of 2, 4-D constant and increasing the concentration of kn from 1 to 4 mg/l (Figure g) leads to formation of brown and friable callus whereas in 1 and 2 mg/l of kn induced compact and brown callus in both stem and leaf explants. Friability increases after increasing the concentration of plant growth regulators. Minimum calluses responses was observed after one week of inoculation (Table 1) while maximum duration

was seventeen days for induction (Table 1) of callus responses in the stem explants. BAP 2.5 mg/l augmented with NAA 0.5 mg/l showed 10 out of 10 culture vessels in both explants, whereas kn 2 mg/l supplemented with 0.2 mg/l 2, 4-D results 8 out of ten culture vessels in stem and leaf explants. BAP showed

100% calluses response and Kn showed 80% response. The moisture content of calluses obtained from both the explants was determined by drying the calluses in incubator (Table 2). The leaf explants showed minimum moisture content 59% and maximum 91% while in stem minimum moisture content

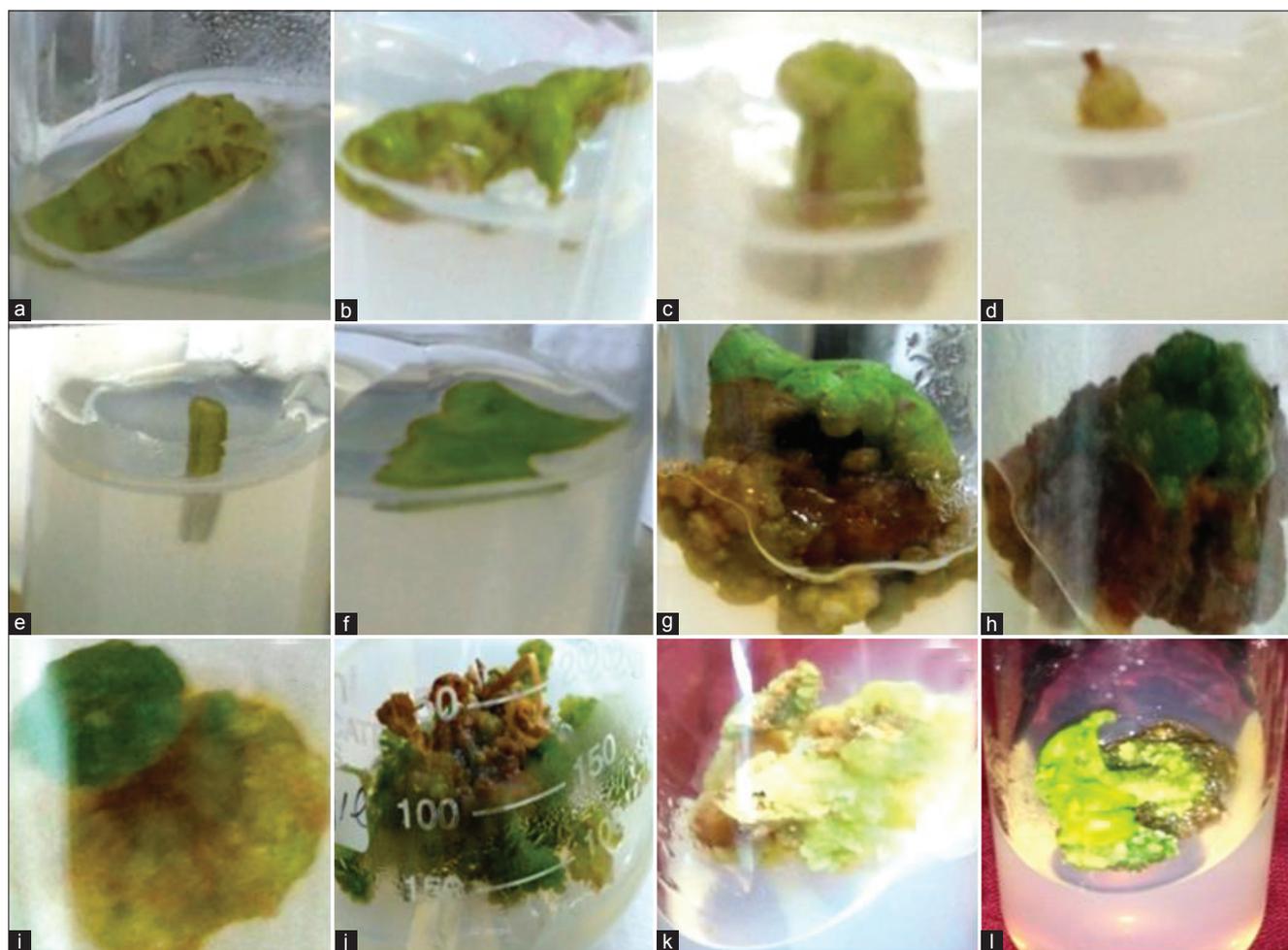


Figure 1: (a-l) different stage of callus induction in B5 media (a), (b), (c) Leaf explants after one week of inoculation, (d), (e), (f) Stem explants after one week of inoculation. (g), brown and friable callus kn 4 mg/l and 2,4-D 0.2 mg/l. (h), (j) green and compact calli BAP 2 mg/l and NAA 0.5 mg/l. (i) brown and compact calli kn 1 mg/l and 0.2 mg/l. (k) 6 week old callus of leaf explants. (l) green callus of leaf.

Table 1: Callus induction in leaf and stem explants in B5 media supplemented with BAP (0.1-2.5 mg/l) and NAA (0.1- 0.5 mg/l), Kn (1-4 mg/l) and 2, 4- D (0.2 mg/l)

Growth regulators				Leaf		Stem	
BAP	NAA	Kn	2,4-D	Type of callus	Day of callus induction	Type of callus	Day of callus induction
0.1	0.1	0	0	Green compact	12	Green compact	13
0.2	0.1	0	0	Green compact	11	Green compact	12
0.3	0.1	0	0	Brown compact	08	Green compact	11
0.4	0.2	0	0	Green friable	09	Brown compact	10
