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BRIEF COMMUNICATION

Establishment of callus and cell suspension cultures of *Centella asiatica*

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Abstract

Methods for induction of callus and cell suspension cultures have been developed for the medicinally important herb *Centella asiatica* (L.) Urban. Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) analysis showed the presence of asiaticoside in the *in vitro* grown leaves, callus and cell suspension cultured cells.

Additional key words: asiaticoside, auxin, cytokinins, high performance liquid chromatography, thin layer chromatography.

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Centella asiatica (L.) Urban (family *Apiaceae*) is an important medicinal plant. Medicinal properties of the plant has led to its over exploitation. Therefore application of tissue culture approaches for rapid multiplication of elite clones and germplasm conservation is of vital importance. Equally important is the generation of bioactive secondary products from this species. *C. asiatica* regeneration has been achieved using leaf derived callus (Patra *et al.* 1998, Banerjee *et al*. 1999), stem segments (Patra *et al.* 1998) and nodal segments as explants (Tiwari *et al*. 2000). A rapid method for multiplication of *Centella asiatica* by shoot tip culture has been developed by us (results not shown). There is no report on suspension culture of this plant. In this paper, we report a protocol for establishment of cell suspension culture for production of asiaticoside (an important bioactive compound).

Centella asiatica (L) Urban was collected around the Tezpur University Campus, Napaam, India. The plants were washed thoroughly under running tap water for 30 min. The excised shoot tips (2 - 3 cm long) were soaked in 1 % *Teepol* (*Sigma*, St. Louis, USA) solution for about 30 min and washed thoroughly under running tap water. Surface sterilization of the shoot tips was done with 0.01 % (m/v) $HgCl_2$ (*Himedia*, Mumbai, India) for 5 min followed by a final rinse (3 - 4 times) with sterile double distilled water.

For callus culture leaves from *in vitro* grown plants were dissected into 2 - 3 equal halves and inoculated in Murashige and Skoog (1962; MS) medium supplemented with different concentrations of 6-benzylaminopurine (BA), α-naphthalene acetic acid (NAA) and 2,4-dichloro-phenoxyacetic acid (2,4-D) with the ventral side of the leaf explants facing the media. The cultures were incubated at 25 ± 2 °C and 55 - 60 % relative humidity (RH) under continuous dark. Friable and light coloured calli (2 - 5 mm in diameter) were subcultured at every 3 weeks interval on the same media.

Suspension culture was done in MS liquid media with the same concentrations of phytohormones. The cultures were incubated under the same conditions as that for callus culture but with continuous shaking (150 rpm). The cultures were maintained through routine transfer of small aliquots of the suspension into fresh medium at every 2 weeks.

Growth measurements of calli were done by recording the fresh mass (FM) and dry mass (DM). Growth of the cells in suspension was measured by packed cell volume (PCV) method (Razdan 2003).

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Abbreviations: BA - 6-benzylaminopurine; 2,4-D - 2,4-dichloro-phenoxyacetic acid; DM - dry mass; FM - fresh mass; HPLC - high performance liquid chromatography; MS - Murashige and Skoog; NAA - α-naphthalene acetic acid; PCV - packed cell volume; RH - relative humidity; TLC - thin layer chromatography.

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For determination of the asiaticoside, leaves from *in vitro* plants, callus and cells from suspension cultures were oven dried at 37 °C and pulverised. Powdered samples (2 g) were soaked in 20 cm^3 of 90 % ethyl alcohol for 48 h and filtered through a fine *Whatman* filter paper. The filtrates were concentrated by vacuum dryer, weighed and used for identification of alkaloid by thin layer chromatography (TLC) (Svendsen and Verpoorte 1983). The crude extract was dissolved in a small volume of ethyl alcohol and fractionated on a precoated silica gel plate with ethyl alcohol:methanol:water (60:12:8, v/v/v) as the solvent system. The chromatograms were treated with iodine solution and asiaticoside was identified by comparing the R_f values of the zones of the samples.

Quantitative analysis of asiaticoside was performed by high performance liquid chromatography (HPLC) (*Waters*, Milford, USA, with *2487 Dual λ Absorbance Detector* and *515 Pump*). The isocratic solvent system was ethanol:water (6:4, v/v), UV detection was at 220 nm. The sample (0.015 cm^3) were injected and run for 10 min at a flow rate of 1 cm^3 min^{-1}. The peaks were identified by comparing the retention time with that of the standard solution of asiaticoside, which was subjected to HPLC separation on a C_{18} column using aceto-nitrile:water (3:7) as solvent and UV detector at 220 nm (Singh 1999).

Data were scored after 14, 21, 28 and 35 d of inoculation for growth of callus. Each mean was based on eight replicates repeated five times each. For quantitative assessment of purified fractions of asiaticoside in leaf, callus and suspension cultured cells by HPLC each mean was based on three replicates repeated three times each.

