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Research Article

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Standardization of protocol for high frequency seed germination and direct regeneration in *Centella asiatica* (L.) Urban: An important medicinal plant

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ABSTRACT

Centella asiatica (L.) Urban, a stoloniferous herb belonging to the family Apiaceae (Umbelliferae), is a slender creeping herb with roots originating from the nodes. The plant commonly called 'Indian pennywort' or 'Gotukola', is used in the preparation of Ayurvedic brain tonic. The plant produces a group of pentacyclic triterpenes known as "Centellosides" that include asiaticoside, madecassoside, thankuniside, sceffoleoside, brahminoside, asiatic acid, madecassic acid etc. Since, these centellosides are mainly present in the aerial parts of the plant, indiscriminate harvest of plants from the natural habitats can be prevented by developing a protocol for a rapid and high frequency regeneration. In the present study, 28.4±1.14 shoots were obtained from single nodal explant on MS media supplemented with 2 mg/l BAP. Also, high seed germination (83%) rate was achieved that would benefit conservation of the medicinally important plant, C. asiatica. Moreover, high frequency regeneration from nodal explants would be a potential tool for the study of metabolic engineering aspects leading to the enhanced production of secondary metabolites.

Key words: *Centella asiatica*, Centellosides, Gibberellic acid, direct organogenesis, BAP (6-Benzylaminopurine), indole-3- butyric acid (IBA) and Murashige and Skoog (MS)

INTRODUCTION

Centella asiatica is one of the most important medicinal herbs, widely used in India and other parts of Asia, commonly called as Asiatic pennywort or Indian pennywort. It is used as a medicinal herb in Indian Ayurveda and traditional medicine of Africa and China. *C. asiatica* produces a group of major compounds known as Triterpenoid saponins. Presences of these compounds in the plant is responsible for its varied medicinal uses *viz*. wound healing, asthma, leprosy, fever, psoriasis, lupus, vein diseases and for relieving anxiety and improving memory [1]. The plant has been reported to have several biological activities like anti-ulcer [2], antitumor [3]; antibacterial [4], antioxidant [5], wound treatment [6, 7], anti-inflammatory [8, 9, 10] , immune modulating [11], anti-proliferative [12], neuroprotective [13] and antigenotoxic [14] properties.

As the herb has immense applications, there is an increased demand for the plant material leading to its destruction in wild habitats. So there is a need to develop high frequency regeneration (more number of plants) in a short span, which can meet the demand as well as assist in conservation of plants. Conventionally *C. asiatica* is propagated by cuttings and seeds. *In vitro* culture techniques have immense potential as an alternative for the continuous supply plants on a large scale for commercial exploitation which is unable to meet the current market requirement.

EXPERIMENTAL SECTION

Plant material

Centella asiatica plants were collected from Rajdhani agro farms, Ghatkesar, Hyderabad.

Culture media and culture conditions

Murashige and skoog [15] medium with sucrose as carbon source (3% w/v) was used in the present study. All the phytohormones were prepared as stocks at a concentration of 1 mg/ml and stored at 4°C. The media pH was adjusted to 5.8 using 1N HCl and 1 N NaOH. The media was prepared and autoclaved at 15 lb pressure and a temperature of 121° C for 20 min. MS media alone and supplemented with different concentration of cytokinins / auxins were used for the study. After inoculation, all the cultures were maintained *in vitro* at 22±2°C temperature with a photoperiod of 16h light/8h dark under cool white fluorescent lights.

Seed germination

Seeds were collected from the plants and thoroughly washed with teepol and surface sterilized with 0.1 % (w/v) mercuric chloride for 2 to 3 minutes. The seeds were inoculated on MS basal media alone and in combination with GA_3 concentrations ranging from 0.25 to 1.5 mg/l. The inoculated cultures were incubated at standard culture conditions (temperature 22±2°C; photoperiod 16h light/8h dark).

Direct organogenesis

Plants were cleaned and washed with teepol followed by washing under running tap water. Nodal explants were separated from the plant and used for direct organogenesis. The explants were treated with 0.1% (w/v) mercuric chloride for four minutes followed by washing with sterile distilled water for 6- 7 times to remove traces of mercuric chloride. After washing, the explants were cultured on MS media supplemented with different concentrations and combinations of cytokinins like kinetin, zeatin and BAP ranging from 0.25 mg/l to 2.5 mg/l and incubated under standard culture conditions. Response of different concentration of cytokinins towards direct regeneration from nodal explants of *Centella asiatica* was evaluated.

