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### Low Cost Alternatives for the Micropropagation of *Centella asiatica*

A.V. Raghu, Gerald Martin, V. Priya, S.P. Geetha and Indira Balachandran  
Center for Medicinal Plants Research (CMPR), Arya Vaidya Sala (AVS),  
Kottakkal-676 503, Malappuram, Kerala, India

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**Abstract:** The study of cost reduction alternatives in micropropagation of *Centella asiatica* was reported. Low cost options adopted in the study were effective in lowering cost of production without compromising the quality of plants. The cost on media ingredients was reduced by using household sugar instead of laboratory grade sucrose and tap water instead of double distilled water. Use of liquid media during multiplication stage also helped in reducing the cost on agar. Rooting of *in vitro* developed shoots under *ex vitro* conditions also helped cost effectiveness.

**Key words:** Liquid medium, *in vitro* propagation, *ex vitro* rooting, cost effective, *Centella asiatica*

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### INTRODUCTION

The major application of plant tissue culture lies in the production of true-to-type high quality planting material that can be multiplied under aseptic conditions on a year round basis any where irrespective of season and weather. Micropropagation is a capital-intensive technology involving energy and labour. This problem has been addressed by inventing reliable cost effective tissue culture methods with out compromising on quality of plants. Cost of chemical inputs, media, energy, labour and capital counts on production cost. The cost of medium preparation can account for 30-35% of the micropropagated plant production. Therefore, low cost alternatives are needed to reduce cost of production of tissue-cultured plants (George, 1993; Anonymous, 2004).

Low cost technology means an advanced generation technology in which cost reduction is achieved by improving process efficiency and better utilization of resources (Savangikar, 2002). Low cost options should lower the cost of production with out compromising the quality of the micropropagules and plants (Anonymous, 2004). The present study aims at developing a cost effective protocol for *in vitro* clonal propagation of *C. asiatica*.

*Centella asiatica* L. (Apiaceae) is an important medicinal plant used in several ayurvedic preparations and is reported to possess antileprotic, antifilarial, antibacterial, adaptogenic, antifeedant and antiviral properties (Warrier *et al.*, 1994). It also used for improving the memory, voice, physical strength, complexion and digestive power and for curing bronchitis, leucoderma, fever, biliousness, asthma, stuttering, anemia, leprosy and small pox (Sivarajan and Balachandran, 1994; Kirtikar and Basu, 1975). The plant contains the bitter compound-vellarin, an alkaloid-hydrocotylin and the glycosides-centalloside and asiaticoside (Anonymous, 1950). The fatty oil consists of the glycerides of oleic, linolic, centoic, linolenic, lignoceric, palmitic and steric acids. In addition, triterpene madasiatic acid, madecassoid and asiaticoside, active in treatment of leprosy, as well as 3-glycosyl quercetin, 3-glycosyl kaempferol and 7-glycosyl kaempferol were also isolated from leaves of *Centella* (Rastogi and Mehrotra, 1993). Due to its high medicinal value this plant has high demand for pharmaceutical industries. The requirement of *C. asiatica* is now being met from the natural population leading to their

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**Corresponding Author:** S.P. Geetha, Center for Medicinal Plants Research (CMPR), Arya Vaidya Sala (AVS),  
Kottakkal-676 503, Malappuram, Kerala, India

depletion. Developing low cost tissue culture techniques can play an important role in the rapid multiplication of elite clones, *in vitro* biomass production and germplasm conservation of *Centella asiatica*.

Earlier *in vitro* propagation through callus cultures (Patra *et al.*, 1998; Josekutty, 1998; Rao *et al.*, 1999), axillary buds (Tiwari *et al.*, 2000; George *et al.*, 2004), shoot tips (Sangeetha and Buragohain, 2003), leaf explants (Banerjee *et al.*, 1999), stolons (Sampath *et al.*, 2001) and somatic embryogenesis (Martin, 2004; Paramageetham *et al.*, 2004) were reported in this species.

The present investigation aims at developing a viable cost effective protocol, which can be used for the true to type mass propagation, *in vitro* biomass production and conservation of *C. asiatica* to meet the pharmaceutical demand. Attempts were made to evolve a low cost micropropagation technology using this medicinal herb as a model system, by adopting low cost substitutes in the media with stationary liquid culture to make the technique more viable.

