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Full Length Research Paper

A rapid two step protocol of *in vitro* propagation of an important medicinal herb *Centella asiatica* Linn.

Chandrakant Tiwari*, Meena Bakshi and Ankur Vichitra

Plant Physiology Discipline, Botany Division, Forest Research Institute, Dehradun, Uttarakhand, India.

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The present investigation aimed at developing rapid micro propagation protocol, which can be used for conservation of *Centella asiatica* and mass multiplication of a valuable medicinal plant to meet out the pharmaceutical demand and its conservation. Attempts were made to evolve a rapid *in vitro* technology to conserve, as well as, mass propagate this valuable medicinal herb in very short duration. The combinations of benzylaminopurine (BAP, 4.0 and 5.0 mg/L) with IBA (0.25 mg/L) show shoot proliferation (83.3±0.16 %). The combination of BAP with indole-3-butyric acid (IBA, 2:0.25) in MS media showed maximum shoot elongation (2.25±0.70). Among the combinations of BAP with IBA and 1-naphthaleneacetic acid (NAA), MS media supplemented with BAP: NAA (4:0.25) show maximum number of shoots per explants (1.2±0.38). BAP (4.0 mg/L) with NAA (0.4 mg/L) shows highest (6.13±0.16) multiplication rate. MS media supplemented with BAP with IBA (4:0.5 and 5:0.5) resulted in shooting, as well as, rooting simultaneously. Micro propagated plantlets were hardened, acclimatized and transferred to the field. Biochemical investigation revealed significantly higher total sugar, as well as, protein contents *in vitro* raised plants than field grown plants however; total starch content was lower in micro propagated micro shoots. This micro propagation procedure could be useful for mass multiplication of superior plant material for field cultivation, as well as, research purpose.

Key words: Axillary shoots proliferation, node culture, axenic, microshoots, benzylaminopurine (BAP), naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA).

INTRODUCTION

In India, approximately 1700 plant species are used in ayurveda, 500 for Siddha, 400 for Unani, 300 for Amchi systems of medicine with substantial overlaps of common plants among these systems. *Centella asiatica* (Bhrami in Hindi; Vallarai in Tamil) is a small herbaceous annual plant of the family *Apiaceae* and is native to India. It is used as a medicinal herb in Ayurvedic medicine for increasing memory power; the plant enjoys considerable reputation in Indian system of medicines as a brain tonic. It is also used in treatment of asthma, bronchitis, dropsy, elephantiasis, gastric catarrh, kidney troubles, leucorrhoea, skin disease and urethritis. It has antibacterial, anti-feedent, anti-filarial, anti-stress, anti-tuberculosis activities and wound healing properties (Chakraborty et al., 1996; Srivastava et al., 1997). In India, *C. asiatica* frequently suffers due to growing modern agriculture, increasing use of herbicides, drastically depleting water level in river, canals and irrigation channels or adding of sewage water in the river cause rapidly eroding natural habitat. The species are facing extinction in natural habitat, and to cope with this adverse condition; the *in vitro* propagation is a boon for mass production and conservation of this valuable medicinal herb.

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

*Corresponding author. E-mail: tiwari.chandrakant08@gmail.com.

The present investigation aimed at developing a rapid protocol, which can be used for large scale production and conservation of *C. asiatica* to meet the pharmaceutical demand.

MATERIALS AND METHODS

Explants and culture initiation

The explants used to initiate aseptic cultures were single nodal segments, 2.5 to 3.0 cm in length. They were collected from medicinal garden, FRI Dehradun, India, in the month of July. The actively growing shoots were collected and nodal segments were prepared with the help of sharp secateurs and washed thoroughly under running tap water to remove the dust particles followed by washing in liquid detergent (labolein) solution with 5 to 10 drops/100 ml of Teepol (Glaxo India Ltd, Mumbai, India) for 5 min and then pre-sterilized in a mixture of fungicides viz. 1% (w/v) Bavistin and 1% (w/v) Blitox for 15 min. Then after, they were disinfected for 10 min in 0.1% (w/v) mercuric chloride and washed in sterile distilled water three times. The sterilized nodes were subsequently cultured singly in culture tubes (25·150 mm) containing shoot initiation medium, that is, 15 ml of MS (Murashige and Skoog, 1962) medium incorporated with 3% sucrose and 0.7% agar. The medium was supplemented with various combinations of BAP and auxins.