Root initiation in regenerated *C. asiatica* plantlets

The developed micro shoots were separated and inoculated on MS media supplemented with different auxins. Different auxins like IAA, IBA, and NAA at concentrations ranging from 0.5 to 2.5 mg/l were used in the study towards root induction.

***Statistical analysis*:** All the data was analyzed using one way ANOVA using Duncan's test ($P<0.05$) and represented as average standard errors.

RESULTS AND DISCUSSION

Medicinal plants grow naturally across the world. Over centuries, man has been identified how to use the plants to maintain health and fight against illness. Thus, they form the basis of approachable and affordable primary health care systems for indigenous and rural populations. Plants produce a wide range of secondary metabolites with therapeutic values. But, these compounds are produced in very low concentrations by plants. The commercial demand for such medicinal plants is leading to their continuous harvest from the wild habitat. Over exploitation from natural habitats, has placed many medicinal plants at the brink of extinction leading to disturbance in the ecological balance. So, there is a need to produce large number medicinal plants, to accomplish the people demand.

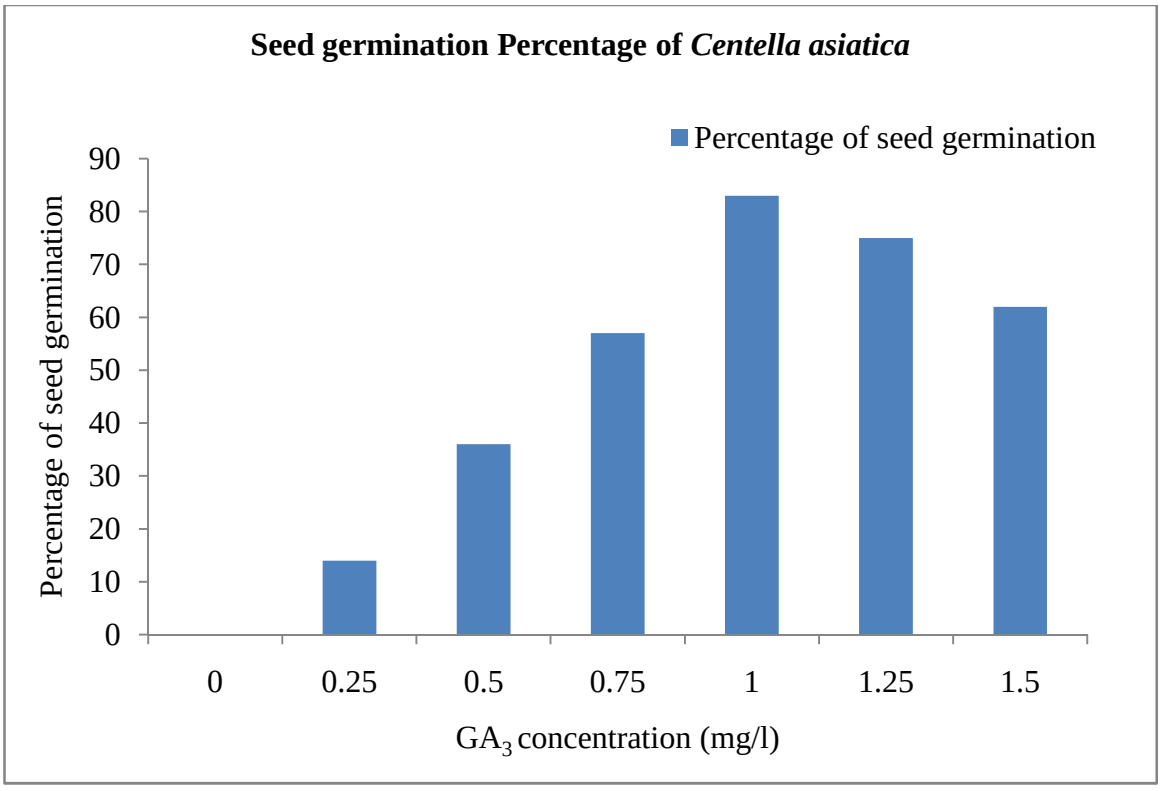
The traditional method of propagation in *Centella asiatica* plants is carried out by vegetative methods *via* nodal (stem) cuttings. Low percentage of seed germination is one of the hindrances for its propagation. Seeds lose their capacity to germinate on storage and the seed viability percentage is also reduced. In an attempt to enhance the seed germination percentage, GA_3 treatment was used in the present study. With the help of GA_3, 83 % seed germination was obtained after 3 weeks, which was otherwise not possible in its absence. Concentrations of GA_3 ranging from 0.25 to 0.75 mg/l were successful in breaking the seed dormancy to a lower extent. GA_3 is well known to be an essential phytohormone in breaking seed dormancy and promoting seed germination [16, 17]. GA_3 is also known to stimulate the production of hydrolytic enzymes which aid in seed germination, thereby increasing seed germination rate. Exogenous GA_3 expresses genes which in turn activate endogenous GA_3 levels which result in promoting seed germination [18]. GA_3 has shown positive response towards improving seed germination in many plants *Arabidopsis* [19]; *Setaria viridis* [20], *Medicago* and *Trifolium* [21], *Prunus avium* [22]. A part from breaking seed dormancy, gibberellins also involved in stem and hypocotyl elongation, flowering, leaf expansion, delay senescence and production of parthenocarpic fruits [18].