## MATERIALS AND METHODS

### Explant Collection and Surface Sterilization

*Centella asiatica* were collected from the plants growing wild in the campus of Centre for Medicinal Plants Research, Arya Vaidya Sala, Kottakkal, Kerala, India (Fig. 1a). The plants were washed thoroughly under running tap water and the leaves and roots were trimmed off from the plant. Nodal pieces were excised from the stolons and kept under running tap water for 30 min. The nodal explants were washed in mild detergent, 1% (v/w) Teepol, for 5 min with constant agitation. The explants were surface sterilized with aqueous mercuric chloride ( $\text{HgCl}_2$ , 0.1%) and Teepol (5 drops/100 mL) for 3 min. The solution was drained off and rinsed 4-5 times with sterile distilled water. The explants were then taken to the laminar airflow chamber. Under aseptic conditions the explants were again treated with mercuric chloride (0.1%) for 3 min followed by thorough washing in sterile distilled water. The explants were trimmed to the appropriate size, making them ready for inoculation.

### Culture Media and Conditions

Throughout the study, different strengths of MS (Murashige and Skoog, 1962) medium were used for experiments. Full strength MS basal medium was used as the initiation medium. After three weeks of inoculation, nodal explants collected from the established cultures were taken out and subcultured in half strength basal medium supplemented with different concentrations and combinations of BA (0.2, 0.5, 1.0, 2.0  $\text{mg L}^{-1}$ ) and auxins (NAA; 0.5  $\text{mg L}^{-1}$  and IAA; 0.5  $\text{mg L}^{-1}$ ) for studying the *in vitro* multiplication responses (Table 1). Sucrose (30%) was used as the carbon source in all the combinations. All media combinations were solidified by adding 0.7% agar.

For establishing commercially feasible micropropagation method, two combinations of half strength and quarter strength MS medium with BA (0.5, 1.0  $\text{mg L}^{-1}$ ) were used (Table 2). In order to reduce the cost on agar, liquid medium was used. Market sugar (house hold cane sugar) was used as a carbon source and tap water was used instead of double distilled water.

The pH of the medium was adjusted between 5.6 and 5.8 using 0.1N HCl or 0.1N NaOH solutions prior to the autoclaving of the medium. Sterilization of the medium was done at a pressure of 15 psi for 20 min and was allowed to cool at room temperature. Jam bottles with polypropylene cap containing 20 mL of medium were used. The cultures were maintained at  $24\pm 2^\circ\text{C}$  under 12 h photoperiod with a light intensity of  $35\text{-}40 \mu\text{mol m}^{-2} \text{sec}^{-1}$  irradiance provided by cool white fluorescent tubes.

The multiplied cultures were taken out, single shoots were excised and they were kept in small plastic cups filled with soil and coir pith for *ex vitro* rooting and kept in humid chamber for providing proper humid conditions.

The experiments were carried out, each with 10 replicates. The percentage response, average number of shoots per explants and average number of leaves per explant was calculated. Cultures were observed daily and the data were recorded at an interval of six days and finally after 4 weeks.

## RESULTS AND DISCUSSION

### Establishment of Cultures

The nodal explants of *Centella asiatica* cultures were successfully established in full strength MS basal medium (Fig. 1b). Only 80% of the cultures could be established and major loss was due to fungal contamination. Emergence of two to three leaves was noticed per culture within 10 days in this hormone free medium. But the cultures did not show any shoot induction response in MS growth regulator free medium. Similar results were reported in the same species (George *et al.*, 2004) and also in other medicinal plants (Patnaik and Debata, 1996; Sahoo and Chand, 1998; Usha *et al.*, 2007). In the present study growth regulator free medium was used to reveal the contamination in culture initiation stage and their elimination. The use of simple salt plus sucrose medium is adopted as a usual method in culture initiation in many laboratories for reducing the cost on contamination (George, 1993).



Fig. 1: (a-f) Shoot multiplication studies in *Centella asiatica*. a: Mother plant, (b) Culture initiation in MS basal medium, (c) Multiple shoots formed in MS media supplement with  $0.5 \text{ mg L}^{-1}$  BA, (d) Multiple shoots formed in MS media supplement with  $0.1 \text{ mg L}^{-1}$  BA, (e) Multiple shoots formed in MS media supplement with  $0.5 \text{ mg L}^{-1}$  BA and  $0.5 \text{ mg L}^{-1}$  IAA and (f) Multiple shoots formed in MS media supplement with  $0.5 \text{ mg L}^{-1}$  BA and  $0.5 \text{ mg L}^{-1}$  NAA

Table 1: *In vitro* responses of nodal explants of *Centella asiatica* in half strength MS medium with various combinations of growth regulators