Axillary shoot proliferation and multiplication

The explants were inoculated in solid MS medium with different concentrations (1 to 5 mg/L) of BAP along with different concentrations of IBA and NAA in tissue culture tubes. A minimum of 24 replicates were maintained for each treatment. Within 10 days, buds were induced in incubated cultures. Elongated axillary buds after 10 days were separated and cultured for multiple shoot proliferation on the same medium. The proliferating shoot cultures were carefully separated into 3 to 4 shoot clusters and used as a unit of propagation, hereafter called a micro-shoots. For further shoot multiplication, five varying levels of NAA (0.1 to 0.5 mg/L) supplemented with 4 mg/LBAP were attempted. The control was devoid of PGRs. The average number of propagules derived from a culture at the end of a subculture cycle of 2 weeks was regarded as the rate of multiplication (fold). A two-way factorial experiment was conducted on MS medium supplemented with different combination of BAP and NAA (0.1, 0.2, 0.3, 0.4 and 0.5 mg/L) to screen out the most effective concentration of plant growth regulator combination for shoot multiplication.

In vitro rhizogenesis

Roots were induced during bud induction, as well as, multiplication since media were already supplemented with auxin. The plantlets with well developed root system were advanced for acclimatization.

Hardening and field transfer of plantlets

Rooted shoots from cultures were taken out from the flasks carefully, without causing any damage to the root system, and the roots were gently washed under running tap water. The plants with washed and cleaned root systems were transferred to autoclaved vermiculite. These plantlets were supplied with half-strength MS medium (without organics) once a week. After two weeks, the bottles were shifted to a mist-chamber with a relative humidity of 80 to 90% and a temperature of 30 ± 2°C. The caps of the bottles were removed and the plantlets were allowed to remain in the bottles for 3 to 4 days before they were transferred to poly bags containing a mixture of sand, farmyard manure and soil in a ratio 1:1:1 (by volume) and shifted to a shade house to acclimatize them to the environmental conditions outside. These plantlets were planted in the field.

Culture condition

Murashige and Skoog (MS) basal medium (consisting of salts, vitamins, 3 % sucrose and 0.7% agar) was used for inoculation. Different plant growth regulators (PGRs) viz. BAP, indole-3-butyric acid IBA and NAA were added at various concentrations to MS medium before the pH of the medium was adjusted to 5.6. Media were autoclaved at 1.06 $kgcm^{-2}$ and 121°C for 15 min. Cultures at all growth stages were incubated under artificial conditions: 25±2°C, 60% RH and a 16 h photoperiod (using white fluorescent tubes) under a photosynthetic photon flux density of 40 $\mu molm^{-2}$ s^{-1}.

Experimental design and statistical analysis

The experiment was designed in a randomized design. Each treatment carried 24 ramets. The experiment was replicated thrice. The recorded numerical information aided in determining the standard error. The data collected was subjected to the analysis of variance (ANOVA); significant differences among the treatments were tested by Duncan's multiple range test (Duncan, 1955) at 5% level and the critical difference (CD) values at P= 0.05 were computed to compare means from various treatments using SPSS (Version 16).

Biochemical estimation

After the sub-cultured plants had grown, they were assessed for biochemical analysis. The plants were taken out from the test tubes, washed, chopped, weighed and were further used for biochemical inference procedure.

Estimation of carbohydrate

Carbohydrate was estimated by anthrone method. About 100 mg of fresh leaf material was taken and homogenize in 10 ml of distilled water. Then 2 ml of 10% trichloroacetic acid was added. This homogenate was centrifuged at 3000 rpm for 5 min. The supernatant was taken and the volume was measured. From this, 0.2 ml of the supernatant was made up to 1 mL with distilled water. To that 4 mL of anthrone reagent (0.2% anthrone in conc. H_2SO_4) was added. This mixture was boiled in a boiling water bath for about 15 min. finally carbohydrate was estimated by UV Spectrophotometer at 625 nm.

