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Comparative analysis of bioactive compounds in different habitat of *Centella asiatica* (L.) Urban: Application for *in vitro* clonal propagation of elite ecotype

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ABSTRACT

Centella asiatica (L.) Urban is a perennial herb with high utility in traditional medicines and pharmaceutical industries. In India the wild populations *C. asiatica* are overexploited at an unrestrained rate to cope up with its high market demands. The aim of this study was to identify potential chemotype of *C. asiatica* from different geographical region in India and optimized *in vitro* micropropagation protocol of the elite ecotype was established for commercial purposes. Five *C. asiatica* accessions were collected from different geographic locations and their active biological components were analyzed. Among these accessions total phenolic and flavonoid content combined with ascorbic acid was found to be significantly higher in CA-4 compared to other ecotypes. The free radical scavenging activity as well as reducing power of the CA-4 ecotype also corroborate the higher phenolic content. Therefore the elite ecotype CA-4 was selected for *in vitro* conservation and micropropagation. Shoot-tips explants was inoculated on Murashige and Skoog basal medium with different concentrations and combinations of cytokinins and auxins. Optimum multiplication was achieved on medium containing 6-benzyl-aminopurine (2.0 mg L^{-1}) and indole-3-acetic acid (0.2 mg L^{-1}). *In vitro* rooting of multiplied individual shoots was achieved on half strength Murashige and Skoog medium supplemented with IBA (1.5 mg L^{-1}), with a maximum of 16.4 ± 0.22 roots. These micropropagated plants were successfully established in soil after hardening with 94.55 % survival rate.

INTRODUCTION

Centella asiatica (L.) Urban, a tropical medicinal plant from Apiaceae family is a perennial herb with creeping stem, rooting at nodes and produce tufts of leaves and flowers. The plant is native to Southeast Asian countries including India, Sri Lanka, China and Malaysia as well as South Africa (Mukherjee and Constance, 1993). In Asiatic countries, *C. asiatica*is used as an ingredient in traditional systems of medicine such as Ayurveda, Siddha and Unani. In accordance with its potential wound healing property, several report described the remarkable protective effect of the plant against several diseases of central nervous system (Jian *et al.*, 2007; Liu *et al.*, 2008). It also involved in wide range of biological activities desired for human health such as anti-inflammatory (George *et al.*, 2009), antiulcer (Cheng *et al.*, 2004), hepatoprotective (Pingale, 2008), anticonvulsant (Sudha *et al.*, 2002), cardioprotective (Gnanapragasam *et al.*, 2004), cytotoxic and antitumor (Lee *et al.*, 2002), antiviral (Yoosook *et al.*, 2000) and antibacterial activities (Zaidan *et al.*, 2005). The antioxidant activity of the plant is comparable to that of commonly utilized plant and it has been reported that it possess very good potential to be explored as a source of natural antioxidants (Jayashree *et al.*, 2003;Tatmiya *et al.*, 2014). It has been reported that *C. asiatica* plant contains a plethora of compounds belonging to wide range of chemical classes. Biological effects of *C. asiatica* have been attributed to the existence of major triterpene derivatives including asiatic acid, madecassic acid, asiaticoside, madecassoside, and brahmic acid (Verma *et al.*, 1999; Schaneberg *et al.*, 2003). The occurrence of several important flavonoid derivatives including quercetin, kaempferol and several important phenolic compounds has also been reported (Subban *et al.*, 2008; Yoshida *et al.*, 2005). However, significant differences in active constituents have been observed between different samples of *C. asiatica* originating from

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different countries (Das and Mallick, 1991; Rouillard *et al.*, 1997; Randriamampionona *et al.*, 2007; Chong and Aziz, 2011). In India, owing to its biological immense activities and pharmaceutical applications, wild genotypes of *C. asiatica* is being collected extensively that could ultimately lead to extinction of important ecotypes/genotypes. The gradual decline in the natural population of the plant species demands efficient conservation efforts in order to ensure continuous and plenty supply of this plant material that are in great demand by the various pharmaceutical industries. One of the possible methods of protection of endangered plant species is multiplying and conservation through *in vitro* cultures. The development of reliable tissue culture protocols are of countless importance for the conservation of this economic important plant species by virtue of producing uniform planting material. Under such circumstances, the objective of present work was to screen for elite ecotypes of *C. asiatica* from different geographical region in India and established optimized micropropagation protocol for commercial and pharmaceutical purposes.