As displayed in Figure 1 (b), maximum seed germination was observed on media supplemented with GA_3 at a concentration of 1mg/l, which was found to be optimum achieving 83% seed germination (Fig 2 b), followed by 0.75 mg/l GA_3 which was able to give a result of 57% seed germination. Concentrations exceeding 1 mg/l of GA_3 caused reduction in seed germination along with blackening of the seeds on elongated incubation period. The germinated seeds were sub cultured and maintained on MS basal media for further experiments.

The present study reports the response of various Cytokinins like BAP, kinetin and zeatin towards direct regeneration. After 2 weeks of inoculation, four to five small nodular structures were formed from the base of each nodal explant on MS media supplemented with BAP and kinetin (Fig 3 (a)). These nodular structures after two weeks developed into small micro shoots (plantlets) (Fig 3 (b)). From each nodular structure nearly four to five new micro shoots developed after 4 weeks of inoculation. Among different cytokinins used, BAP was found to be good achieving 92 % regeneration frequency, with maximum number of shoots (28.4±1.14) at 2 mg/l. Lower concentrations also gave a positive response (21±4.63), with the next good choice being 1.5 mg/l BAP. Higher concentrations (>2 mg/l) were unable to yield better results, in turn leading to reduction in the number of shoots this could be due to the hyperhydricity observed at higher concentration of cytokinins [23, 24, 25].

Kinetin and zeatin were also found to induce shoot regeneration to a lesser extent. Kinetin produced a maximum of 18±0.70 shoots at 1.5 mg/l, whereas, zeatin gave 13.6±1.34 shoots at 1.5 mg/l concentration (Table 1). Among the various cytokinins, BAP was found to be most successful in inducing high frequency regeneration in *Centella asiatica*. BAP stimulates cell division and induces growth in plants. MS media supplemented with BAP found to be good towards regeneration of *Centella asiatica*. BAP has shown to produce good response towards shoot induction and regeneration in various plant systems like *Arachis hypogaea* [26], *Trichosanthes cucumerina* [27], *Mentha arvensis* [28]; and *Camellia nitidissima* [29].

The newly emerged shoots were separated and used for further tissue culture experiments. Among the different auxins used for the study, IBA was found to be more effective in comparison to IAA and NAA (Table 2). IBA was able to induce a maximum of 43.2±0.44 roots from the newly emerged shoots after 2 weeks of inoculation, at a concentration of 2mg/l (Fig 3 (f)). Lower and higher concentrations, beside the optimum (2mg /l) were able to induce root development but to a lower extent. The efficacy of IBA in root induction was evident in many plant species *viz Cassia sophera* [30], *Embelia ribes* [31], *Gossypium hirsutum* [32] and *Clitoria terneta* [33].



