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BRIEF COMMUNICATION

In vitro regeneration of medicinal plant *Centella asiatica*

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Abstract

This paper describes multiple shoot regeneration from leaf and nodal segments of a medicinally important herb *Centella asiatica* L. on Murashige and Skoog's (MS) medium supplemented with a range of growth regulators. The highest number of multiple shoots was observed on MS augmented with 3.0 mg dm^{-3} N^6-benzylaminopurine (BAP) and 0.05 mg dm^{-3} α-naphthaleneacetic acid (NAA). Leaf explant showed maximum percentage of cultures regenerating shoots (81.6 %), with the highest shoot number (8.3 shoots per explant) and the shoot length (2.1 cm) whereas, nodal explant showed less number of shoots with callus formation at the base cut end. Successive shoot cultures were established by repeatedly sub-culturing the original explant on a fresh medium. Rooting of *in vitro* raised shoots was best induced on half strength MS supplemented with 0.5 mg dm^{-3} indole-3-butyric acid (IBA) with highest percentage of shoot regenerating roots (76.8 %) with 3 - 4 roots per shoot. Plantlets were acclimated in *Vermi-compost* and eventually established in soil. Contents of chlorophyll, total sugars, reducing sugars and proteins were estimated in leaf tissue from both *in vivo* and *in vitro* raised plants. Chlorophyll content was higher in *in vivo* plants, whereas other three components were higher in *in vitro* plants.

Additional key words: biochemical parameters, direct regeneration, leaf explant, growth regulators.

Centella asiatica (L.) Urban syn. *Hydrocotyle asiatica* (L.) of family *Apiaceae* is a valuable medicinal and aromatic herb distributed throughout tropical and subtropical region such as India and Srilanka. It contains several triterpene saponins (*e.g.*, asiaticoside), sapogenins, glycosides, and alkaloids hydrocotylin (Duke and Yensu 1985, Glasby 1991). The requirement of *Centella asiatica* is now met from natural population, leading to their gradual depletion. For the above, the tissue culture techniques can play an important role in the rapid multiplication of elite genotypes and germplasm conservation of *Centella asiatica*. *In vitro* plant regeneration has been reported in *C. asiatica* through callus culture from leaf explants (Banerjee *et al.* 1999, Rao *et al.* 1999), from stem node explants (Hossain *et al.* 2000) and through somatic embryos (Martin 2004). Different biochemical parameters like chlorophyll, sugar and protein contents were studied for this medicinally important plant earlier by Hossain *et al*. 2000. In this paper, we are going to describe a procedure for high frequency plant regeneration from different nodal segments and leaves.

Young *in vivo* shoots with 5 - 6 nodes of *Centella asiatica* L. Urban were collected from the university garden. Explants such as, 1.0 cm long nodal segment (part of the stem without leaf but with axillary bud) and whole leaf without petiole were excised and washed thoroughly under running tap water with 5 % (v/v) *Teepol* for 20 min then surface sterilized with 0.1 % (m/v) mercuric chloride (*Hi-media*, Mumbai, India) for 4 min., followed by five times thorough washing with sterile double distilled water. The explants were aseptically inoculated in different combinations of basal media: Murashige and Skoog (1962; MS) medium and Gamborg *et al.* (1968; B_5) medium. Both media supplemented with 30 g dm^{-3} sucrose (*Hi-media*) and 0.8 % (m/v) agar (*Hi-media*) were used with 0.5 - 5.0 mg dm^{-3} N^6-benzylaminopurine (BAP) and 0.05 mg dm^{-3} α-naphthaleneacetic acid (NAA) singly or in combination. The pH of the medium was adjusted to 5.8 prior to

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Abbreviations: BAP - N^6-benzylaminopurine; B_5 medium - Gamborg *et al.* (1968) medium; IBA - indole-3-butyric acid; MS medium - Murashige and Skoog (1962) medium; NAA - α-naphthaleneacetic acid.
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the addition of agar. The media were dispensed to 100-cm^{-3} Erlenmeyer conical flasks (*Borosil*, Bangalore, India), 30 cm^3 of media per flasks, capped with aluminum foil and autoclaved at 121 °C and 104 kPa for 20 min. All cultures were maintained in a culture room at temperature of 25 ± 1 °C, relative humidity of 57 ± 2 %, and 16-h photoperiod with irradiance 35 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes (*Philips*, Bangalore, India). The growth responses of different explants were studied at weekly intervals. The original explant was repeatedly (3 times) sub-cultured on shoot multiplication medium after each harvest of shoots.