MATERIAL AND METHODS

Plant material

The Plant of *C. asiatica* was collected from five different ecotypic regions of India. The localities, geographic positions and altitudes of the collection spots were represented in Table 1. The leaves of the collected plants were used for biochemical analysis.

Table 1: *C. asiatica* ecotypes collected from different geographic location.

Sl No.	Code Name	Area of study	Altitude (meter)	Geographical location
1	CA-1	Rudraprayag, Uttarpradesh	692.942	78° 981" E, 30° 284" N
2	CA-2	Darjeeling, West Bengal	2121.941	88° 262" E, 27° 036" N
3	CA-3	Kamakha hill, Assam	235.048	91° 704" E, 26° 166" N
4	CA-4	Calcutta, West Bengal	13.953	88° 363" E, 22° 572" N
5	CA-5	Konarak, Orrisa	12.0	86° 094" E, 19° 887" N

Determination of total phenolic and flavonoid content

Total phenolic content was measured following Folin–Ciocalteau method (Singleton *et al.*, 1999). The total flavonoid content was determined following the aluminum chloride colorimetric assay (Chang *et al.*, 2002). Total phenolic and flavonoid content was expressed as mg g^{-1}fresh weight (fw).

Estimation of ascorbic acid

The ascorbic acid was estimated by using Folin phenol reagent using different concentrations of standard ascorbic acid (Jagota and Dani, 1982). The amount of ascorbic acid was expressed as mg g^{-1}fw.

Estimation of reducing sugar and protein

The amount of reducing sugar was estimated by dinitrosalicylic acid method (Miller, 1959). The total protein content was determined following the method reported by Bradford (Bradford, 1976). The reducing sugar and total protein content was expressed as mg g^{-1}fw.

Determination of antioxidant capacity

Free radical scavenging by the use of the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical

The antioxidant activity of the ethanolic extracts was determined according to the DPPH method (Brand-Williams *et al.*, 1995). Briefly, 50 µL of different concentrations of ethanolic extract was mixed with 1950 mL of 6.34×10^{-5}M DPPH radical solution in ethanol. The mixture was allowed to stand for 30 min in the dark. The absorbance of the solution was measured at 517 nm. The ascorbic acid was used as standard. Free radical scavenging activity was calculated using the following formula:

$$\% \text{ inhibition} = [(A_B - A_E)/A_B] \times 100$$

Where, A_B and A_E are the absorbance at 30 min of the blank and the sample, respectively.

The antioxidant activity was calculated as IC_{50} (µg mL^{-1}), the extract dose required to cause a 50% decrease of the absorbance at 517 nm. A lower IC_{50} value corresponds to a higher antioxidant activity. The antiradical activity was expressed as 1/ IC_{50}.

Table 2: Correlation of antiradical activity ($1/IC_{50}$) with total phenolic and flavonoid content

Assays	Correlation R^2	
	Phenolics	Flavonoids
DPPH	0.898	0.624
ABTS	0.935	0.689
FRAP	0.873	0.592

Free radical scavenging by the use of the ABTS radical

The free radical scavenging activity was studied using the ABTS radical cation decolorization assay (Re *et al.*, 1999). ABTS radical cation was produced by reacting 7.0 mM ABTS solution with 2.45 mM potassium persulfate and the mixture was kept in dark at room temperature for 16 h. For the analysis, the solution was diluted in double distilled water to an absorbance of 0.7 (±0.02) at 734 nm.

