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A quick bud breaking response of a surface model for rapid clonal propagation in *Centella asiatica* (L.)

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Present investigation was planned to evaluate time period of bud breaking in *Centella asiatica* with different concentration of plant growth regulators, a medicinal herb distributed throughout the worldwide. For the study, concentrations were designed for response surface model describing bud breaking growth in optimum conditions. A combination of BAP (2 mg/L) + gibberellic acid (GA_3, 0.5 mg/L) was achieved at a best initial bud breaking at 8th hour. Longest time period taken for bud breaking was shown in combination of BAP (0.5 mg/L) + naphthalene acetic acid (NAA, 0.5 g / L) and BAP (0.1 mg/L) + adenine sulphate (0.5 mg/L) which was recorded at 84th hour. Half strength MS media was supplemented with IBA alone (2 mg/L) and in combination with IAA (0.5 mg/L) to attain an early *in vitro* rooting. Their interactions observed were statistically significant ($P < 0.05$).

Key words: *Centella asiatica*, bud, plant growth regulator, medicinal plant.

INTRODUCTION

Centella asiatica (L.) Urban, synonym *Hydrocotyle asiatica* (Family: Apiaceae) is a small perennial herb, commonly known as Mandukparni. In India, this species is mostly found in the swampy areas up to an altitude of 600 to 1800 m asl (Patra et al., 1998). Medicinally, *C. asiatica* used as memory enhancer and in the treatment of chronic diseases, mental disorders and neuropharmacological disorders like insomnia, insanity, depression, psychosis, epilepsy and stress (Chopra et al., 1980). The major bio-active ingredients in the plant are the triterpenes, asiatic acid, madecassic acid and their glycosides such as asiaticoside and madecassoside (Zheng and Qin, 2007). Due to the presence of these active ingredients, it possesses antileprotic, antifilarial, antibacterial, antifeedant, adaptogenic and antiviral properties (Warrier et al., 1994). The roots contain many polyacetylenic compounds, the major compound being 8-acetoxyfalcarinol (Loc and Tam, 2010).

Over-exploitation of *C. asiatica* from natural habitats for medicinal purposes causes depletion of plant population. There has been an increase interest in *in-vitro* culture techniques for mass multiplication of important species to overcome the pressure of over-exploitation and to restore species diversity (Patra et al., 1998; Tiwari et al., 2000; Bhandari et al., 2010). However, till date *in-vitro* technique has been applied only for < 20% of medicinally important species (Shukla et al., 1999). *In-vitro* propagation of *C. asiatica* was also carried out through leaf explants (Banerjee et al., 1999), axillary buds (George et al., 2004), stolons (Sampath et al., 2001), shoot tips

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Abbreviations: MS, Murashige; **BAP,** 6-benzylaminopurine; **NAA,** naphthaleneacetic acid; **GA3,** gibberellic acid; **IBA,** indole-3-butyric acid, **Kn,** kinetin; **IAA,** indole-3 acetic acid; **PGR,** plant growth regulator; **ANOVA,** analysis of variance.

(Sangeetha et al*.,* 2003), callus cultures (Patra et al*.,* 1998; Rao et al*.,* 1999) and somatic embryogenesis (Martin, 2004)*.* Besides all, there is information available on the methods to initiate early bud breaking, shoot formation and root initiation. Therefore, present study was designed to understand the effect of different PGRs in alone and in combination for bud breaking, shoot formation and root initiation. Present study will be useful in producing quality planting material with in short duration.

MATERIALS AND METHODS

Ex- plant selection

For the Rapid clonal propagation of *C. asiatica,* explants were collected from the Herbal garden (1545 m asl) of Herbal Research and Development Institute, Mandal, Gopeshwar (Chamoli) Uttarakhand, India; It is bounded by North Latitude 30° 27' 13.40" and East Longitude 79° 16' 21.61".

