Standardization of an Efficient Protocol for In Vitro Micropropagation of *Centella asiatica* L. – An Important Medicinal Plant

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Abstract

The present study was undertaken to evaluate the most suitable and efficient media of low concentration growth regulators for shoot induction and subsequent multiplication in *Centella asiatica* L. an important and valuable medicinal plant. The best shoot induction ($82 \pm 2.2\%$) was found in MS medium supplemented with 0.5 mg/l BAP + 0.1 mg/l NAA from axillary meristem explant. Combination of 0.5 mg/l BAP + 0.5 mg/l NAA and 0.5 mg/l BAP + 0.5 mg/l KN gave the most effective shoot multiplication from initiated meristem cultures. The elongated shoots were rooted in MS medium supplemented with different concentrations of auxins (NAA and IBA). Lower concentration of IBA was more suitable for root induction when compared to NAA. The success of plant tissue culture for *in vitro* culture of *Centella asiatica* was encouraged by acclimatization of the plantlets in the field conditions. About 95% plantlets survived under field conditions.

1 Introduction

Centella asiatica (Linn) commonly known as Indian pennywort, naturally found in India throughout subtropical and tropical regions up to the height of six hundred meters. It is considered as an important medicinal herb has been used from thousands of years in China, Nepal, Shrilanka, Madagascar and India. In India the plant is distributed at high altitudes of 1550m in Sikkim and also at 1200 meters in Mount Abu (Rajasthan).

Centella asiatica L. urban synonym *Hydrocotyle asiatica* L. included in the family Apiaceae (Umbelliferae), an important major herb used for treating nerves and revitalizing brain cells also known as Brain tonic in India. Reports reveal that *C. asiatica* is in large demand and captured the international medicinal plant trade. Due to which the natural vegetation of this important plant is continuously depleted, because of over exploitation and lack of large scale cultivation the species now has been listed in threatened and endangered plant species by the International Union for Conservation of Nature and Natural Resources (IUCN)^{1,2}.

The plant material and its products are being used for health care since ages. Indigenous knowledge is valued and recognized as a rich natural resource in the custody of our society. *C. asiatica* is an important and highly valued plant in the traditional Indian system of medicines for treating various ailments like asthma, ulcers, skin diseases, as a brain tonic for improving memory, kidney problems, arthritis and many more health problems³.

Earlier reports shows *in vitro* propagation of *Centella asiatica* through organogenesis mainly from different explants such as shoot tips, axillary nodes, leaves and callus⁴⁻⁵. The present study was aimed to develop a simple, rapid and efficient protocol of plant regeneration from nodal explants of *Centella asiatica*.

2 Materials and methods

2.1. Preparation and sterilization of explants

Young and healthy plants of *Centella asiatica* were collected from Government medicinal plants nursery, Sanjeevini, Bhopal. Plants were grown in pots containing soil and farmyard manure (1:1) in the Botanical garden of College. Young healthy shoots of *Centella asiatica* were selected for present experiment as

explants. Nodal parts having axillary meristems were isolated and cleaning thoroughly under running tap water for 25 min followed by immersing in 10% (v/v) detergent solution labolene (Qualigens fine Chemicals, Mumbai, India) for 30 minutes. Followed by washing thrice with water and rinsed finally with double distilled water. The washed explants were surface sterilized with 0.1% (w/v) HgCl₂ for 5-7 minutes under aseptic conditions (inside laminar air flow) and washed five times with sterile double distilled water to remove traces of HgCl₂ before inoculation.

2.2. Medium and culture condition

2.2.1 To standardize shoot induction experiment of *Centella asiatica*

For shoot initiation surface sterilized nodal explants from the shoots of *Centella asiatica* were inoculated on Murashige and Skoog's (MS)⁶ media supplemented with different concentrations of BAP (0.1-1.0 mg/l) (6-benzylaminopurine), KN (Kinetin) either alone or with combinations of NAA (α -naphthalene acetic acid) (0.1-0.5 mg/l). The cultures were incubated at 25 $\pm$ 2 °C under 16 hours photoperiod provided by cool white fluorescent tubes (Phillips India). The percentage of bud break and shoots formation was analyzed after one week culture.