Estimation of protein by Bradford method

Protein determination was made according to the Bradford method (Bradford, 1976) and using a standard curve prepared with bovine serum albumin. Briefly, Coomassie blue, in response to protein concentration, reacts with basic amino acid residues, especially arginine. Leaf samples (0.1 g) were homogenized with 0.5 ml of phosphate buffer at pH 7.5 and centrifuged at 12000 rpm for 15 min at room temperature. The supernatant was recovered samples were diluted 1:100 and read in triplicate in a spectrophotometer at 595 nm.

Estimation of starch

Starch content was determined by anthrone method. About 0.1 g of leaf material was macerated with 2 ml 50% alcohol and then centrifuged. Then the supernatant was discarded. After that, 1ml of

1 N H_2SO_4 and 1 ml of distilled water were added to the pellets. Then the mixture was boiled for 30 min. and cooled. Then mixture was centrifuged. Then the filtrate was measured and made up to 5 ml with distilled water. From that 0.5 ml of the supernatant was taken and added with 0.5 ml of distilled water and 4 ml of anthrone reagent (0.2% in conc. H_2SO_4). Then the mixture was heated for 30 min. and cooled. Finally the starch was determined by UV Spectrophotometer at 630 nm wavelength.

RESULTS AND DISCUSSION

In vitro shoot induction

For the present study, nodal explants were taken for shoot induction. Uninodal segments of mature plants have been however used in most cases. Nodal explants of *B. Monnieri* were propagated *in vitro* using shake cultures (Tiwari et al., 2000); nodal explants were also used for *Eclipta alba* (Gawde and Paratkar, 2004*);* shoot tip, nodal and intermodal segments were reported in *phyllanthus amarus* (Ghanti et al., 2004). Stem and leaf explants of green house green plants were used for the regeneration from callus cultures of *C. asiatica,* (Patra et al., 1998). Banerjee et al. (1999) used 5 to 6 months old glass house grown plants of *C. asiatica* for *in vitro* multiplication from leaf explants. Tiwari et al. (2000) reported micro propagation of *Centella* using nodal segments for clonal propagation of *C. asiatica.* Among various cytokinins, BAP is the most often used, cytokinin, particularly, in commercial micro propagation establishments where cost and ease of handling are major considerations (Zaerr and Mapes, 1982; Thomas and Blakesley, 1987). It was reported that shoot induction as well as multiplication of *C. asiatica* were better in BAP than Kinetin (Karthikeyan et al., 2009).

In the present study, BAP (cytokinin) was taken in combination with auxin. Bud break along with root induction was achieved in 7 to 10 days of inoculation followed by multiple shoot formation. Bud break on BA supplemented MS media after two weeks of inoculation followed by shoot proliferation, and further two weeks for root induction, was reported by Karthikeyan et al. (2009). Rapid micropropagation protocol of *C. asiatica* through callus proliferation on MS media supplemented with combination of cytokinin and auxin was reported by Thangapandian et al. (2012).The data pertaining to the effect of different combinations of BAP and auxins viz. IBA and NAA on shoot induction and proliferation of *C. asiatica* in a two-way factorial experiment is presented in Table 1. Maximum bud break (83.33±0.16) was achieved in BAP 5 and 4 mg/L+ IBA 0.25 mg/L. The treatment of BAP and auxin in combination and their interactions had statistically significant effects ($P < 0.05$) on the rate of shoot proliferation. In all the treatments, the shoot induction increased from lower to higher concentration that is, 5 and 4 mg/L of BAP. Significant variable rate of shoot proliferation was obtained in different concentrations of BAP. The maximum shoot induction (90%) was observed with BAP 5 and 4 mg/L + IBA 0.25 mg/L, as well as, 4 mg/LBAP+ 0.5 mg/LIBA, which was significantly higher than other combinations. Maximum shoot proliferation through callus culture was obtained on MS medium supplemented with 2.0 mg/L kinetin and 4.0 mg/L NAA by Patra et al. (1998) in *C. asiatica.*