** Response was taken after 3 weeks of culture.*

Fig 1: Seed germination percentage of *Centella asiatica*

Fig 2: (a) **(b)**

Fig 2: (a) Seeds of *Centella asiatica* (b) *Centella asiatica* seed germination on MS media supplemented with 0.1 mg/l GA_3 after 3 weeks of inoculation

Table 1: Effect of different concentration of cytokinins on direct regeneration of nodal explants of *Centella asiatica*

Cytokinins	Shoot regeneration frequency (%)	No. of shoots per explants (Mean)	Response
Kinetin (mg/l)			
0.25	**40**	**3±0.70**	+
0.5	**45**	**8.8±0.44**	++
1	**60**	**11±0.70**	+++
1.5	**85**	**18±0.70**	++++
2	**80**	**13.6±2.19**	+++
2.5	**75**	**11.6±1.194**	+++
Zeatin (mg/l)			
0.25	**45**	**7±2.44**	++
0.5	**55**	**9.2±1.78**	++
1	**60**	**11±2.12**	+++
1.5	**80**	**13.6±1.34**	+++
2	**65**	**9.8±1.30**	++
2.5	**40**	**9±1.58**	++
BAP (mg/l)			
0.25	**50**	**10±1.58**	++
0.5	**68**	**12±2.12**	+++
1	**88**	**13.8±1.64**	+++
1.5	**90**	**21±4.63**	+++
2	**92**	**28.4±1.14**	+++++
2.5	**85**	**19.4±1.81**	+++

** Response was taken after 4 weeks of culture.*
+++++ Very good; +++ Good; ++ Moderate; + Poor

Table 2: Effect of various auxins towards root induction of *Centella asiatica*

Auxins	Rooting frequency (%)	No. of roots produced per explants	Response
IAA (mg/l)			
0.5	**40**	**8±0.70**	+
1	**56**	**11±1.58**	++
1.5	**63**	**16.6±0.89**	++
2	**87**	**27.4±1.14**	+++
2.5	**76**	**17.8±0.83**	++
IBA (mg/l)			
0.5	**60**	**15.2±1.09**	++
1	**85**	**23.6±0.54**	+++
1.5	**90**	**31.6±0.89**	++++
2	**98**	**43.2±0.44**	+++++
2.5	**93**	**36.6±0.89**	++++
NAA (mg/l)			
0.5	**25**	**6.2±1.30**	+
1	**40**	**12.8±0.83**	++
1.5	**68**	**22.8±1.30**	+++
2	**50**	**15±1.41**	++
2.5	**45**	**12.6±1.51**	++

