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In vitro flower induction and multiple shoot regeneration studies in *Centella asiatica* from nodal and leaf explants

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Abstract: *In vitro* regeneration of clones was achieved from explants like leaf and nodal segments on MS medium containing different concentrations and combinations of auxins and cytokinins. *In vitro* flowering and multiple shoot induction was noticed from nodal explants cultured on MS medium supplemented with BA 1.0 mg/L+IAA 1.5 mg/L. And the same was observed from leaf explants supplemented with MS medium+ BA (1.0 mg/L).The regenerated shoots were transferred onto MS medium fortified with IBA for root induction. The regenerated plantlets have been successfully established in soil and they were shifted to field condition.

Keywords: *In vitro* flowering, Benzyl Adenine (BA), Indole butyric acid(IBA), Indole acetic acid(IAA)

Introduction

Interest in herbal products and drugs derived from plants has increased tremendously in recent years. *Centella asiatica* is a small, annual herb plant of the subfamily Mackinlayoideae of family Apiaceae, and is native to India, Sri Lanka, Indonesia, and other parts of Asia**.** The herb is known as Brahmi in Unani and Mandookaparni in Ayurveda (Plant Profile for *Centella asiatica*, 2012)**.** It is a slender, tender aromatic herb which has numerous creeping stoloniferous stems up to 2 m long. Shoots are striated and often reddish in appearance. The leaves are 1-3 from each node of the stem, petioled, 2-6cm long, glabrous on both sides. Flowers are fascicled umbels, each umbel consisting of 3-4 white to pink, sessile flowers (Zheng, 2007; Satake et *al*., 2007; *Centella asiatica*, 2007). *C.asiatica* shows amplitude of variation in growth traits with respect to soil type in controlled environmental conditions and soil type (Anjana Devkota and Pramod Kumar Jha, 2009)

Being famous traditionally as health and memory enhancer (Silviya Rajakumari Jared, 2010), its bioactive compounds, Pentacyclic tri terpenoids (Jacinda T. James *et al*, 2009) like Asiatic acid, asiaticoside, madecassoside and centelloside etc., are responsible for its therapeutic ability. Leaves of *C. asiatica* are used in medical aids, which improve general mental ability of mentally retarded children (Appa Rao MV *et al*, 1973)

Modern research has found that *C. asiatica* possess anti-bacterial (Patchanee Yasurin *et al,* 2012), anti-microbial, cytotoxic and anti-oxidant acyiviyies (Ullah, M.O *et al,* 2009) anti-malarial and wound healing properties (Randriamampionona *et al.,* 2007)**.** Asiaticoside has anxiolytic**,** (Liang *et al.,* 2008) anti-inflammatory, antioxidant, antiulcer, and wound-healing properties (Kimura *et al.,* 2008).

Asiaticoside and madecassoside may be effective in treating arthritis (Liu *et al.,* 2008). Genetic diversity of traditional medicinal herbs and plants are vulnerable by extinction as a consequence of over exploitation, environment unfriendly harvesting techniques, loss of growth habitats and uncontrolled trade of medicinal plants (Venugopal Gaddaguti *et al* 2012). Therefore, development of a rapid clonal multiplication of this medicinal herb has become imperative in order to reduce the existing pressure on natural populations and supply constant plant materials for pharmaceutical industry (Tiwari et al 1998; Shrivastava et al 1999; Stough et al 2001 and Sumathi et al 2002). Also an efficient protocol is necessary for maintaining an *In vitro* line of this plant for further manipulations like *In vitro* flowering to improve centelloside, asiaticoside yield and other such parameters. The present paper deals with the development of a reproducible protocol for *In vitro* flowering and shoots regeneration.

Materials and Methods

Plant Material:

Centella asiatica plants were collected from Seshachalam forest, Tirupati, Andhra Pradesh, India and were successfully planted in university herbal garden for further use. Species identification was done with the help of Dr. N. Yasodamma, Professor, Department of Botany, Sri Venkateswara University, Tirupati and specimen is maintained in the dept. of Biotechnology, KL University. For the initial experiments, healthy nodal and leaf explants were collected from two months old plant.

Selection and Surface sterilization of explants:

To determine appropriate explants to be used, healthy shoot tips, nodal segments (0.8-1.0 cm) with dormant auxiliary buds, leaf (0.6 cm) explants were excised from stock plants grown in

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the KL University garden. We found the leaf and nodal explants are appropriate as it was responding well under *In vitro* conditions showing flowering and multiple shooting.