0.5	0.2	0	0	Brown friable	08	Brown compact	09
1	0.2	0	0	Brown friable	07	Brown compact	08
2	0.5	0	0	Green friable	13	Green friable	07
2.5	0.5	0	0	Green friable	07	Brown compact	07
0	0	0.2	1	Brown compact	08	Brown compact	08
0	0	0.2	1.5	Brown compact	11	Brown compact	10
0	0	0.2	2	Brown compact	09	Brown compact	14
0	0	0.2	2.5	Brown compact	13	Brown friable	10
0	0	0.2	3	Brown friable	17	Brown friable	11
0	0	0.2	4	Brown friable	15	Brown friable	09

was 60% and maximum 95%. The minimum fresh weight was (0.27 mg) and dry weight (0.03 mg) for leaf and maximum fresh and dry weight of leaf explants were (0.27 mg) and (0.03 mg) respectively. The minimum fresh and dry weight was (1.06 mg) and (0.16 mg) for explants and maximum fresh and dry weight (3.78 mg) and (0.42 mg) respectively. BAP has been better plant growth regulators as compared to kn and showed better growth. Stem explants were better explants over leaf explants for callus induction. No callus responses were obtained without plant growth regulators.

Effect of different combination and composition of BAP and NAA, Kn and 2,4-D on callus induction and type of callus on leaf and stem explants.

Multiplication of Shoots

Calluses derived from stem and leaf explants were further inoculated in shooting media. BAP (0.5- 1.5 mg/l) fortified with NAA (0.1-0.5 mg/l) in B5 media leads to formation of shoots in *Centella asiatica*. BAP 1.5 mg/l and NAA 0.5 mg/l has maximum shoot length 2.01 cm whereas BAP 0.1 mg/l and NAA 0.2 mg/l have minimum shoot length of 0.49 cm. Number of shoots per explants was minimum at BAP (0.1 mg/l) and NAA (0.2 mg/l) while maximum at BAP (1.5 mg/l) and NAA (0.5 mg/l) (Table 3 Figure 2). It has been observed that low concentration of plant growth regulators showed poor growth and high concentration showed green shoots with branching. After transfer of callus in shooting media shoots were regenerated after four week of inoculation.