The individually grown *in vitro* shoots were transferred to 30 cm^3 culture tubes containing the rooting medium [½ MS + 0.5 - 2.0 mg dm^{-3} indole-3-butyric acid (IBA) or 0.5-2.0 mg dm^{-3} NAA] and placed in an inclined manner in culture room at the above conditions as for shoot regeneration. After root initiation the rooted shoots were transferred to auxin free ½ MS medium for elongation of roots. The rooted shoots were transferred to the plastic pots containing autoclaved *Vermi-compost* (*Ranjan Agrotech*, Bhubaneswar, India) mixed with *Soilrite* mix (*Karnataka Explosives*, Bangalore, India) (1:1), moistened with ¼ MS media and covered with polyethylene bags. The potted plants were maintained inside a plant growth chamber (*SICO-IIC*, New delhi, India) set at 25 ± 1 °C, 85 - 90 % relative humidity and 16-h photoperiod with irradiance of 50 μmol m^{-2} s^{-1} for a period of one week. Then the plants were transferred to larger earthenware pots containing garden soil and kept under shade for another week before transferring out door.

Each experiment was repeated three times and consisted of 10 flasks (2 explants per flask). Data were analyzed using analysis of variance (*ANOVA*) for completely randomized design (CRD). Duncan's Multiple Range Test (DMRT) (Gomez and Gomez 1984) was used to separate the means for significant effect.

Leaf tissue from *in vivo* (seedlings grown in greenhouse) and *in vitro* grown plants (21 d after *ex vitro* transfer) were taken for biochemical analysis. The content of chlorophyll was measured according to (Arnon 1949), total sugars according to (Nelson 1944), reducing sugars by method of Dreywood (1946) and proteins by method of Lowry *et al.* (1951).

Of the two different basal media MS found superior than B_5 (Table 1). Two different explants (leaf without petiole and nodal segments) failed to regenerate shoots on MS or B_5 basal media devoid of growth regulators. Incorporation of BAP and NAA single or in combination to the basal media was essential to induce shoot proliferation. Similar response has been observed in *Aristolochia indica* (Soniya and Sujitha 2006), *Centella asiatica* (Banerjee *et al.* 1999, Rao *et al.* 1999, Hossain *et al.* 2000, Martin 2004), *Ocimum basilicum* (Sahoo *et al.* 1997), and *Vitex trifolia* (Hiregoudar *et al*. 2006). The morphologic responses exhibited in form of shoot and root is specific according to auxin/cytokinin ratio (Ali *et al.* 2002). Out of eight combinations of growth regulators tested MS + 3.0 mg dm^{-3} BAP + 0.05 mg dm^{-3}

Table 1. Effect of phytohormones supplemented to different basal media for shoot regeneration from leaf explant (without petiole) or nodal segment of *Centella asiatica* L. Data pooled from 3 separate experiments each with 10 flasks containing 2 explants per flask. Mean values within the column with same superscript are not significantly different ($P<0.05$; Duncan's New Multiple Range Test). No response was observed on media without phytohormones.

Explant	BAP + NAA [mg dm^{-3}]	Cultures regenerating shoots [%] MS	Cultures regenerating shoots [%] B_5	Number of shoots [explant^{-1}] MS	Number of shoots [explant^{-1}] B_5	Shoot length [cm] MS	Shoot length [cm] B_5
Leaves	0.5 + 0.05	13.3g	-	2.1e	-	0.6e	-
	1.0 + 0.05	20.0e	18.3d	3.0d	1.1e	0.9d	0.5de
	1.5 + 0.05	25.0d	19.8cd	3.5cd	2.8c	1.1cd	0.8cd
	2.0 + 0.05	36.6b	31.6b	4.7b	3.3b	1.4b	1.2b
	3.0 + 0.05	81.6a	33.3a	8.3a	3.9a	2.1a	1.4a
	4.0 + 0.05	30.0c	28.4c	3.8c	1.9d	1.3bc	1.0c
	5.0 + 0.05	15.0f	-	1.0f	-	0.3f	-
Nodal segments	0.5 + 0.05	7.8f	-	1.1e	-	0.3d	-
	1.0 + 0.05	11.3e	9.4e	1.5d	1.1cd	0.9c	0.5c
	1.5 + 0.05	21.3d	12.7d	1.8cd	1.3cd	1.1c	0.7c
	2.0 + 0.05	40.1b	23.4b	2.3b	2.0b	1.3b	0.9b
	3.0 + 0.05	46.3a	28.4a	3.4a	2.7a	2.0a	1.3a
	4.0 + 0.05	38.1bc	21.3bc	2.1bc	1.9bc	1.1c	0.9b
	5.0 + 0.05	36.3cd	18.8c	1.3de	1.1cd	1.0a	0.5c