The media for clonal propagation was prepared by following Murashige and Skoog (1962), All Chemicals used for the research purpose were purchased from HiMedia Laboratories (Mumbai, India), and growth regulators were purchased from Sigma Chemical Co. (St Louis, MO) and HiMedia Laboratories, India. Cultures were established for the bud explants on MS medium (Murashige and Skoog, 1962) containing 58 mM sucrose and gelled with 0.7% (w/v) agar. The pH of medium was adjusted between 5.6 to 5.8 using 0.1 N HCL or 0.1 N NaOH solution prior to the autoclaving at 121°C and a pressure of 15 psi for 20 min then allowed to cool at room temperature. The explants thoroughly washed with running tap water for 15 to 20 min, then treated with 1% (v/v), Tween 20 solution and subsequently for 15 min with a sodium hypochlorite solution (0.5% active chlorine) in laminar air flow cabinet and finally the explants were washed thoroughly with autoclaved distilled water for several times to remove the traces of sodium hypochlorite. In support of surface disinfection, bud segments were trimmed from the cut ends in appropriate size, and cultured.

Culture conditions and *in-vitro* establishment of plantlets

For establishment of cultures, the surface disinfected explants were inoculated on full strength MS (Murashige and Skoog, 1962) basal medium having 3% of sucrose, semi-solidified with 0.7% (w/v) agar and supplemented with different concentrations of plant growth regulator viz. BAP (0.1 to 2 mg/L), adenine sulphate (0.1 to 0.5 mg/L) and gibberellic acid (0.5 mg/L). Half strength of MS medium supplemented with growth regulator IBA (0.5 to 2.0 mg/L) and NAA 0.1 to 0.5 mg/L was attempt for rooting. Each hormonal combination was tried in three replicates. 250 ml (Borosil, India) Conical flasks containing 20 ml of medium were used. Cultures vessels were used for incubated at 25 ± 1°C under a 16/8 h light/dark photoperiod with light provided by cool-white fluorescent lamps (Philips India, Mumbai, India) at a light intensity of 1000 lux. The multiplied cultures were taken out; every single shoot was excised and kept in small plastic cup filled with a mixture of soil: sand (1:1) for *ex-vitro* rooting.

Multiple shoots from bud induction

The explants were inoculated in semi-solid MS medium with concentrations (0.1, 0.2, 1.5 and 2 mg/L) of BAP in alone, with combination of BAP (0.5 and 2 mg/L) along with gibberellic acid in 0.5 mg/L, BAP (0.1, 0.5,1 and 2 mg/L) with adenine sulphate 0.5 mg/L and BAP (0.5 to 2 mg/L) with the combination of NAA (0.5 mg/L). Sub culturing was carried out at periodic intervals of three weeks.

Rooting of microshoots

Developed shoots having one or two nodes were excised and transferred to root induction medium comprising of ½ strength MS medium with 3% sucrose and supplemented with different concentrations of IBA (0.5, 1, 1.5 and 2.0 mg/L) in alone and (0.1, 0.2, 0.5, 1.5 and 2.0 mg/L) with IAA (0.5 mg/L) in combination. Number of roots per shoot and root length was score in alternate day.

Hardening and acclimatization

For *in vitro* hardening, rooted shoots were transferred to ¼ MS strength medium having 3% sucrose devoid of PGR for seven days in flasks. Thereafter, they were transferred to polybags containing a mixture of soil: sand: FYM manure (1:1:1) and kept for two weeks in mist-chamber under controlled condition (tamp-25°C ± 2°C), humidity (65% ± 5%). Acclimatized plants were later shifted to soil in pots in agronet-shade house for one week and after that in field.

Statistical analysis

The data collected was subjected to the analysis of variance (ANOVA); using MS Excel 2007 for calculating the significance among different treatments and time of bud breaking and time of root initiation values at $P < 0.05$ were computed to compare means from various treatments.