Fifty microliter of the ethanolic extract of different concentrations was added to 1950 µL of ABTS solution and the absorbance was recorded after incubation of 30 min at 30 °C. The different amount of ascorbic acid was used as standard. The percentage of inhibition of ABTS+• was calculated using above formula (eq 1). The antiradical activity was expressed as $1/IC_{50}$.

Ferric reducing antioxidant power (FRAP) assay

Antioxidant activity was also determined by ferric reducing power using a spectrophotometer at 700 nm (Oyaizu, 1986). Briefly, 1 mL of extract was mixed with 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide.

The mixture was incubated at 50 °C for 20 min. Then 2.5 mL of 10% trichloroacetic acid was added and centrifuged at 3000 rpm for 20 min. Then 1 mL supernatant was added to the mixture of 0.5 mL of 0.1% $FeCl_3$ and 2.5 mL of double distilled water. The absorbance was measured at 700 nm after incubation for 10 min. Different concentrations of ascorbic acid was used as control. Increase in absorbance was interpreted as increased ferric reducing

activity. The IC_{50} value (μg/mL) is the concentration giving an absorbance of 0.5. The antiradical activity was expressed as $1/IC_{50}$.

In vitro propagation

In vitro adventitious shoot induction and elongation

For *in vitro* propagation purpose MS basal medium was used (Murashige and Skoog, 1962). The pH of the medium was adjusted to 5.6 before the addition of agar. The medium was autoclaved at 1.04 kg/cm pressure and 121°C for 18 min. The shoot tips (8-10 mm) were used as explant. The explants were implanted in culture tubes (25 mm × 150 mm) containing MS basal medium supplemented with 0.8% agar, 3.0% sucrose and different concentrations of 6-Benzylaminopurine [BAP], 6-(γ,γ-dimethylallylamino) purine [2iP], kinetin [Kn] alone or in combination with three different concentrations of 1-Naphthaleneacetic acid [NAA] (Table 3). For shoot elongation, cytokines were totally withdrawn and only 0.5 mg L^{-1} IAA were supplemented. The cultures were incubated inside the growth chamber maintained at 24±2°C under a 16 hrs photoperiod with a photosynthetic photon flux density of approximately 50 $\mu molm^{-2}s^{-1}$ emitted from cool fluorescent tubes (Philips India Ltd.). The cultures were sub-cultured in their respective fresh media at every 4 weeks intervals.

In vitro root induction

For *in vitro* root induction, we follow the method previously optimized by the present laboratory (Haque and Ghosh, 2013a; Haque *et al.*, 2015). Elongated adventitious shoots were cut into about 2.5 cm pieces and implanted on half strength MS media supplemented with different concentrations of IBA or NAA. MS medium devoid of growth regulator served as control. The percentage of rooting, mean root length and total number of roots were calculated after 4 weeks.

Acclimatizing and field evaluation of regenerated plantlets

Rooted plantlets (about 6-8 cm) were transferred to small earthen pots containing 'Soilrite' (Keltech Energies Ltd., Bangalore, India) and protected with transparent polythene bags to maintain 90-99% relative humidity and were kept in 25 ± 2°C temperature and 16-h photoperiod for 25 to 30 days. Thereafter, the acclimatized plants were relocated on earthen tubs containing a mixture of soil and vermin compost (3:1 ratio) and maintained inside the poly-green house (30 ± 2°C temperature and relative humidity of 60-65%) for another 3 months. Finally, the plantlets were transferred to the field under full sunlight**.**

Statistical analyses

A randomized block design was used to set up all the experiments. The data presented are means ± standard deviation of 3 biological replicates and are examined by analysis of variance (ANOVA) to detect significant differences between means and those differing significantly ($p<0.05$) are compared using Duncan multiple range test (DMRT) at the same (5%) probability level using SPSS software (17.0.0, 2008, SPSS Inc.).

RESULT AND DISCUSSION

Comparative analysis of bioactive compounds

The variation of major bioactive compounds of *C. asiatica* due to different agro-climatic conditions of were analyzed. It was found that total phenolic content of CA-4 ecotype was significantly higher compared to the other ecotypes (Fig. 1A). The total phenolic content in different ecotype were found to be in following order: CA-4 > CA-5 > CA-3 > CA-1 > CA-2. The flavonoid content also differ significantly among different ecotypes (Fig. 1B).