Root Induction

After shooting, 1.5 -2 cm long shoots were transferred in full strength B5 media augmented with BAP 0.1 mg/l and NAA

0.1-0.5 mg/l for root induction. The results have been presented in (Table 4 and Figure 3). It has been shown that 0.1 mg/l BAP fortified with NAA 0.5 mg/l showed 100 % rooting, highest root per shoot 11.6 cm and mean root length 1.86 cm. However percentage of rooting, root length and number of roots per shoot varied among the different media composition. It has been observed that increasing the concentration of NAA enhances the percentage rooting, number of roots per shoot as well as root length. No results of rooting were observed in BAP augmented with IBA.

Acclimatization of Plantlets

For acclimatization well developed plantlets were isolated from rooting media and washed with lukewarm double distilled water for removal of agar and subjected to hardening. Plantlets were transferred in soil mixture containing vermiculite sand and soil (1:1:1) in plant growth chambers for 2 week (Figure 4). The plantlets were transferred into earthen pots into greenhouse, potted in 3:1 vermicompost and soil for another 2 week. After then plants were kept outside under full sunlight which is resulted into 90% survival.

Analysis of Phenol

Subsequent analysis of total phenolic content was determined *In vivo* leaf, stem and invitro grown 40 days old leaf and stem calluses. The percentage yield of total ethanolic alkaloid contents were 11.8%, 12% and 21% in powder tissues of stem, leaf and invitro raised calluses respectively. Calluses contain more alkaloid contents as compared to the explants. The phenolic content of calluses was more as compared to leaf and stem explants. The callus contained 0.67 mg/gm dw of phenolic while leaf 0.63 mg/gm dw and stem 0.59 mg/gm dw respectively.

Table 2: Growth of leaf and stem calluses were observed by measuring callus fresh weight and dry weight in (gm) from randomly selected samples of different concentration and combination of plant growth regulators

B5 media with PGR (mg/l)	Leaf			Stem		
	Fresh Weight (gm)	Dry weight (gm)	% moisture content	Fresh Weight (gm)	Dry weight (gm)	% moisture content
BAP(0.2)+NAA(0.2)	0.27	0.11	59	3.09	0.18	94
BAP(0.4)+NAA(0.2)	0.33	0.06	81	3.37	0.24	92
BAP(0.1)+NAA(0.2)	0.68	0.09	86	3.78	0.23	93
BAP(2.5)+NAA(0.5)	0.35	0.03	91	3.25	0.16	95
Kn(1)+2,4-D (0.2)	1.51	0.17	88	2.13	0.31	85
Kn(2)+2,4-D (0.2)	2.36	0.27	88	1.74	0.16	90
Kn(2.5)+2,4-D(0.2)	1.47	0.24	86	1.96	0.38	80
Kn(4)+2,4-D (0.2)	1.76	0.37	78	1.06	0.42	60

Table 3: Shooting B5 media augmented with combination of BAP (0.5-1.5 mg/l) and NAA (0.1 mg/l)

BAP (mg/l)	NAA (mg/l)	Shooting Percentage	Shoot length (cm)	No. of shoots per explants	Remarks
0.5	0.1	41	1.05±0.12	1.38±0.12	Poor growth
0.5	0.1	28	0.95±0.07	1.62±0.16	Poor growth
0.1	0.2	31	0.49±0.22	0.80±0.13	Green shoots
0.1	0.2	43	1.15±0.25	1.46±0.17	Green shoots
1.5	0.5	29	0.15±0.14	1.45±0.17	Green shoots with branching
1.5	0.5	67	2.01±0.10	2.25±0.32	Green shoots with Branching

Means were represented as means ± SE (10 explants per culture vessels repeated three times)