** Response was taken after 2 weeks of culture.*
+++++ Very good; ++++ Good; +++ Moderate; ++ Poor; + Low

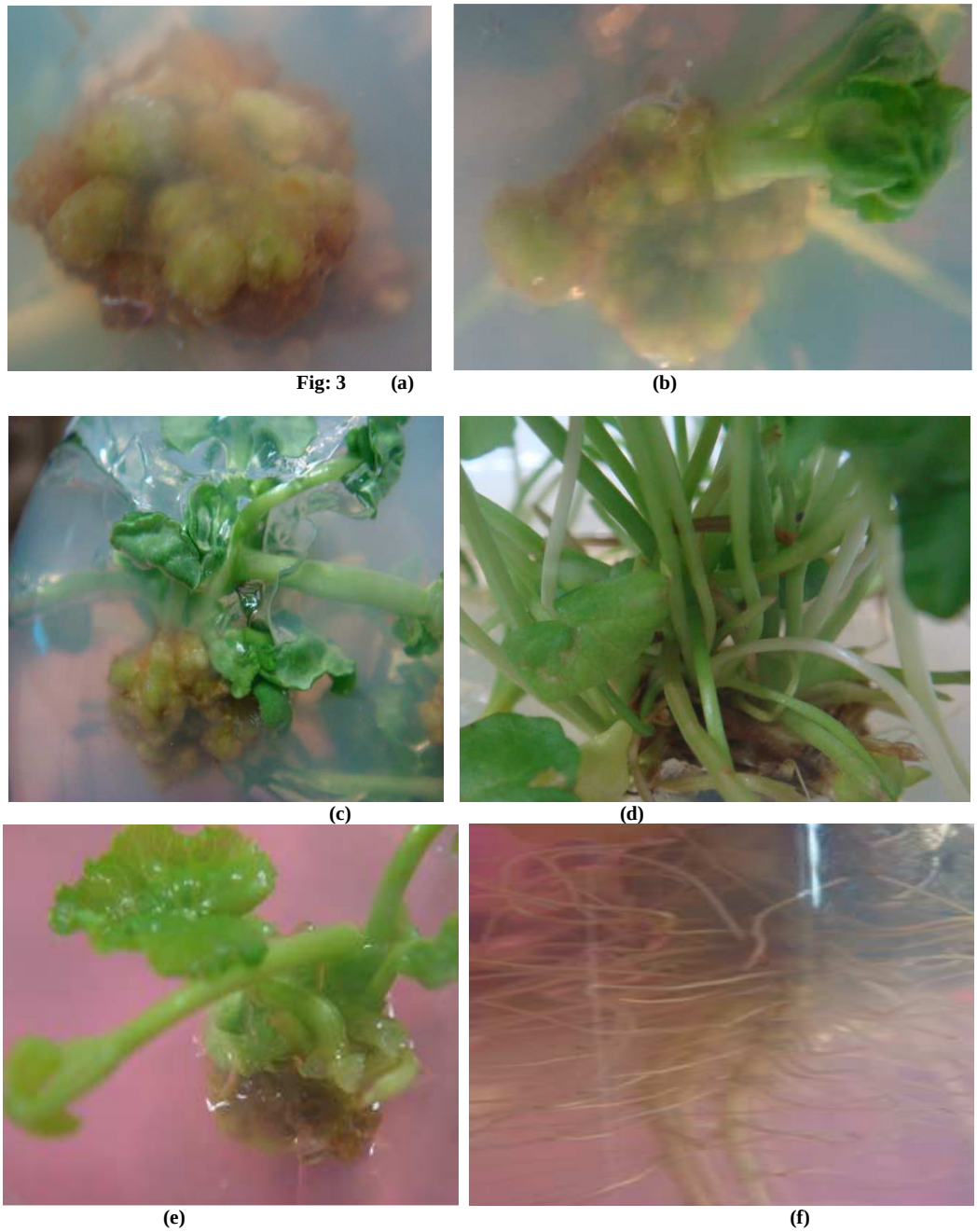


Fig: 3 (a) (b) (c) (d) (e) (f)

Fig: 3: a) Induction of small nodular structures (NS) at base of the nodal explant of *Centella asiatica* after two weeks of initiation b) Emergence of of microshoots from base of nodal explant c) Development of more micro shoots from each NS d) Number of multiple shoots emerged from single nodal explant e) Independently growing new plantlets of *C. asiatica* on MS basal media f) Root induction on MS media supplimented withn IBA (2mg/l)

Chandra sekhar et al [34] reported induction of 22 shoots in *Centella asiatica* using 4% fructose and BAP (1.5mg/l) and Kn (1mg/l), as against the 28.4±1.14 shoots obtained from MS basal media supplemented with 2mg/l BAP after 4 weeks of culture. In this study, a single nodal explant gave rise to 28 - 32 new plantlets. These plantlets were separated carefully and subcultured every fortnight onto MS media. Maximum rooting of (43.2±0.44) of microshoots were observed on 2 mg/l of IBA after two weeks of inoculation. These complete plantlets can be acclimatized and used for plantation in the fields that will help in bulk havesting of plants without disturbing the natural habitats. By using the protocol from the present study, high number of shoots (28.4±1.14) can be developed from a single explant during a short span leading to the generation of huge quantity of biomass that can be used for the betterment of mankind.

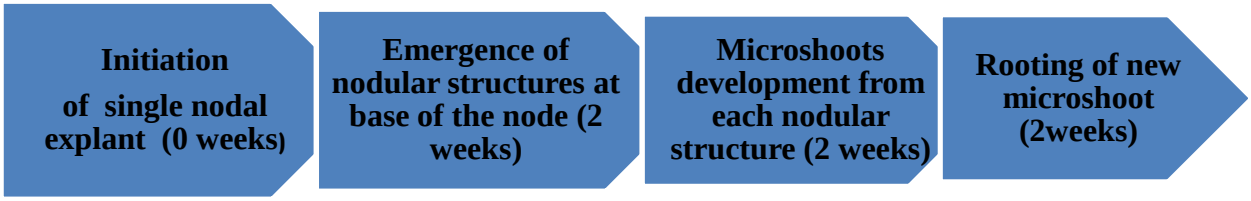


Fig 4: Over all process of direct organogenesis of *Centella asiatica*

CONCLUSION

A successful seed germination protocol was developed for the *Centella asiatica*, which can be used for its conservation and rapid multplication. High frequency regeneration protocol was developed for mass multiplication of the plant in less time period and limited space, which could be used for the *in vitro* experiments involving secondary metabolite enhancement as well as their extraction from the plant.

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