The total flavonoid content in different ecotype were found to be in following order: CA-5 > CA-4 > CA-1 > CA-3 >CA-2. The ascorbic acid content was found to be highest in CA-4 ecotype followed by CA-5 (Fig. 1C). Although the total protein content was comparable in three samples (CA-4, CA-5 and CA-3) but reducing sugar were found to be significantly higher in CA-4 and CA-5 compared to others (Fig. 2). The reducing sugar in different ecotype were found to be in following order: CA-4 > CA-5 > CA-3 > CA-2 > CA-1.

Table 3: Influence of plant growth regulators and their interaction on morphogenic response from nodal explants of *C. asiatica* on MS growth medium.

SL No.	MS media with growth regulators (mg l^{-1})				Percentage of response	No. of multiplied shoots / nodal explant	Micro shoot length (cm)
	BAP	Kn	2iP	NAA			
1	1.0	0.0	0.0	0.0	63.20	5.1 ±0.20^{c}	2.0 ±0.42^{c}
2	2.0	0.0	0.0	0.0	75.45	8.5 ±0.20^{g}	2.7 ±0.25^{e}
3	3.0	0.0	0.0	0.0	72.50	7.3 ±0.25^{f}	2.1 ±0.26^{c}
4	0.0	1.0	0.0	0.0	51.23	4.6 ±0.43^{a}	1.5±0.25^{a}
5	0.0	2.0	0.0	0.0	68.25	7.2 ±0.34^{f}	2.5 ±0.25^{d}
6	0.0	3.0	0.0	0.0	62.26	6.6 ±0.21^{d}	2.1 ±0.10^{c}
7	0.0	0.0	1.0	0.0	58.24	4.9 ±0.22^{b}	1.7 ±0.09^{b}
8	0.0	0.0	2.0	0.0	75.24	7.3±0.15^{f}	2.6 ±0.09^{de}
9	0.0	0.0	3.0	0.0	60.20	6.8 ±0.20^{e}	2.0 ±0.14^{c}
10	2.0	0.0	0.0	0.1	87.47	11.0 ±0.23^{i}	3.8±0.13^{f}
11	2.0	0.0	0.0	0.2	90.25	12.5 ±0.15^{j}	4.5±0.17^{h}
12	2.0	0.0	0.0	0.3	86.56	10.8 ±0.13^{h}	4.1±0.20^{g}

Values are mean ± SE of three independent experiments each with 12 replicates. Treatment means followed by same letter within column are not significantly different from each other ($P < 0.05$) according to ANOVA and DMRT.