Growth regulators (mg L <sup>-1</sup> )			Percentage of response	Mean No. of shoots	Mean No. of leaves
BA	NAA	IAA			
0.2			90	2.7 <sup>bc</sup>	1.0 <sup>a</sup>
0.5			90	5.2 <sup>ab</sup>	3.0 <sup>c</sup>
1.0			90	6.3 <sup>a</sup>	5.0 <sup>d</sup>
2.0			100	3.8 <sup>d</sup>	2.2 <sup>bc</sup>
0.2	0.5		70	1.0 <sup>a</sup>	1.2 <sup>a</sup>
0.5	0.5		90	2.0 <sup>b</sup>	1.0 <sup>a</sup>
1.0	0.5		90	2.3 <sup>b</sup>	1.8 <sup>b</sup>
2.0	0.5		90	2.0 <sup>b</sup>	1.2 <sup>a</sup>
0.2		0.5	80	2.2 <sup>b</sup>	1.9 <sup>b</sup>
0.5		0.5	90	2.8 <sup>b</sup>	2.6 <sup>c</sup>
1.0		0.5	90	5.5 <sup>a</sup>	4.5 <sup>d</sup>
2.0		0.5	90	2.0 <sup>b</sup>	1.8 <sup>b</sup>

Observations were made after 4 weeks of culture. Values are Mean±SE of three independent experiments each with 12 replicates. Treatment means followed by same letter(s) within column are not significantly different from each other ( $p = 0.05$ ); comparison by LSD multiple range test

### Effect of Growth Regulators

The cultures established in the initiation medium were further used as source of nodal explants for the experimental trials. The explants were subcultured in half strength MS medium fortified with different concentrations of BA (0.2, 0.5, 1.0 and 2.0 mg L<sup>-1</sup>) alone and in combination with NAA (0.5 mg L<sup>-1</sup>) and IAA (0.5 mg L<sup>-1</sup>) for studying the various *in vitro* responses (Table 1). The percentage response ranged from 70-100. Cultures in media with BA alone showed good response in the number of shoots/explant but did not favor shoot elongation (Fig. 1c and d). Among the combinations of BA with auxins, IAA showed good response than NAA in the case of number of shoots/explant (Fig 1e and f). And this combination also showed little superiority in shoot elongation. BA at 0.5 and 1.0 mg L<sup>-1</sup> alone and BA (1.0 mg L<sup>-1</sup>) with IAA (0.5 mg L<sup>-1</sup>) showed good shoot multiplication and the difference were not significant among the trials. Maximum number of leaves was observed in all the combinations of BA and IAA. Rooting was noticed in all the trials but the percentage of response was very low (20%).

Similar to our observations, George *et al.* (2004) also reported that BA alone showed good shoot induction in *C. asiatica*. The use of BA as an efficient cytokinin for the axillary bud multiplication has been reported in several plants such as *Artemisia annua* (Usha and Swami, 1998), *Heracleum candicans* (Wakhlou and Sharma, 1998), *Tinospora cordifolia* (Raghu *et al.*, 2006). BA with IAA combinations showed superiority to NAA combinations, which was in agreement with the report by Tiwari *et al.* (2000) in the same plant. The potential of BA in combination with IAA was demonstrated in *Plumbago rosea* (Harikrishnan and Hariharan, 1996) and *Alpinia calcarata* (Agretious *et al.*, 1996).

### Effect of Low Cost Substitutes

Trials for developing commercially feasible micropropagation method was initiated by subculturing the nodal explants established in the initiation medium into half strength as well as quarter strength MS medium with BA (0.5, 1.0 mg L<sup>-1</sup>). Percentage of culture response (80%) was same in both the treatments. Difference in the strength of basal medium and cytokinin concentration showed considerable variation in morphogenic responses (Table 2). BA at higher concentration (1.0 mg L<sup>-1</sup>) showed effective shoot proliferation (Fig. 2a and b). Rooting was negligible in both treatments.

Direct transfer of excised shoots from *in vitro* established cultures to sand alone and sand with coir pith indicated that sand with coir pith was most favorable medium for *ex vitro* rooting. Eighty percent of the shoots rooted in this medium within two weeks. New leaf emergence was noticed and hardened plants were well established with 100% success (Fig. 2c and d).