The treatment of IBA (0.5 mg/l) along with BAP resulted in shoot as well as root induction whereas other combinations failed to induce rooting during shoot induction. Maximum shoot number per explant (1.2±0.38) was recorded when MS media was supplemented with 4 mg/L BAP + 0.5 mg/L NAA followed by 4 mg/L BAP + 0.5 mg/L NAA (1.1±0.35), which were significantly higher than others while maximum shoot length (2.25±0.70) was observed on MS media supplemented with 2.0 mg/LBAP + 0.25 mg/L of IBA, which was significantly higher than others. Rooting on MS media supplemented with lower concentration of auxin (NAA, 1 mg/L) was reported by Singh and Bhati (2011). In present study, rooting was achieved on two combinations viz. 4.0 mg/L BAP + 0.5 mg/L IBA (1.25 ±0.25) and 5.0 mg/L BAP + 0.5 mg/L IBA (2.0 ±0.63). For multiplication of shoots further, sub culturing was performed on MS medium supplemented with combinations of BAP (4mg/L) and varying concentrations of NAA (Figure 1A to D).

Shoot multiplication

Rooted as well as non-rooted micro shoots (aggregate of 3to4 shoots) were transferred to multiplication media supplemented with combinations of BAP and lower concentration of NAA (0.1 to 0.5 mg/L). Initial sprouting and differentiation of shoots and their growth required a medium with relatively higher concentration of BAP and NAA at a lower concentration instead of IBA (Banerjee et al., 1999). The data pertaining to the effect of different combinations of BAP and NAA on the shoot multiplication of *C. asiatica* in a two-way factorial experiment is presented in Table 2. Different concentrations of NAA had different effects on the rate of shoot multiplication. The treatment NAA 0.4mg/L had statistically significant effects ($P < 0.05$) on the rate of shoot multiplication. It was observed that the maximum rate of shoot multiplication (6.13±0.16) was obtained on medium supplemented with 4.0 mg/L of BAP and 0.4 mg/L of NAA, which was significantly higher than the shoot multiplications obtained with other NAA combinations, in three weeks period (Figure 2A, B and C). The longest shoots (5.2±0.10) were obtained on the same medium that is 4.0 mg/L of BAP and 0.4 mg/L of NAA which was significantly higher than the other treatments. Significant decrease in mean shoot length was observed upon the addition of NAA beyond 0.4 mg/L (Table 2).

In vitro rooting of micro shoots

In vitro rooting was achieved during shoot induction

Table 1. Effect of different combinations of BAP, IBA and NAA on percent shoots induction, number of shoots per explants length of shoots (cm) and Root length (cm) from nodal segments of *Centella asiatica* cultured on MS media. (Observations recorded after 2 weeks of inoculation).

Treatment	Concentration (mg/L)	Shoot induction (% ± SE)	Number of shoots per explants(Mean ± SE)	Shoot length(cm) (Mean ± SE)	Root length(cm) (Mean ± SE)
Control	-	-	-	-	-
BAP+IBA	1:0.25	33.3±0.21^{ab}	0.4±0.25^{ab}	0.98±0.62^{abcd}	-
	2: 0.25	50.0±0.22^{ab}	0.5±0.23^{ab}	2.25±0.70^{d}	-
	3: 0.25	50.0±0.22^{ab}	0.56±0.25^{ab}	1.93±0.86^{cd}	-
	4: 0.25	83.3±0.16^{b}	0.59±0.11^{ab}	1.3±0.26^{ab}	-
	5: 0.25	83.3±0.16^{b}	0.58±0.11^{ab}	0.87±0.18^{abcd}	-
BAP+IBA	1:0.5	-	-	-	-
	2: 0.5	-	-	-	-
	3: 0.5	-	-	-	-
	4: 0.5	83.3±0.16^{b}	0.4±0.08^{b}	1.6±0.32^{bcd}	1.25 0.25^{b}
	5: 0.5	66.7±0.21^{b}	0.5±0.16^{ab}	1.4±0.45 ^{abcd}	2.0 ±0.63^{c}
BAP+ NAA	1:0.25	33.3±0.21^{ab}	0.45±0.28^{ab}	0.54±0.34^{abc}	-
	2: 0.25	33.3±0.21^{ab}	0.62±0.39^{ab}	0.71±0.45^{abc}	-
	3: 0.25	50.0±0.22^{ab}	0.72±0.32^{ab}	0.33±0.15^{ab}	-
	4: 0.25	66.7±0.21^{b}	0.95±0.30^{ab}	0.25±0.08^{ab}	-
	5: 0.25	66.7±0.21^{b}	0.62±0.20^{ab}	0.83±0.26^{abc}	-
BAP+ NAA	1:0.5	33.3±0.21^{ab}	0.35±0.22^{ab}	1.1±0.69^{abcd}	-
	2: 0.5	33.3±0.21^{ab}	0.50±0.32^{ab}	0.9±0.57^{abcd}	-
	3: 0.5	50.0±0.22^{ab}	0.98±0.44^{b}	1.3±0.60^{abcd}	-
	4: 0.5	66.7±0.21^{b}	1.2±0.38^{b}	0.65±0.20^{abc}	-
	5: 0.5	66.7±0.21^{b}	1.1±0.35^{b}	0.4±0.13^{ab}	-
$CD_{(0.05)}$ treatment		0.0095	0.062	0.025	
$CD_{(0.05)}$ concentration		0.016	0.108	0.013	