Table 4 :Rooting B5 media fortified with BAP (0.1 mg/l) and NAA (0.1 mg/l)

BAP (mg/l)	NAA (mg/l)	Rooting %	No. of roots/per shoot	Mean length of roots(cm)
0.1	0.1	17±1.5	8.125±1.36	0.20±0.01
0.1	0.2	28±2.25	10.28±1.45	0.48±0.09
0.1	0.3	30±3.5	7.21±1.03	0.42±0.04
0.1	0.4	36±4.5	5.05±0.86	0.42±0.08
0.1	0.5	100±07	11.56±1.64	1.86±0.30
0.1	0.6	80±6.5	6.08±0.96	1.74±0.25

Means were represented as means ± SE (10 explants per culture vessels repeated three times)

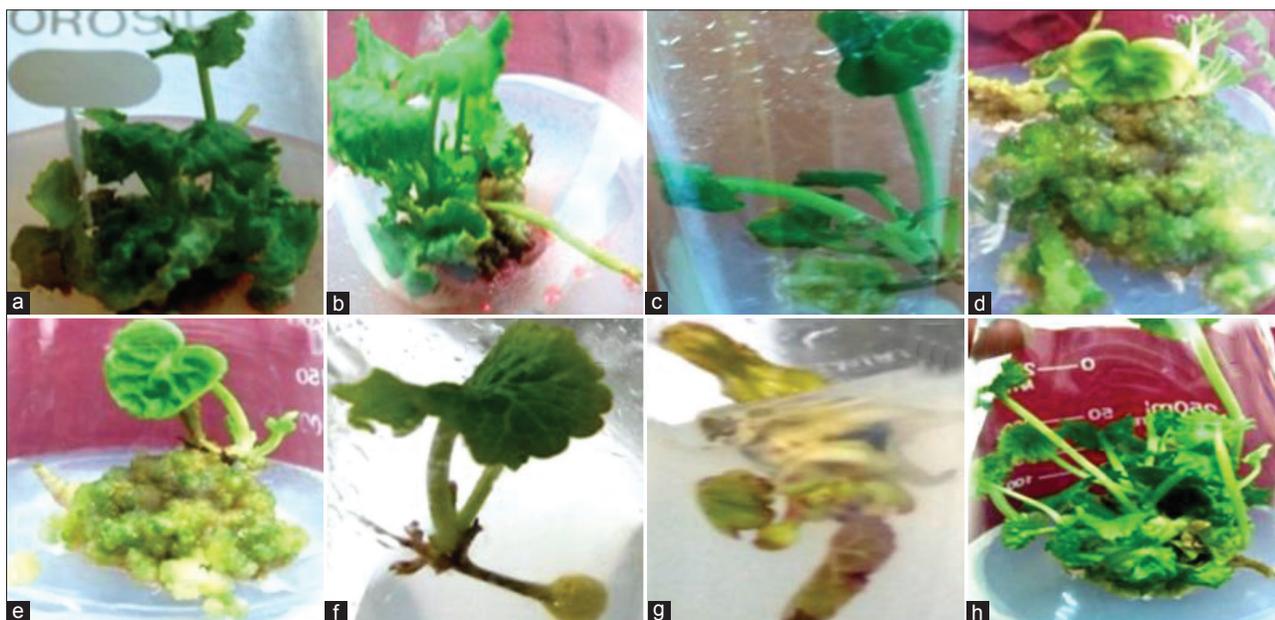


Figure 2: (a-h) Different stages of indirect regeneration in *Centella asiatica*

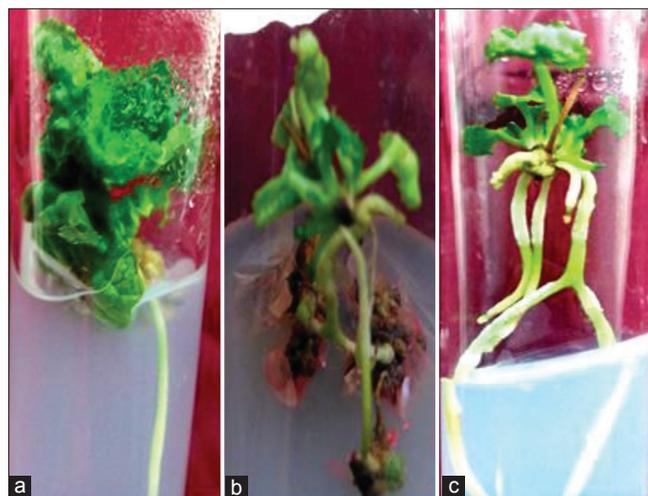


Figure 3: (a-c) Different types of roots developed in B5 media

DISCUSSION

Micropropagation protocol represents minimum cost techniques, by which we can obtain high quantity of plants. In micropropagation by using phytohormones reduced expenses and enhances fast and effective plant production. The present investigation standardized a protocol for callus

induction and regeneration from stem and leaf explants in *Centella asiatica*. This experiment determined a protocol for shoot proliferation and root emergence from the callus. The combination of BAP and NAA was essential for callus induction as well as plant regeneration [2, 18-26]. The effectiveness of BAP and NAA was shown by various researchers in *Centella asiatica* [30]. BAP and Kn showed significant roles in callus induction [21]. Combination of Kn with 2, 4-D showed good results which is similar to previous findings [27, 28]. But the characteristics of callus were different. The culture conditions, age, and type of explants may contribute to these differences. The combination of cytokinin and auxin are very important for callus induction and multiplication of shoots from the explants. No regeneration was observed without the growth regulators [18]. Similar regeneration was reported in combination of BAP and NAA in B5 media [15, 24]. Phenols are the important constituents of medicinal plants that possess hydroxyl groups, which are mainly responsible for antioxidant properties in any plants. Various researches have been conducted on polyphenols and stated that polyphenols showed an inhibitory effect against cancer and mutation. The callus contains more amounts of phenolics as compared to explants and leaf has a high amount of phenolic compounds as compared to stems. Callus cultures have the ability to attain significant amounts of phenolic compounds as compared to the counterparts of *In vivo* grown plants. Callus grows in nutrient-rich media, which is exposed to more carbon