C. asiatica plants were grown *in vitro* following a protocol developed in our laboratory (results not shown). Young leaves from the shoot tips of these plants were inoculated in MS medium supplemented with different concentrations of BA, NAA and 2,4-D. Swelling of the leaf explants appeared in almost all the media. Callusing had been achieved 28 and 21 d after inoculation in MS_1 and MS_2 media, respectively. Rapid increase in the callus mass was observed in the MS_2 medium. 2,4-D was reported to be an effective hormone for callus formation in other members of *Apiaceae* (Sugano and Hayashi 1967). In the present study however, best result for calli induction was found with the MS_2 medium supplemented with 1.0 mg dm^{-3} BA + 1.0 mg dm^{-3} NAA (Table 1). The callus turned green and friable 35 d after inoculation and was subsequently subcultured at every 3 weeks interval.

The growth of callus was measured by recording FM and DM. The growth curve was in the exponential phase upto the eighth week of cultivation. FM and DM of the calli increased after 4 weeks of incubation by 124.15 % and 270 % in comparison with the initial FM and DM. This callus turned green and friable in the MS_2 medium on the fourth week and was suitable for further subculture or establishment of suspension culture.

Table 1. Growth of callus derived from *Centella asiatica* leaves under different combinations of growth regulators [mg dm^{-3}]. Growth was determined 14, 21, 28, and 35 d after inoculation.

Media	BA	NAA	2,4-D	Time [d]	Callus score	Callus morphology
MS_1	0.5			14	-	-
		1.0	-	21	-	-
				28	++	slightly yellowish
				35	++	slightly yellowish
MS_2	1.0	1.0	-	14	-	-
				21	++	slightly yellowish
				28	+++	green and compact
				35	++++	green and friable
MS_3	0.5	-	1.0	14	-	-
				21	-	-
				28	+	yellowish
				35	+	turned necrotic
MS_4	1.5	-	1.5	14	-	-
				21	-	-
				28	+	yellowish
				35	+	-

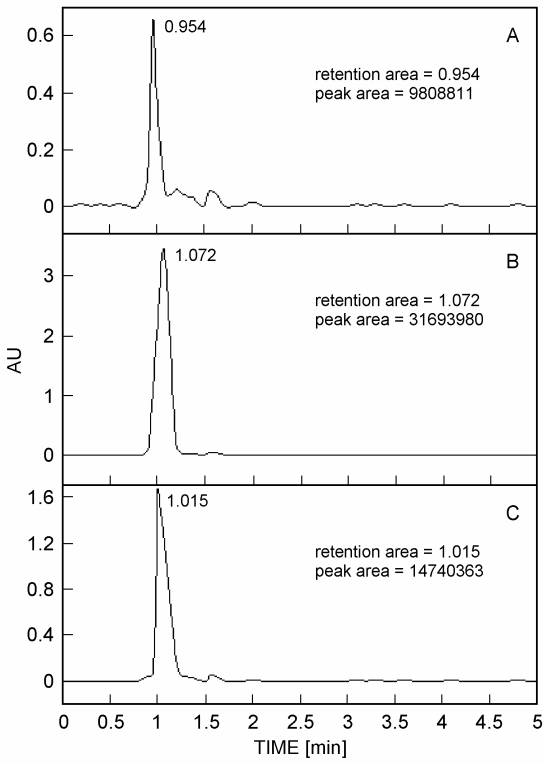

Fig. 1. HPLC separated chromatograms of asiaticoside in *in vitro* regenerated leaf (*A*), callus (*B*) and suspension cultured cells (*C*) of *Centella asiatica* using C_{18} column at 220 nm absorbtion mode showing retention time of 0.954, 1.072 and 1.015 min. AU - arbitrary units.

The growth curve of the cell suspension of *Centella asiatica* (L.) Urban was seen with an initial lag phase

upto 10 d of incubation followed by a steep rise in the growth rate until the third week. The cells in suspension culture grow with a higher rate than the cells in callus. The growth of the cell mass in suspension in MS_2 medium increased by 268.54 % (FM), 946.78 % (DM) and 373.68 % (PCV) of the FM and DM of the inoculum. The growth curve in cell suspension was eventually found to decline in the fourth week followed by callus senescence.

The asiaticoside in the suspension-cultured cells was found to be present in higher amount [494.62 mg g^{-1}(d.m.)] than in callus and leaf [190.48 and 125.0 mg g^{-1}(d.m.)] samples, respectively. It can be concluded therefore, that by suspension culture, not only large amount of calli could be obtained within a short period but also the alkaloid concentration could be increased in the cells. In two-stage processes, biomass and product formation are negatively correlated (Dicosmo and Misawa 1996). It is therefore essential to produce as much metabolically active biomass per unit volume by cell suspension culture as possible in order to achieve the higher possible production of bioactive compound.

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