RESULTS AND DISCUSSION

Initial study on *C. asiatica* was undertaken by Patra et al. (1998), Banerjee et al. (1999) and Tiwari et al. (2000). Tiwari et al. (2000) reported that initiation of nodal culture is better using different combination of plant growth regulators. The results of the present study on bud initiation, bud establishment and root initiation in MS medium supplemented with various combinations of growth regulators are presented in Tables 1 to 3. The earliest bud breaking in this study was achieved in BAP (2 mg/L) + GA_3 (0.5 mg/L). Initiation of bud breaking within 8 h of *in-vitro* culture in *C. asiatica* was reported first time in present study. Achieving early bud breaking is of importance as it produces quality planting material vis a vis reduces time and efforts. Different combinations of PGRs have also been reported to initiate bud breaking (Sen and Sharma, 1991). The longest time period (84 h) taken for bud breaking in present study was noticed in combination of BAP (0.5 mg/L) + NAA (0.5 mg/ L) and BAP (0.1mg/L) + adenine (0.5 mg/L) which are presented in Tables 2 and 3.

BAP alone at higher concentration (2 mg/L) seems to initiate early bud breaking. Similar observations in

Table 1. Morphogenetic response of *C. asiatica* buds cultured on MS medium supplemented with different concentrations of BAP and Gibberellic acid (Bud breaking is found positively significant LSD = 24.25 (P < 0.05).

BAP (mg/L)	Gibberellic acid (mg/L)	Time of bud breaking (h)
Control	-	0
0.1		72
0.1		60
0.1		66
0.2		60
0.2		72
0.2		72
0.5	0.5	36
0.5	0.5	38
0.5	0.5	40
1.5		40
1.5		46
1.5		42
2	0.5	8
2	0.5	12
2	0.5	14
2		24
2		18
2		16

t-value= 2.02, LSD = 24.25 (P < 0.05)

Table 2. Morphogenetic response of *C. asiatica* buds cultured on MS medium supplemented with different concentrations of BAP and Adinine (Bud breaking is found positively significant LSD=19.36; P < 0.05).

BAP (mg/L)	Adinine sulphate (mg/L)	Time of bud breaking (h)
Control	-	0
0.1	0.5	84
0.1	0.5	80
0.1	0.5	78
0.5	0.5	72
0.5	0.5	68
0.5	0.5	66
1	0.5	48
1	0.5	44
1	0.5	40
2	0.5	24
2	0.5	20
2	0.5	18

t-value = 2.13, LSD =19.36; (P < 0.05).

Table 3. Morphogenetic response of *C. asiatica* buds cultured on MS medium supplemented with different concentrations of BAP and NAA (Bud breaking is found positively significant LSD=19.36; P < 0.05).

BAP (mg/L)	NAA (mg/L)	Time of bud breaking (h)
Control	-	0
0.5	0.5	84
0.5	0.5	80
0.5	0.5	76
2	0.5	48
2	0.5	46
2	0.5	42

t-value= 2.03, LSD=19.36; P < 0.05

Ocimum basilicum was found by Pattnaik and Chand (1996). In addition, BAP was found more efficient over kinetine (Kn) in *in-vitro* shoot proliferation in different species (Purohit, 1994; Martin, 2003). In *Swertia chirata*, BAP with higher concentration have optimal response for shoot proliferation (Chaudhuri, 2007; Pant et al., 2010).

In present study, BAP in combination with GA_3 (with different concentrations) significantly ($P < 0.05$) enhance the rate of bud breaking, shoot proliferation and root initiation (Table 1, Figure 1A-E). Sharma and Sharma (2010) attributed this to the stimulating effects of various hydrolytic enzymes activities thus increasing availability of nutrients for growth. The result of the effects of BAP and GA_3 on shoot proliferation in present study was found comparable to the earlier reports. However, Tiwari et al. (2013) reported improved bud breaking using high concentration of BAP (5 mg/L) and improved root formation in combinations of BAP (4.0 mg/L) and IBA (0.5 mg/L). Karthikeyan et al. (2009) described the rapid clonal propagation through auxiliary shoot proliferation in *C. asiatica*. The shoot elongation with the treatment of BAP and GA_3 might be due to cell enlargement and increase in normal cell division (Karivartharaju and Ramakrishnan, 1985). Earliest root initiation was achieved alone in IBA (2 mg/L) and in combination of IBA (2 mg/L) and IAA (0.5 mg/L) (Tables 4 and 5).