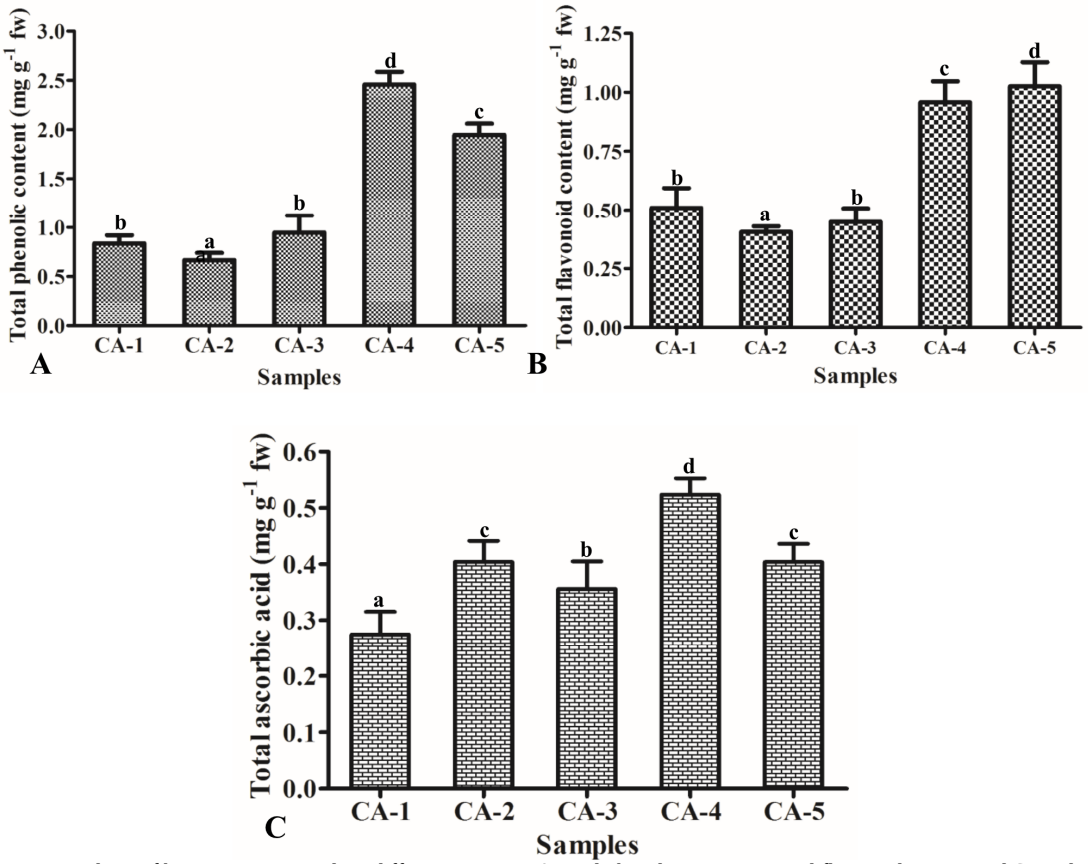

Fig. 1: Comparative analysis of bioactive compounds in different ecotypes. A. total phenolic content B. total flavonoid content and C. total ascorbic content. Bars are mean ± SD; bars followed by same alphabets are not significantly different at p≤0.05 according to ANOVA and DMRT.

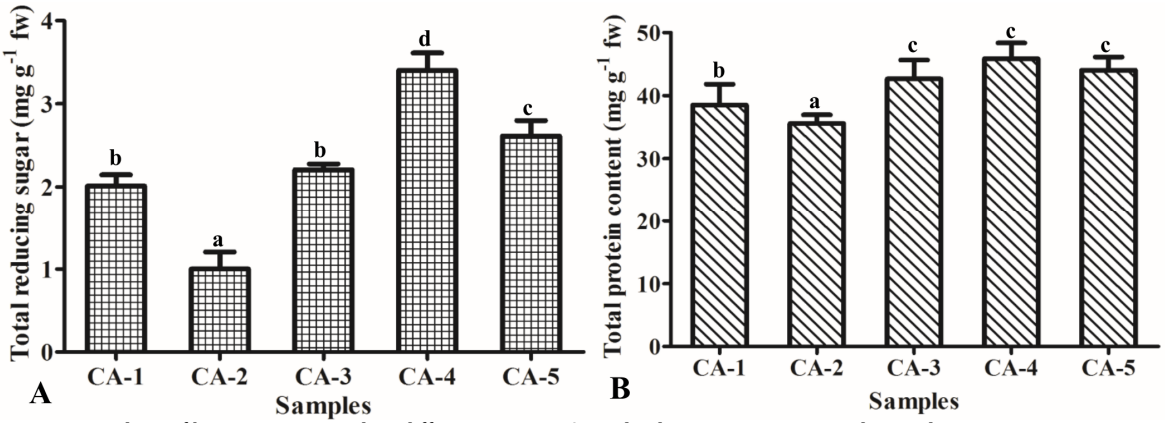

Fig. 2: Comparative analysis of bioactive compounds in different ecotypes. A. total reducing sugar content and B. total protein content. Bars are mean ± SD; bars followed by same alphabets are not significantly different at p≤0.05 according to ANOVA and DMRT.