2.2.2 To standardize shoot multiplication experiments of *Centella asiatica*

To standardize the media for shoot regeneration/ multiplication experiment, initiated and regenerated shoots from explants nodal segments were separated, isolated and transferred on fresh medium for rapid multiple formation of shoot. Experiments were carried out to check the effect of different concentration of cytokinin like BAP (6 benzylaminopurine) and KN (kinetin) either alone or with the addition of auxins like IBA (Indole-3-butyric acid), and NAA (indole-3-acetic acid) in the concentration of 0.5 mg/l. The responding growth of cultures was observed at weekly interval. The parameters were taken as the average number and length of shoots multiplied per culture.

2.2.3 To standardize the medium for rooting of multiplied shoots of *Centella asiatica*

Final stage of the *in vitro* micropropagation process of *Centella asiatica* is the experiment of root formation.

After continuous process of shoot multiplication the shoots isolated from multiplication medium and shifted to the auxins rich medium. Hence, the regenerated shoots were transferred to the medium containing different Auxins like IAA (indole-3-acetic acid) NAA (α -naphthalene acetic acid) and IBA (Indole-3-butyric acid) in the concentrations of 0.5-1.0 mg/l. The growth parameters were observed as per percentage of root initiation, length in centimeters (cm) and number of roots formed.

2.2.4 Hardening and acclimatization

Rooted plantlets were separated out from rooting medium and washed in running tap water to remove agar and were then transferred to pots containing a mixture of sterilized sand: soil: vermicompost (1:1:1). The potted plantlets were kept under transparent polythene to maintain humidity. Hardening was continued for 2 weeks with mild irrigation at 2-day interval and supplied with ¼ strength MS inorganic solution twice a week during the initial period until they were successfully acclimatized. After one month, the surviving plants were transferred to garden soil.

2.3 Statistical analysis

Experiments were setup in a Randomized Complete Block Design (RCBD) and each experiment was repeated two times. Data were recorded on the percentage of response, number of shoots per explants, number of roots and root length per shoot. Means and Standard errors were carried out for each treatment.

3 Results and Discussion

3.1 To standardize shoot induction experiment of *Centella asiatica*

Selection of a best plant material for conducting successful results is the prerequisite of all experiments. Axillary and apical meristems from young shoots of *Centella asiatica* were collected in the month of August to march were proved the best explants collection period in the present experiment. For surface sterilizing 0.1% HgCl₂ (Mercuric chloride) solutions treatments given to sterilize the explants, it was observed that treatment for three to five minutes without any pre treatment with alcohol gives eighty percent sterile culture establishment.

For the standardization of shoot initiation of *Centella asiatica* nodal segments explant shows best response in every medium used to study the effect of different growth regulators like cytokinins and Auxin on MS media in the concentration of 0.1 mg/l to 1.0 mg/l). The percentage of bud break, and shoot induction was observed as a result on different medium tried.

C. asiatica is an herbaceous plant gives very good response in tissue culture studies. The buds starts break after three to four days of inoculation and induced shoot with a short duration of ten to fifteen days in all medium tried. It was observed that the cytokinins 6-benzylamino purine (BAP) and Kinetin (KN) when used without any additional hormones gives satisfactory shoot induction. Highest bud break and shoot initiation (80%) of 4.0 $\pm$.1 centimeters within ten days was reported in the medium having 0.5 mg/l BAP compare to same amount of Kinetin.

A synergetic effect was observed in bud break and shoot induction in the medium having auxin with both cytokinins from nodal segment explants. All medium initiated shoots but the medium having low amount of (0.5mg/l) BAP and (0.5mg/l) NAA shows best results, initiated maximum (90.5) percentage and highest number (5.0 $\pm$ 0.4) of shoot in the time duration of ten

days. Initiated shoots obtained vigorous health length of 6.5 ± 0.5 in this medium only in the duration of ten to fifteen days. Results obtained are shown in the Table 1 and Fig 1.