Values are given as mean ± standard error (SE) of three replicates; each replicate consisted of 24 ramets. Mean followed by the same letter are not significantly different at the 5% level (Duncan's multiple range test).

Table 2. Effect of different combinations of BAP and NAA on rate of multiplication and length of shoots (cm) from propagules* of *Centella asiatica* cultured on MS media. (Observations recorded after six weeks of culture on MS media).

Treatment	Concentration (mg/L)	Shoot length(cm) (Mean±SE)	Multiplication rate (Mean±SE)	Shoot no. (Mean ± SE)
CONTROL	-	1.05±0. 07^{a}	1.05±0. 07 ^{a}	1.46±0. 17 ^{a}
	4:0.1	3.43±0.11^{b}	2.11±0.05^{b}	2.0±0. 07 ^{b}
	4: 0.2	3.8±0.13 ^{b}	3.43±0.22 ^{c}	3.7±0.10 ^{c}
BAP+ NAA	4: 0.3	4.38±0.24 ^{c}	5.36±0.19 ^{d}	4.16±0. 09 ^{d}
	4: 0.4	5.2±0.10 ^{d}	6.13±0.16 ^{e}	5.33±0.15 ^{f}
	4: 0.5	3.66±0.12 ^{b}	5.08±0.13 ^{d}	4.55±0. 11 ^{e}
$CD_{(0.05)}$		0.171	0.356	

Values are given as mean ± standard error (SE) mean ± SE of three replicates; each replicate consisted of 10 cultures. Mean followed by the same letter are not significantly different at the 5% level (Duncan's multiple range test).