Comparative analysis of antioxidant potential of different ecotypes

The ethanolic extract of the *C. asiatica* plant collected from different agro-climatic conditions showed scavenging activity over DPPH free radicals. A dose dependent increase in scavenging activity was recorded with all the samples. The antiradical activity was expressed as 1/ $IC_{50.}$ A higher value corresponds to a higher antioxidant activity. The antiradical activity of five different ecotype was found to be in following order: CA-4 > CA-5 > CA-3 > CA-1 > CA-2. The antiradical activity of CA-4 over DPPH radical was found to be 1.287 ± 0.043 (Fig. 3). The ethanolic extract also showed ABTS radical scavenging activity. The scavenging activity was also found to be in following order: CA-4 > CA-5 > CA-3 > CA-1 > CA-2.

The reducing power of ethanolic extract was also significantly higher in CA-4 compared to other samples (Fig. 3). It has been reported that there is a positive correlation between the total phenolic content and antioxidant activity (Miliauskas *et al.*, 2004; Gulati *et al.*, 2012). A highly positive correlation between

the DPPH (R^2 = 0.898) and ABTS (R^2 = 0.935) values and total phenolic content clearly indicated that phenolic compounds could be one of the major components responsible for antioxidant activity (Table 1). Our results clearly revealed that the higher phenolic content in the CA-4 ecotype coincided with higher antioxidant activity. The variation of the bioactive compounds including antioxidant potential due to discrepancy in agro-climatic conditions were also reported in several other plants (Siddhuraju and Becker, 2003; Gull *et al.*, 2012; Kundu *et al.*, 2015).

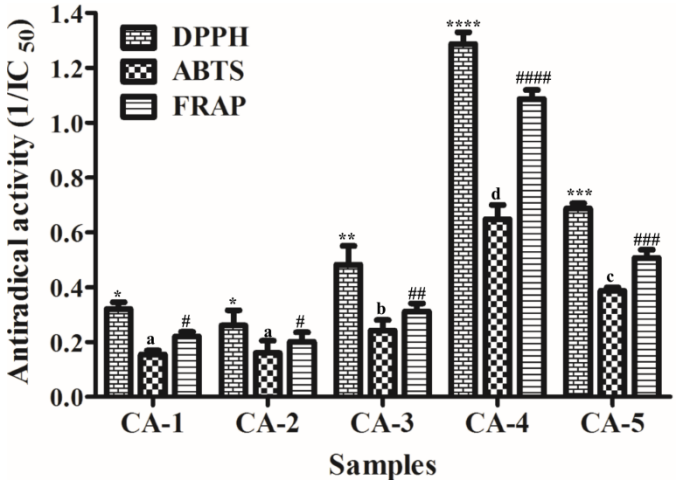

Fig. 3: Comparative analysis of antiradical activity in different ecotypes. Bars are mean ± SD; bars followed by same symbols are not significantly different at p≤0.05 according to ANOVA and DMRT.

Effects of plant growth regulators for optimal proliferation

Maximum of 8.5 ± 0.20 shoots were induced in MS medium containing 2.0 mg L^{-1} BAP after 45days of culture (Fig. 1A). Out of three cytokinin tested BAP prove to be more efficient than KIN and 2iP for shoot proliferation (Table 3). The efficacy of BAP alone on multiple shoot bud differentiation has been established in number of reports (Loc *et al.* 2005; Zhang *et al.* 2010; Haque and Ghosh, 2013b). NAA in low concentration along with optimum concentration of BAP (2.0 mg L^{-1}) had beneficial effect on multiplication rate and elongation of multiplied shoots of *C. asiatica*. Maximum 12.5 ± 0.15 shoots from each explant were induced in MS basal medium supplemented with 2.0 mg L^{-1} BAP with 0.2 mg L^{-1} IAA after 45 days of culture (Fig. 4B). Maximum elongation of shoots reaching 4.5 ± 0.17 cm was recorded on 2.0 mg L^{-1} BAP + 0.2 mg L^{-1} IAA after 45 days of culture (Table 3).