After selection of nodal segment and leaves as ideal explants for our experimentation, we have chosen them for further studies on the effect of growth hormones like Benzyl Adenine (BA) and Indole Acetic acid (IAA).

All explants were first washed under running tap water for 15 minutes next immersing in 10% Extran detergent solution (Qualigens India Ltd.) for 15 min and then rinsing with distilled water for 15 minutes to remove detergent foam and transferred to Laminar air flow chamber. The explants were surface sterilized with 0.1 %(w/v) $HgCl_2$ solution for 2min and finally washed with autoclaved double distilled water for 2-3 times to remove traces adhere with explants. Now the explants were cut to the required size and inoculated onto culture medium. All the explants were placed horizontally on the medium, and the leaves were placed with their dorsal side in contact with the medium.

Culture Medium and Conditions:

The culture medium used for the explants selection was Murashige and Skoog (MS) medium (1962) supplemented with 3% (w/v) sucrose and pH was adjusted to 5.7 with 1N NaOH or HCl before addition of 0.8% (w/v) agar (Hi media, India) and enriched with varying concentrations of BA and IAA were used further to determine the optimum growth regulator levels. The concentrations tested for BA and IAA were 0.5, 1.0, 1.5, 2.0 and 2.5 mg/lit. Molten media were dispensed into test tubes (Borosil, India) and closed with non-absorbent cotton plugs and media were autoclaved at 121^0C at 15p.s.i pressure for 15 min. The cultures were maintained at 25±2◦C under a 16 hour photoperiod of 50µmol m^{-2} s^{-1} irradiance provided by cool white fluorescent tubes.

Results

Shoot Proliferation and flower induction:

MS basal medium supplemented with various concentrations of BA or IAA ranging from 0.0 to 2.5mg/lit individually or in combination, were used for culture initiation and multiplication of shoots. After 2 weeks of inoculation pink coloured flower buds formation was observed along with shooting. 3weeks after inoculation all the cultures were transformed to the fresh medium. The mean number of flowers shoots and their lengths were evaluated after 4 weeks of inoculation.

Statistical Analysis:

The experiments followed by completely randomized design and were done at three times. Ten explants per replicate were used per treatment. Data were analyzed by one way ANOVA and the mean values from treatments were compared by using Turkey's HSD test at p = 0.05 with SPSS ver.13.0. The results are expressed by means ±SE of three experiments.

Discussion

Centella asiatica plants were efficiently regenerated from field-grown young plants on MS medium supplemented with different types of growth hormonal concentrations were used to study their effect on shoot multiplication and flower induction from nodal and leaf explants. In the present study, growth regulators were used in combination with 1.0mg/l BA and 0.5-2.5 mg/l IAA or 0.5-2.5 mg/l of BA for flower and multiple shoot induction. The flower induction response with respect to the test concentrations of growth hormones exhibit apparent difference and the data were presented in Table 1&2. Of the combination (BA with IAA and BA alone) were tested, On the other hand MS media supplemented with (BA1.0 + IAA 1.5 mg /l) showed high shooting percentage and more number of shoots (3.85), shoot lengths (4.00cm) and number of floral buds (6.55) per nodal explants, Whereas MS media supplemented with BA 1.0mg/l found to exhibit (3.15) number of shoots, shoot length (3.37) and number of floral buds (6.20) per leaf explants four weeks after ideal cultural conditions. From the results it is evident that the BA plays a key role in flower induction in *Centella asiatica*. Medium without growth regulator (control) gave no regeneration response and explants swelled and became necrotic 2 weeks after inoculation. Number of shoots and length of the multiple shoots per explants and number of flowers are found to be significantly reduced in all combinations of growth hormones tested either above or below optimised concentrations and also reported in several medicinal plants.

Conclusion

In the present study, we established a competent and reliable micro propagation protocol for *In vitro* regeneration and flower induction of *Centella asiatica*, from nodal and leaf explant. This ensures large scale propagation of the targeted plants, which is important for the sustainable supply of plant materials to the pharmaceutical industries and for conservation of elite germplasm. Our results also designate that multiple shoots, floral bud induction and regeneration in *Centella asiatica*, regulated by appropriate cytokinin and auxin concentration significantly influence large scale multiple shoots and flowers formation.