Table 1: Effect of MS media and growth regulators on shoot initiation of *Centella asiatica*

Media Code	MS+Auxin/cytokinin (mg/l)			% age of bud break	No. of days required	Mean No. of shoots produced $\pm$ SE	Mean shoot length in cm $\pm$ SE
	BAP	NAA	KN				
Cl ₁	0.1	-	-	50.0	10	1.0 $\pm$ 0.4	2.0 $\pm$ 0.3
Cl ₂	0.5	-	-	80.0	10	3.0 $\pm$ 0.1	4.0 $\pm$ 0.1
Cl ₃	1.0	-	-	70.1	10	2.0 $\pm$ 0.5	3.0 $\pm$ 1.3
Cl ₄	-	-	0.5	40.0	15	1.1 $\pm$ 0.3	2.5 $\pm$ 0.9
Cl ₅	0.5	0.1	-	80.0	10	5.1 $\pm$ 0.6	6.0 $\pm$ 0.1
Cl ₆	0.5	0.5	-	90.5	10	5.0 $\pm$ 0.4	6.5 $\pm$ 0.5
Cl ₇	-	0.5	0.5	50.0	15	2.3 $\pm$ 0.5	2.5 $\pm$ 0.2
Cl ₈	-	0.5	1.0	60.5	15	3.0 $\pm$ 0.1	2.0 $\pm$ 0.5
Cl ₉	1.0	0.5	-	80.0	10	5.0 $\pm$ 0.0	4.0 $\pm$ 0.1
Cl ₁₀	-	0.5	1.0	60.5	15	2.0 $\pm$ 0.1	2.0 $\pm$ 0.5

Values are expressed in Mean $\pm$ SEM

Fig 1: Different stages of micropropagation of *Centella asiatica*; 1 & 2 - Shoot induction from axillary meristems of *Centella asiatica* on MS 0.5 BAP; 3 & 4 - Shoot induction from axillary meristems in MS + 0.5 BAP + 0.5 NAA after 10 days; 5 - Elongated multiple shoots on MS 0.5 BAP+0.5 NAA after 20 days; 6 - Root induction on MS +0.5 mg/l IBA after 15 days; 7 & 8 - Hardened Tissue culture raised plantlets of *Centella asiatica*

3.2 To standardize shoot multiplication experiments of *Centella asiatica*

For standardize best medium having appropriate growth regulator which promote shoot multiplication various combination and concentration has tried, the initiated shoots when transferred to the fresh medium starts multiplying within five to seven days in every supplement of growth substance. According to the best medium for shoot induction BAP and KN with NAA and IBA is further used for shoot multiplication. It was observed that the highest shoot multiplication was seen in the medium containing cytokinin with auxin tried. The addition of BAP with NAA in

amount of 0.5 mg/l shows better response (90%) compare to the medium having KN with NAA. An average of 9.8 ± 0.5 number of shoots multiplied having the average length of 7.2 ± 0.8 cm in the 20 days culture have reported in the medium having 0.5 mg/l BAP and NAA. The cultures shows good shoot multiplication, elongation and rapid proliferation of shoot buds in the minimum concentration of cytokinin while it forms callus when the addition of cytokinin was exceeds up to 2.0 mg/l.

Results obtained in other tried medium were shown in the Table 2 and Fig 1.

Table 2: Effect of MS media and growth regulators on shoot multiplication of *Centella asiatica*

Media code	MS+Auxin/cytokinin (mg/l)				%age of shoot multiplication	No. of days	Mean No. of shoots	Shoot length in cm $\pm$ SE
	BAP	KN	NAA	IBA				
Cm ₁	0.5	-	0.5	-	90.2	20	9.8 ± 0.5	7.2 ± 0.8
Cm ₂	0.5	0.5	0.5	-	76.0	20	5.0 ± 0.08	6.3 ± 0.9
Cm ₃	0.5	-	--	0.5	70.0	30	6.3 ± 0.9	3.0 ± 0.2
Cm ₄	1.0	-	-	0.5	50.5	20	5.7 ± 0.1	5.3 ± 0.9
Cm ₅	1.0	-	0.5	-	75.5	25	8.0 ± 0.7	7.0 ± 0.5
Cm ₆	1.0	0.5	0.5	-	66.4	30	7.6 ± 0.5	4.6 ± 0.1
Cm ₇	1.0	0.5	-	0.5	70.0	30	7.9 ± 0.9	4.0 ± 0.6
Cm ₈	-	1.0	0.5	-	40.0	30	3.3 ± 0.5	2.3 ± 0.0
Cm ₉	2.0	-	-	0.5	60.0	40	6.5 ± 0.9	4.6 ± 0.4
Cm ₁₀	2.0	-	0.5	-	40.9	35	3.0 ± 0.1	2.8 ± 0.8