Table 2: *In vitro* responses of nodal explants of *Centella asiatica* in commercially feasible treatments

Treatments	Response (%)	Mean No. of shoots	Mean No. of leaves
½ MS+ 0.5 mg L <sup>-1</sup> BA	80	4.0±0.7 <sup>b</sup>	2.3±0.3 <sup>b</sup>
½ MS+ 1.0 mg L <sup>-1</sup> BA	85	5.9±0.9 <sup>c</sup>	4.6±0.7 <sup>c</sup>
¼ MS+ 0.5 mg L <sup>-1</sup> BA	80	3.1±1.2 <sup>a</sup>	1.6±0.4 <sup>a</sup>
¼ MS+ 1.0 mg L <sup>-1</sup> BA	80	4.4±0.2 <sup>b</sup>	2.2±0.9 <sup>b</sup>

Observations were made after 4 weeks of culture. Values are Mean±SE of three independent experiments each with 12 replicates. Treatment means followed by same letter(s) within column are not significantly different from each other (p = 0.05); comparison by LSD multiple range test

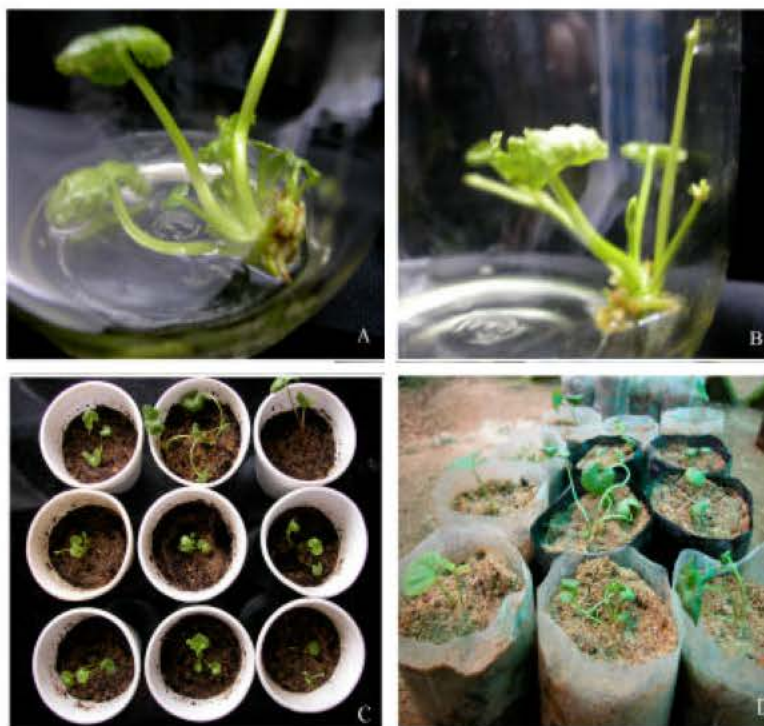


Fig. 2: Low cost micropropagation system in *Centella asiatica*; (A) Half strength MS medium with 0.5 mg L<sup>-1</sup> BA + 30 g L<sup>-1</sup> house hold sugar (cane), (B) Quarter strength MS medium with 0.5 mg L<sup>-1</sup> BA + 30 g L<sup>-1</sup> house hold sugar (cane), (C) *Ex vitro* rooted plants and (D) Hardened plants in polythene bags

The composition of culture media used for shoot proliferation and rooting has a tremendous influence on production costs. Sucrose and agar add significantly to the media cost. Use of household sugar and liquid medium help in reducing cost of production to certain extent, without compromising quality of plants. Water is the main compound of all tissue culture media. Distilled water produced through electrical distillation is very expensive and use of alternative water sources can be used to lower the cost of medium. The type of culture vessel influences the efficiency of transfer during subculture and production of propagules per unit area. Proper choice of media and containers can reduce the cost of micropropagation (Prakash *et al.*, 2002). In the present study we used tap water instead of distilled water for media and jam bottles as culture vessels instead of test tubes for laboratory scale multiplication.

Liquid media are ideal in micropropagation for reducing plantlet production costs and for automation (Etienne and Berthouly, 2002). In this study, the use of liquid medium and the low cost

substitutes such as market sugar and tap water did not adversely affect the proper growth of the plant. The shoot multiplication rate obtained in the low cost options was not less when compared to the other *in vitro* multiplication trials. Ganapathi *et al.* (1995) used tap water, commercial-grade sugar and reduced the salt components in medium for banana plantlet production and achieved a maximum cost reduction of 31.2%. Use of liquid media, culture vessel and low cost substitutes for mass propagation was successful in several other species (Sujatha and Chandaran, 1997; Varshney *et al.*, 2000; Kodym and Arias, 2001; Konstas and Kintzios, 2003; Kadota and Niimi, 2004; Piatezak *et al.*, 2005; Hung *et al.*, 2006).

Successful *ex vitro* rooting further reduced the cost of micropropagation. George *et al.* (2004) and Tiwari *et al.* (2000) reported the easiness of field establishment *C. asiatica*. Successful *ex vitro* rooting and field establishment was reported in several other species (Sujatha and Chandran, 1997; Geetha *et al.*, 2005).

The cost effectiveness by the use of alternate media constituents established during this study using *C. asiatica* shoot culture as a model system can be utilized in any other species.

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