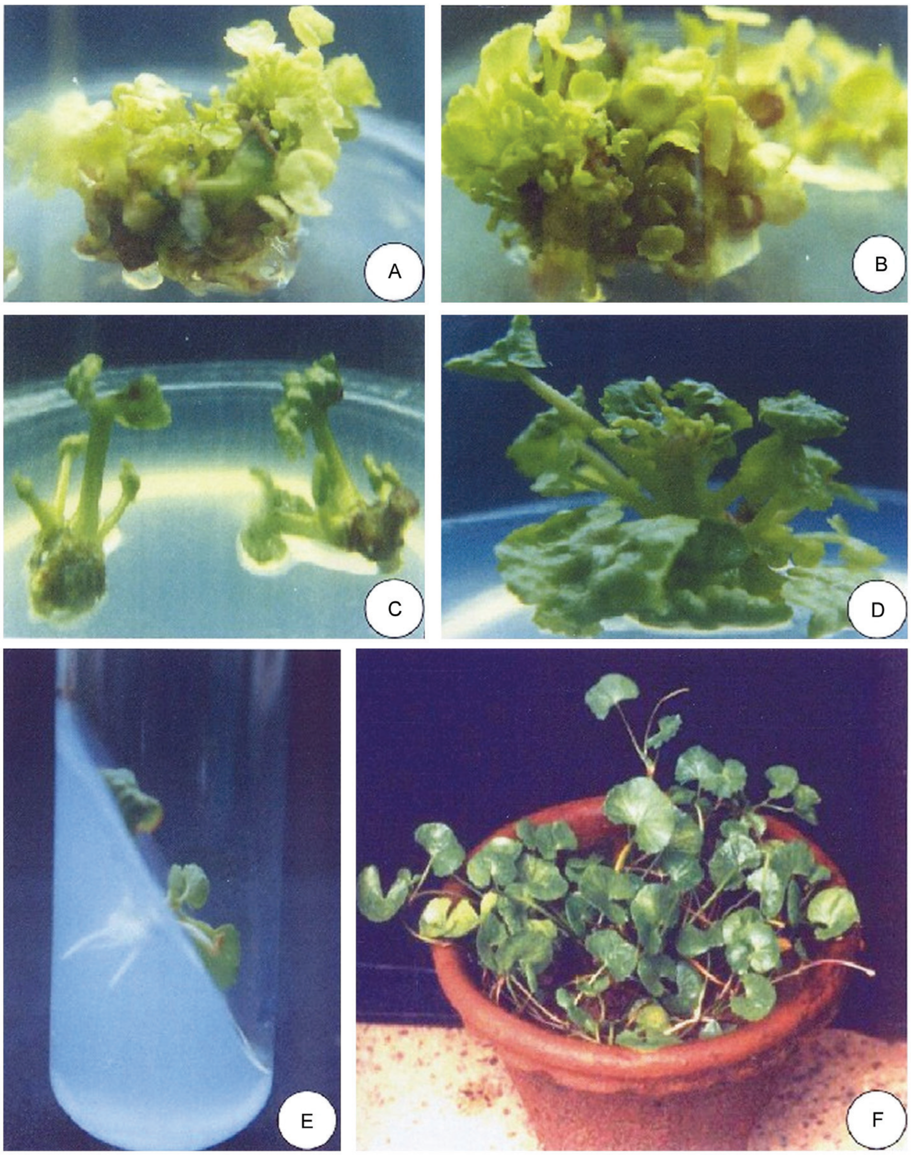

Fig. 1. *A* - Multiple shoot initiation from leaf explant on MS + 3.0 mg dm^{-3} BAP + 0.05 mg dm^{-3} NAA at 12 d of culture. *B* - Elongation of *in vitro* shoots on the same medium at 27 d of culture. *C* - Multiple shoot initiation from nodal explant on MS + 3.0 mg dm^{-3} BAP + 0.05 mg dm^{-3} NAA with slight callus at the base after 15 d of culture. *D* - Elongation of *in vitro* shoots of on the same medium at 27 d of culture. *E* - A rooted shoot on ½ MS + 0.5 mg dm^{-3} IBA after 12 d of culture. *F* - An acclimated plant in garden soil.