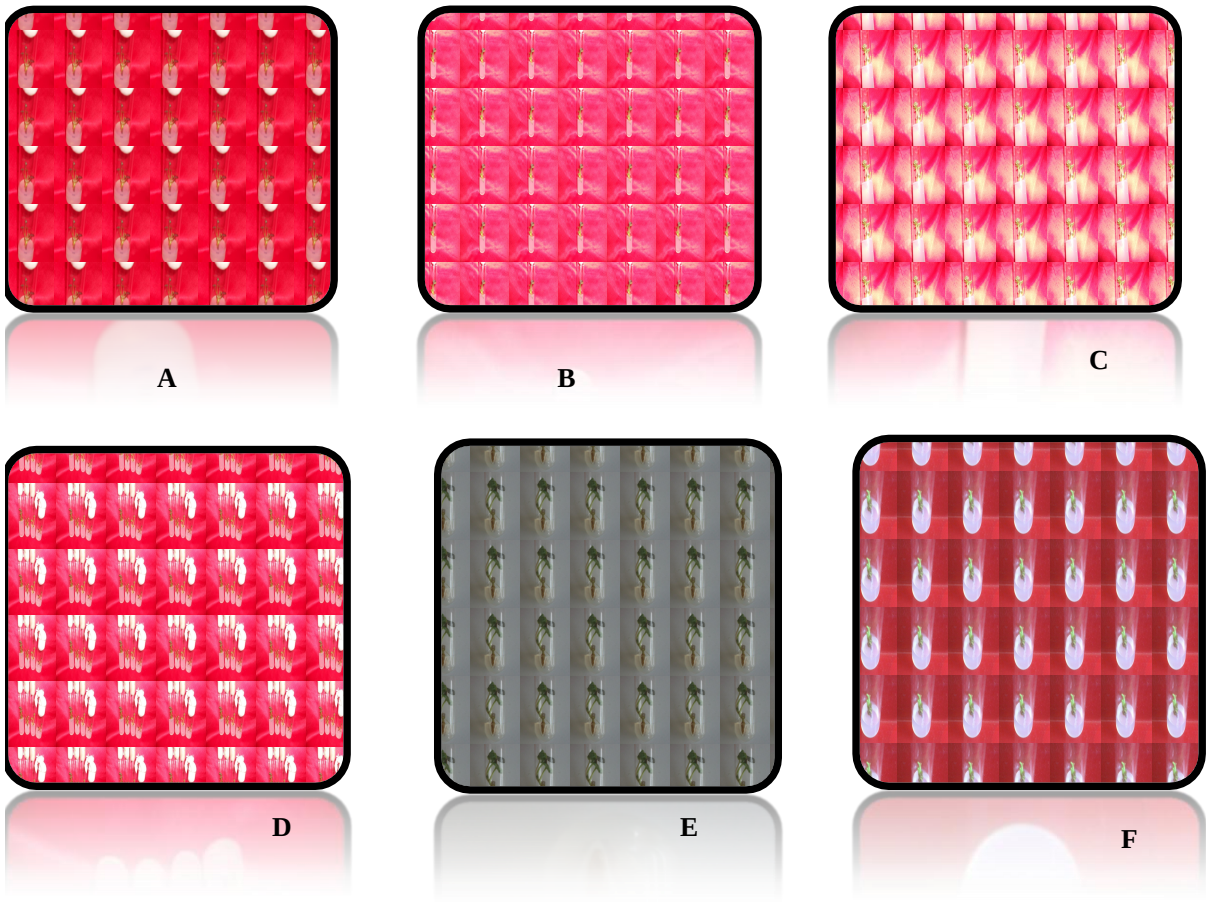

Figure 1. A to D, Bud break in nodal explants of C. *asiatica;* **E and F**, shoot as well as root induction in nodal explants of *C. asiatica.*

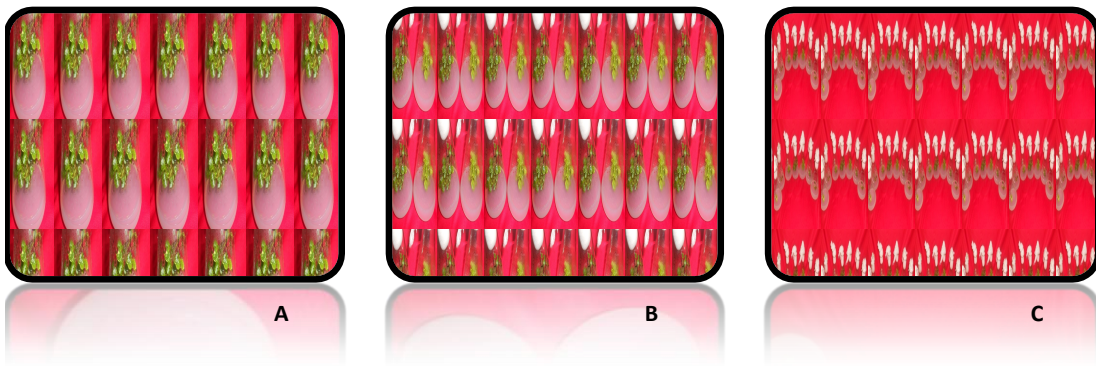

Figure 2. A, B and C, Multiplication (2nd subculture) of microshoots of C. *asiatica.*

therefore same had been followed for rooting. Maximum mean root number (2.0 ± 0.63) was achieved on 0.5 mg/L IBA along with 5 mg/L BAP supplemented full strength MS media. A well developed root system was obtained after three weeks of inoculation of nodal explants (Figure 1E and F).