***In vitro* rooting of regenerated shoots and acclimatization of plantlets**

The micro-shoot tips (about 2–3 cm) with two to three leaves were harvested from the medium and used for rooting. The rooting response differed depending upon the concentration of NAA and IBA as well as the strength of the basal medium (Table 4). Rooting percentage was significantly lower in full-strength as well as one-quarter MS medium compared to the half strength MS medium.

Therefore ½ MS media with different concentrations of NAA and IBA were used for further analysis. The average root length was also affected significantly by the IBA and NAA concentrations. The earliest (14.2±0.40 days) and highest percentage (97.8%) of root induction with maximum of roots (16.4±0.22) per explants was achieved on ½ MS medium containing 1.5 mg L^{-1} of IBA. The optimistic role of IBA on *in vitro* root induction was previously reported in many plants (Haque and Ghosh, 2013c).

A total of 55 out of 60 (91.66 %) *in vitro* rooted plantlets were successfully acclimatized for 20 to 25 days (Fig.4E). Thereafter, the acclimatized plants were transplanted on earthen tubs containing a mixture of soil and vermin compost (3:1 ratio) for next 3 months with 94.55% (52 out of 55) survival rate. The majority of the micropropagation protocols do not deals with concern of the acclimatization process or they only mention that the acclimatization was tested with success (Haque and Ghosh 2014), but we studied it thoroughly not only on acclimatization, but also up to the flowering of field-grown micropropagated plants. Recently, similar approach has been reported in few other important medicinal plants like Aloe, Bacopa, Tylophora, Curcuma, where all the micropropagated plants have studied up to the flowering stage (Haque and Ghosh, 2013a, b, c; Jose and Thomas, 2015).

Table 4: The effect of various concentrations of auxins treatments on root induction.

Nutrient medium (3% Sucrose)	Concentrations of auxins (mg L^{-1})		Percentage of root induction	No. of roots induced / micro shoot	Days taken for root induction
	NAA	IBA			
MS	0.0	0.0	51.0	3.8 ± 0.14^{a}	22.2 ± 0.49^{j}
½ MS	0.0	0.0	65.0	6.6 ± 0.26^{c}	19.3 ± 0.30^{i}
¼ MS	0.0	0.0	58.0	5.1 ± 0.23^{b}	17.6 ± 0.23^{h}
½ MS	0.5	0.0	74.0	8.3 ± 0.21^{d}	16.4 ± 0.30^{e}
½ MS	1.0	0.0	77.2	10.4 ± 0.26^{e}	15.2 ± 0.3^{c}
½ MS	1.5	0.0	82.2	13.8 ± 0.32^{i}	14.2 ± 0.31^{a}
½ MS	2.0	0.0	80.5	12.0 ± 0.24^{g}	17.2 ± 0.31^{g}
½ MS	0.0	0.5	88.2	10.7 ± 0.16^{f}	16.8 ± 0.25^{f}
½ MS	0.0	1.0	92.8	13.1 ± 0.28^{h}	14.8 ± 0.40^{b}
½ MS	0.0	1.5	97.8	16.4 ± 0.22^{k}	14.2 ± 0.40^{a}
½ MS	0.0	2.0	83.5	14.6 ± 0.20^{j}	16.2 ± 0.36^{d}

Values are mean±SE of three independent experiments each with 12 replicates. Treatment means followed by same letter within column are not significantly different from each other (P < 0.05) according to ANOVA and DMRT.