Values are expressed in Mean $\pm$ SEM

In this present study it was observed that the initiated shoot were multiplied even in minimum time and minimum hormonal requirement.

3.3 To standardize the medium for rooting of multiplied shoots of *Centella asiatica*

Healthy shoots initiated roots in all media having auxins with in 15-25 days from the axillary nodes and also from the shoot cluster, shown in the Table 3 and Fig 1.

In the present investigation it was reported that in every auxin tried gives satisfactory results and the best (95%) root induction was observed in the medium having IBA 0.5 & 1.0 mg/l followed by the auxin IAA in minimum time period which obtains the length of 7.0 ± 0.6 cms. The initiated roots are white, thin and slender in morphology in all media tested.

Thus the medium MS having auxin IBA and IAA (0.5 mg/l) is standardized as a root induction protocol for the medicinal plant *Centella asiatica*. In the present study it was observed that a low concentration growth regarding media is standardized as an efficient micropropagation protocol.

3.4 Hardening and acclimatization

Tissue culture raised plantlets were transferred to the natural compost containing the combination of sand, vermicompost and garden soil in the percentage of 25:50:25 and were maintained in the green house under high humidity. After 20-25 days the plants were hardened and produce new leaves with the survival rate 80-90 % in the month of September to February while it was only 40-60% in the hot month of march to July. All the hardened plants survived up to maturity in the soil mixture of sand and vermicompost (Fig 1).

4 Discussion

Our results of surface sterilization are same as the results describe by Tejavathi and Shailaja (1999)⁷ carried out for surface sterilization of explants of brahmi by mercuric chloride (0.1%) for the time duration of three minutes. In the present experiments of shoot induction highest bud break and shoot initiation within minimum time was reported in the medium having 0.5 mg/l BAP compare to same amount of other cytokinin Kinetin. Medium having low amount of (0.5mg/l) BAP and (0.5mg/l) NAA shows

best results, initiated maximum percentage and highest number of shoot in the time duration of ten days.

Table 3: Effect of MS media and growth regulators on root induction of *Centella asiatica*

Media code	Media composition (mg/ l).			% age of root formation	No. of days	Mean root length in cm±SE
	IAA	IBA	NAA			
Cr ₁	0.5	-	-	90.0	15	4.0±0.4
Cr ₂	-	0.5	-	95.0	15	7.0±0.6
Cr ₃	-	-	0.5	70.2	20	6.2±0.8
Cr ₄	1.0	-	-	70.0	20	5.5±0.0
Cr ₅	-	1.0	-	80.0	20	6.0±0.6
Cr ₆	-	-	1.0	70.0	25	4.0±0.2

Values are expressed in Mean±SEM

Results obtained were same as the observation reported for best shoot induction and multiplication from nodal explants of *Centella* by using growth regulators BAP and IAA from nodal segments and best root formation on IBA^{8,9}. Rozita *et al.*, (2004) reported the effect of auxins for promote best shoot formation on the medium added with BAP with IAA¹⁰. Nodal explants proved the best explants for direct shoot formation and highest rate of multiplication as previously reported by Gurwinder *et al.*, (2014) on the medium having combination of BAP and Kinetin and best rooting in the 1.0 mg/l NAA containing medium¹¹.

5 Conclusion

In conclusion, an efficient protocol for micropropagation of an important medicinal plant was developed in this study by testing various concentrations of growth regulators and culture conditions. This protocol provides a successful and rapid technique that can be used for mass propagation of elite species and could permit genetic transformation studies, which so far have been limited.

6 Conflict of interests

None

7 Author's contributions

SK, TT and SK performed whole experimental procedures. Both authors read and approved the final manuscript.

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