Hardening and acclimatization

The plantlets with well developed root and shoot systems were transferred to a 500 ml glass jars containing sterilized vermiculite presoaked with half strength MS nutrient medium (Sucrose-free). The jar bottles were

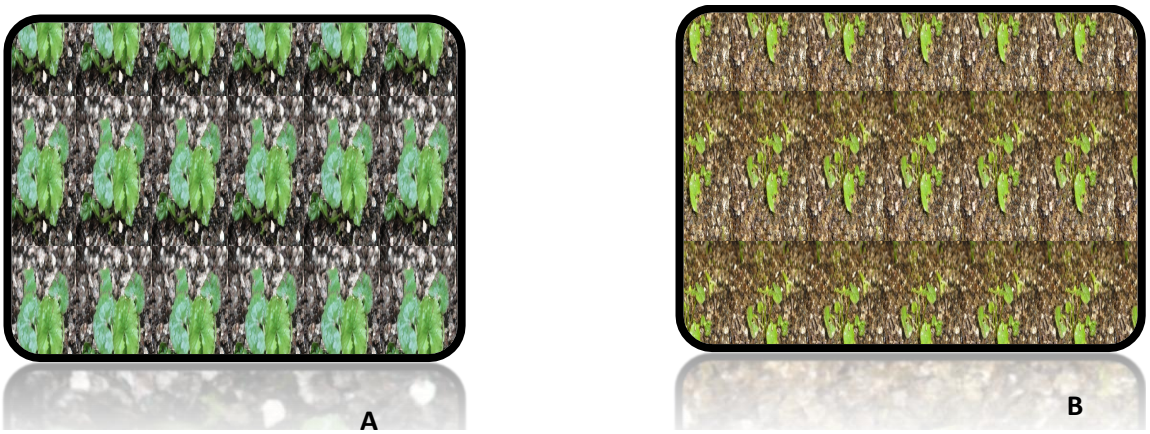

Figure 3. A and B, Hardened and Acclimatized plantlets of C*. asiatica* under shade house.

Table 3. Biochemical estimation of mother and cloned plants.

S/N	Biochemical	Mother plant	*In vitro* raised plant
1.	Total Carbohydrate (µg/100 mg sample)	75.2 ± 0.2	99.35 ± 0.36
2.	Starch (mg/500 mg sample)	0.67 ± 0.23	0.42 ± 0.28
3.	Total Protein (mg/500 mg sample)	20.71 ± 0.8	31.2 ± 0.07

initially kept for two weeks in culture room. More than 90% of the plantlets survived when subjected to two weeks hardening in the culture room. These plantlets were shifted to plastic pots containing soil, sand, and FYM (farmyard manure) in 1:1:1 ratio and were placed in the mist chamber for one week and subsequently shifted to shade house for acclimatization to external environment (Figure 3A and B). On the one hand, simultaneous rooting and shooting reduces cost of propagation and on the other it reduces time. Therefore, it is a very efficient protocol for conservation as well as mass propagation.

Biochemical estimation

In vitro raised plants were biochemically tested against field grown mother plants for estimation of carbohydrate, protein and starch contents. The total carbohydrate, starch as well as protein contents of field grown mother plant samples were estimated as 75.2 ± 0.2 µg/100, 0.67± 0.23 mg/500 mg and 20.71± 0.8 mg/500 mg, respectively whereas 99.35 ± 0.36 µg/100, 0.42 ± 0.28 mg/500 mg and 31.2 ±0.07 mg/500 mg, respectively in *in vitro* raised plant samples.

Indra et al. (2011) documented biochemical characterization of mother plant and tissue culture plants of *Cassia siamea*. Higher carbohydrate content in micropropagated plants in this study is in agreement with their results. The increase in the total carbohydrate is due to the higher concentration of carbon source, which is provided by the addition of sucrose to the medium for the micropropagation of *Pogostemen cablin* (Tawer et. al., 2010). Higher amount of protein in *in vitro* raised plants is in agreement with the works of Ali et al. (2010) in *Stevia rebaudiana* as well as Kumaraswamy et al. (2010) in *Spilanthes acemella*. Significant differences among two samples imply probability of superiority of *in vitro* raised plants over mother (field grown) plants which in turn explain advanced growth of micropropagated plants over mother plants. The findings of present study imply short term propagation of *C. asiatica* that is, within two month of inoculation on account of root as well as shoot development simultaneously, which either takes 5 to 6 months with normal micro propagation protocol. This protocol will not only help in short term mass propagation of this valuable medicinal herb but also reduce cost of the technique by reducing multistep technique to only two steps (Table 3).

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