Thus, it is concluded that *in-vitro* micro-propagation offer rapid clonal multiplication of elite clones and further helps in dissemination fulfilling the need of vis a vis to quality planting material. BAP (2 mg/L) in combination with GA_3 (0.5 mg/L) is recommended for effective and earliest bud breaking. Likewise, IBA (2 mg/L) is recommended for earliest rooting in *C. asiatica*.

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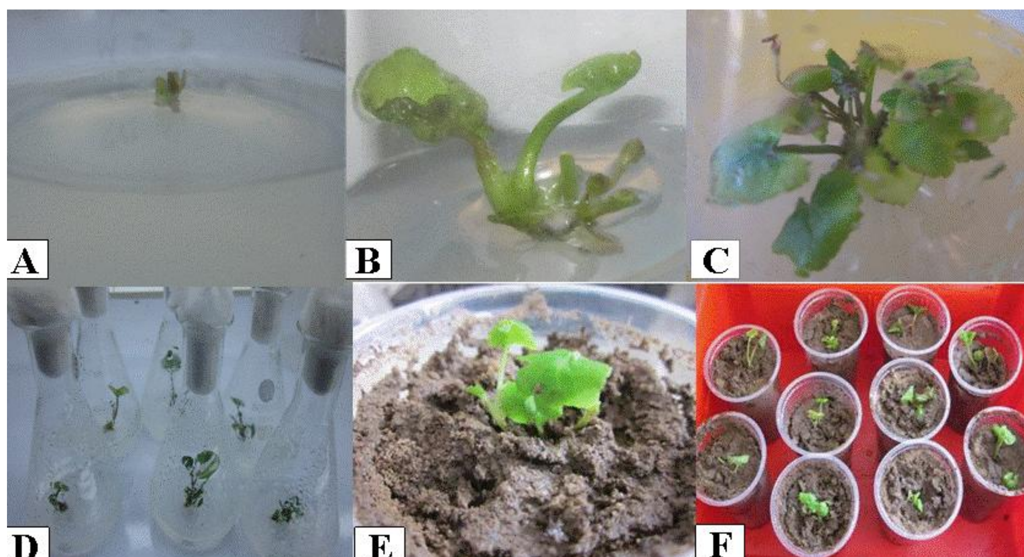

Figure 1. In vitro regeneration of *Centella asiatica,* via bud explants; **(A)** axillary bud induction on nodal segment MS medium+BAP (2 mg/L)+gibberellic acid (0.5 mg/L), **(B)** bud induction on nodal segment, **(C)** culture establishment, **(D)** multiplication of shoots in BAP (0.5 mg/L)+NAA 0.1 mg/L, **(E)** rooted plantlet containing a mixture of soil : sand : manure (1:1:1), **(F)** plantlet in soil for hardening containing a mixture of soil : sand : manure (1:1:1).

Table 4. Morphogenetic response of root initiation explants of *C. asiatica* cultured on half strength MS medium supplemented with different concentrations of IBA (Root initiation is found positively significant LSD=3.16; ($P < 0.05$).

IBA (mg/L)	Time of root initiation (Days)
Control	0
0.5	16
0.5	14
0.5	15
1	15
1	14
1	13
1.5	12
1.5	11
1.5	11
2	10
2	9
2	8

t-value= 2.07, LSD=3.16; ($P < 0.05$)

Table 5. Morphogenetic response of root initiation explants of C. asiatica cultured on half strength MS medium supplemented with different concentrations of IBA and IAA (Bud breaking is found positively significant LSD=3.12; ($P < 0.05$).

IBA (mg/L)	IAA (mg/L)	Time of root initiation (Days)
Control	0	0
0.1	0.5	18
0.1	0.5	16
0.1	0.5	16
0.2	0.5	18
0.2	0.5	16
0.2	0.5	16
0.5	0.5	15
0.5	0.5	14
0.5	0.5	13
1.5	0.5	12
1.5	0.5	11
1.5	0.5	10
2	0.5	10
2	0.5	9
2	0.5	9

t-value= 2.02, LSD=3.12; ($P < 0.05$)

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