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Table.1: Nodal explants with various concentrations of BA in combination with IAA

Growth Hormone (mg/lit)	No. of shoots	Shoot length (cm)	No. of floral buds
MS+BA 1.0+IAA 0.5	1.400 ^{a} ±0.275	0.925^{a}±.1859	1.550^{a}±0.3032
MS+BA 1.0+IAA 1.0	2.600^{b}±0.3583	2.750^{b}±.3778	3.700^{b}±0.5135
MS+BA 1.0+IAA 1.5	3.850^{c}±0.3266	4.021^{c}±.3402	6.550^{c}±0.5255
MS+BA 1.0+IAA 2.0	2.050^{b}±0.3515	2.300^{b}±.3996	3.100^{b}±0.5424
MS+BA 1.0+IAA 2.5	1.150^{a}±0.2643	0.750^{a}±.1720	0.000^{NS}±0.0000

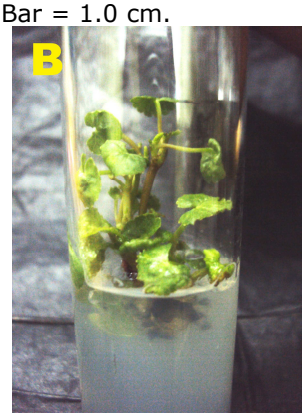
Table.2: Leaf explants with various concentrations of BA

Growth Hormone (mg/lit)	No. of shoots	Shoot length (cm)	No. of floral buds
MS+BA 0.5	1.5560^{a}±0.2760	1.056^{a}±0.1813	1.500a±0.2760
MS+BA 1.0	3.1500^{c}±0.3346	3.372^{c}±0.3042	6.200^{c}±0.5424
MS+BA 1.5	2.7050^{b}±0.3706	2.800^{b}±0.3828	3.450^{b}±0.4696
MS+BA 2.0	2.2045^{b}±0.3811	2.175^{a}±0.3721	3.000^{b}±0.4968
MS+BA 2.5	1.1108^{a}±0.2502	0.925^{a}±0.1995	1.230^{a}±0.0039

Values are means ± SE. (n = 15 in triplicate). Means followed by same letters do not differ significantly at p ≥0.05 by Tukey's HSD test.

Figure.1: [A-C] *In vitro* nodal propagation of *Centella asiatica*-developmental stages.

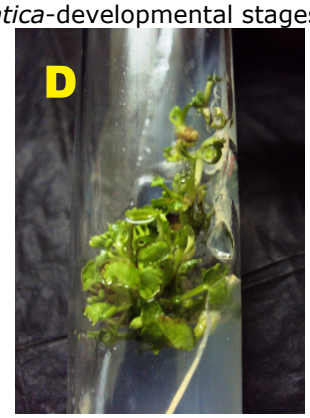
(A) Single nodal explant with induced growth of nodal buds on MS medium supplemented with 1mg/l of Benzyl Adenine (BA) and 1.5mg/l of Indole acetic acid (IAA) two weeks after inoculation. Bar = 1.0 cm.



(B) Shoot multiplication on MS medium supplemented with BA1.0+IAA 1.5 mg/lit after 4 weeks of culture. Bar = 0.8cm.

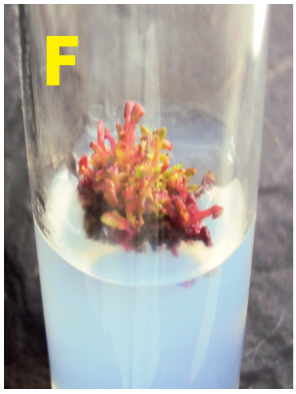
(C) Regenerated shoots with well-developed flowers cultured on MS medium supplemented with BA1.0+IAA 1.5 mg/lit after 4 weeks of culture. Bar = 0.5 cm.

Figure.1: [D-F] *In vitro* leafy propagation of *Centella asiatica*-developmental stages.



(D) Leaf explants showing shoot multiplication on MS medium supplemented with BA1.0 mg/lit after 4 weeks of culture. Bar = 0.8cm.

(E) Leaf explants with floral bud initiation on MS medium with BA1.0 mg/lit after 10days of culture. Bar = 0.3cm.



(F) Leaf explants with well-developed floral buds on MS medium with BA1.0 mg/lit after 3 weeks of culture. Bar = 0.4cm.

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