Table 2. Comparison of chlorophyll content, total sugar content, reducing sugar content and protein content [mg g^{-1}(f.m.)] between *in vivo* and *in vitro* regenerated leaves of *Centella asiatica*. Means denoted by the same letters are not significantly different (P = 0.05) using Duncan's Multiple Range Test.

Time [week]	Chlorophyll		Total sugars		Reducing sugars		Proteins	
	in vivo	*in vitro*	*in vivo*	*in vitro*	*in vivo*	*in vitro*	*in vivo*	*in vitro*
2	1.10	0.90	1.40	1.65	0.37	0.58	1.5	1.8
4	1.3	0.95	1.52	1.85	0.42	0.63	1.6	2.4
6	1.55	1.20	1.73	1.91	0.53	0.76	1.8	2.8
8	1.75	0.84	1.90	1.60	0.70	0.93	2.0	1.8

NAA showed the highest response irrespective of explant. Leaf explants showed the highest percentage of shoot development (81.6), maximum shoot numbers per explant (8.3) and the longest shoots (2.1 cm) after 27 d of inoculation of explants (Table 1, Fig. 1*A*,*B*) whereas, nodal segments showed less number of shoots (3.4) on the above medium (Table 2, Fig. 1*C*,*D*) at the same day. Periodic sub-culture increases the number of shoots. According to Banerjee *et al*. 1999 leaf segments devoid of petioles showed maximum 80 % sprouting of stunted shoot buds on MS + 2.0 mg dm^{-3} BAP + 0.1 mg dm^{-3} IBA. These stunted shoot bud were elongated when transferred to MS + 3.0 mg dm^{-3} BAP + 0.05 mg dm^{-3} NAA instead of IBA. But in this paper, shoots with roots were obtained directly on above mentioned medium. Stem node explants showed multiple shoot formation on MS + 1.0 mg dm^{-3} BAP + 0.5 mg dm^{-3} NAA (Hossain *et al*. 2000) with a lower number of shoots than ours. The frequency of shoot regeneration was markedly declined at higher concentrations of growth regulators (Table 1). Similar response was observed in *Cajanus cajan* (Singh *et al.* 2002), *Glycine max* (Kaneda *et al*. 1997) and *Ocimum basilicum* (Sahoo *et al.* 1997).

No root formation was observed when *in vitro* raised shoots were cultured on ½ MS medium devoid of auxins. Rooting of *in vitro* regenerated shoots was achieved on half-strength MS supplemented with either NAA or IBA. IBA at 0.5 mg dm^{-3} induced the highest frequency of rhizogenesis (76.8 %), with maximum root number (3.6 roots per shoot) and longest roots (3.9 cm) (Fig. 1*E*). Similar response was observed by Banerjee *et al*. 1999, Hossain *et al*. 2000 and Sahoo *et al.* 1997. The plantlets regenerated *in vitro* were successfully acclimatized in *Vermi-compost* and eventually in soil (Fig. 1*F*). 61 % of plantlets survived following transfer to *Vermi-compost* and 73 % of these plants survived transfer to soil.

Four different biochemical parameters were studied in leaves of *in vivo* and *in vitro* grown plants with respect to time intervals such as contents of chlorophyll, total sugars, reducing sugars and proteins (Table 2). While the chlorophyll content per fresh mass unit of *in vitro* regenerated leaves was lower, the contents of sugar and proteins were higher than those in leaves of *in vivo* grown plants. In contrast, Hossain *et al.* (2000) did not observed any significant differences among chlorophyll a, chlorophyll b, carotenoids and soluble protein contents in leaves of *in vitro* regenerated and natural plants.

The protocol reported herein for *in vitro* plant regeneration of *Centella asiatica* from leaf explant will considerably facilitate large scale propagation and conservation of this medicinally important plant.

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