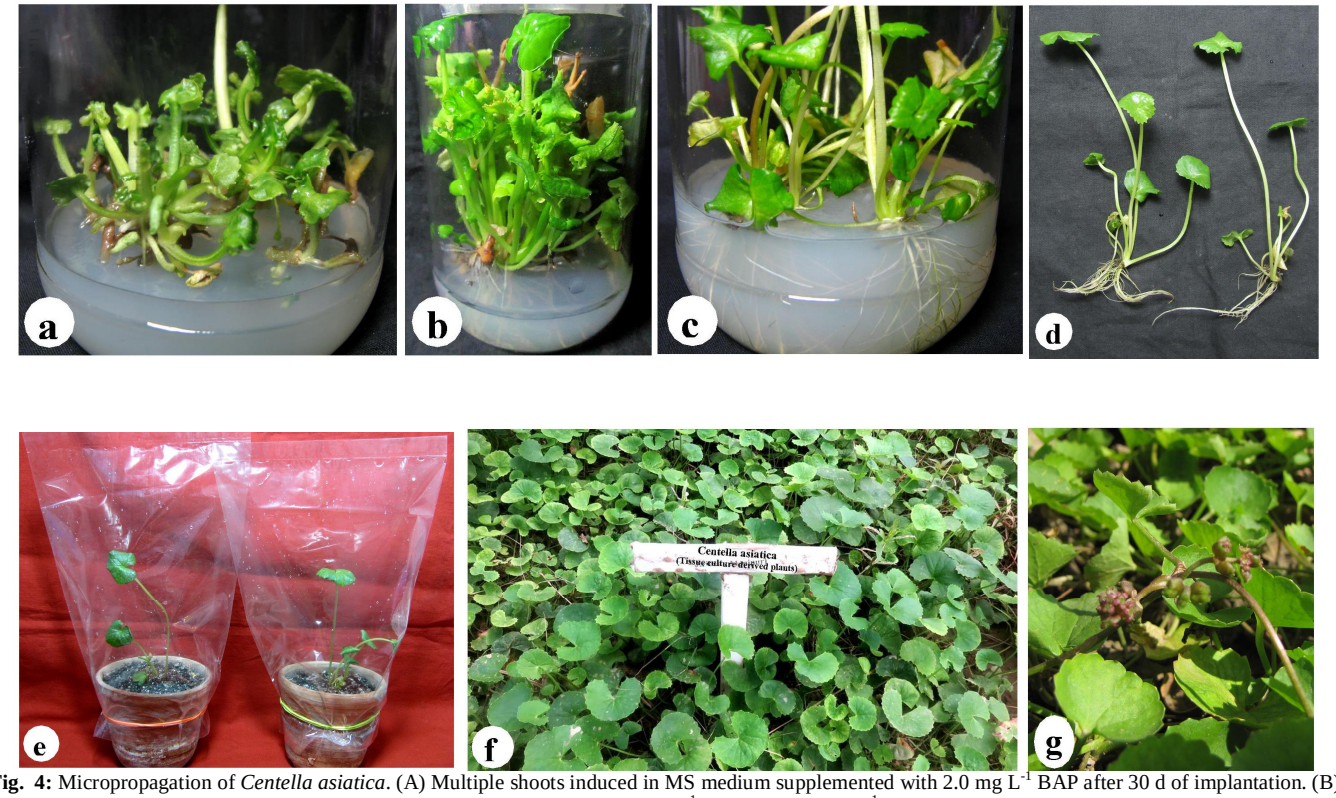

Fig. 4: Micropropagation of *Centella asiatica*. (A) Multiple shoots induced in MS medium supplemented with 2.0 mg L^{-1} BAP after 30 d of implantation. (B) Elongated multiplied shoots after 60 d of initial implantation on BAP 2.0 mg L^{-1} and NAA 0.2 mg L^{-1} containing MS medium. (C) *In vitro* rooting in MS medium supplemented with 1.5 mg L^{1} IBA after 30 d of implantation. (D) *In vitro* derived complete plantlet ready for hardening. (E) Hardening of regenerated plants. (F) Tissue culture derived regenerated plant after 6 months of field transfer. (G) Regenerated plant bearing flowers after 9 months of field transfer.

CONCLUSION

The Elite ecotype of *C. asiatica* identified based on active biological components could be commercially cultivated and utilized in traditional medicines and pharmaceutical industries. This information will also be helpful in restriction of the uncontrolled collection of the plant from the wild that could lead to complete elimination of certain economic important ecotypes. This study provides platform that could be exploited in enhancing the production of bioactive metabolites in *in vitro* conditions.

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