Figure 4: *In vitro* regenerated plantlets in B5 media of *Centella asiatica*

influx than the *In vivo* grown, it might attribute to influence metabolic flux for the synthesis of increased level of phenolics. Phenolics compounds have redox properties which are also responsible for antioxidant properties. Among the three plant parts (leaf, stem, and callus) of *Centella asiatica*, the callus showed maximum amounts of total phenolics compound. Plant tissue culture techniques are very important tools for secondary metabolites production. Callus culture has been mostly used for secondary metabolites production against the intact plants. Several researchers have been reported secondary metabolite production through the *in vitro* culture in various medicinal plants [31-33].

CONCLUSION

The callus induction and regeneration of plantlets were easily maintained in B5 media fortified with auxin Viz: NAA, 2, 4-D and cytokinin BAP and NAA. The experiment revealed that combination and composition of plant growth regulators are responsible for callus induction as well as regeneration of plantlets. Combination of BAP and NAA are the best for callus induction over the combination of Kn and 2, 4-D. For callus induction stem is better explants as compared to leaf explants, however leaf contains high amount of phenolics relative to stem. Callus showed more amounts of phenolics as compared to both the explants. Higher of plant growth regulators enhances callus induction as well as shoot multiplication and root induction. B5 medium augmented with BAP and NAA are responsible for shooting and rooting. Rooted plantlets were transferred into field where they achieved 90% establishment successfully. The investigation may be concluded that a simple and efficient protocol was developed for rapid callus induction and regeneration in *C. asiatica*. The determination of phenolics suggested that the callus possessed high amounts of metabolites as compared to explants. This micropropagated protocol might be helpful in conservation of *C. asiatica* which is under threatened species due to overexploitation.

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