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In Vitro Propagation of Valuable Medicinal Plant *Centella asiatica* with an Approach to Study Its Phytochemical Properties by Thin Layer Chromatography

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ABSTRACT

An efficient *in vitro* propagation protocol was developed by using nodal and leaf explants for the conservation and mass multiplication of a valuable medicinal plant *Centella asiatica* to meet out the pharmaceutical demand. The medium supplemented with various concentrations of auxines in combination with cytokinines shows good response for callus induction. Best callus induction with frequency 83.33% and 86.66% observed with BAP 0.5mg/l and NAA 0.3mg/l in both node and leaf explants respectively. The induced calli from leaf explants of *Centella asiatica* were found to bear growing buds and tender leaves. The shoot induction occurred within 2-3 weeks of culture from calli. Among the various combinations of phytohormones used, MS+BAP 2.0 mg/l and NAA 0.5mg/l was found to be optimum for leaf explants with 80.88% shoot induction factor, shoot length 5.3 cm and number of five shoots in 16 days. The rate of shoot bud proliferation ability was maintained up to 6th subculture period on MS medium supplemented with BAP 4.0 mg/l and NAA 0.5mg/l by regular subculture at every 3 weeks was found to be most conducive with 95.77% shoot induction factor, 6.6 cm shoot length and 7 number of shoots. Inclusion of NAA, IAA or IBA (0.5–25 mg/l) with 2 % sucrose induced the rooting within 3 weeks of culture. The highest percentage of rooting was observed 87.77% with MS medium supplemented with IAA 2.5 mg/l in combination with NAA 0.5mg/l and root length 6.1 cm and number of roots 33 in 22 days. Micro propagated plantlets were hardened, acclimatized and transferred to the field. The phytochemical tests ascertains the presence of various phytoconstituents like Alkaloids, Carbohydrates, Glycosides, Flavonoids, Steroids, Phenolic Compounds, Saponins, Terpenoids, Tannins, Quinines, Proteins and TLC confirms the presence of amino acids.

INTRODUCTION

Centella asiatica L. is a valuable medicinal herb belonging to the family Apiaceae. It is distributed throughout the tropical and subtropical countries like India, Srilanka and Bangladesh. It is an evergreen perennial creeping herb with hollow or solid stem, alternate leaves, and epigynous, small, bisexual or staminate flowers, commonly found in moist place (Oyedeji and Afolayan, 2005). The plants possess antileprotic, antifilarial, antifeedant, adaptogenic, antiviral, antibacterial properties (Gurib-Fakin *et al.*, 1997) and also anti-tumour activity (Babu *et al.*, 1995). It is also reported to possess insecticidal (Stuart, 1982) and mutagenic properties (Yen *et al.*, 2001). The plant contains several triterpene saponins namely asiaticoside, sapogenins, asiatic acid, madecassic acid, adecassoside, vellarin, glycosides and centelloside (Duke and Ayensu, 1985; Glasby, 1991). It is rich in minerals such as calcium, magnesium, potassium, phosphorus and Aluminium (Herbert *et al*., 1994; Brinkhaus *et al.*, 2000). It has been used to treat leprosy, wound, cancer, fever, syphilis, acne, allergies (Inamdar *et al*., 1996) abscesses, headache, asthma, bronchitis, catarrh, convulsions, dysentery, eczema, gonorrhea, hypertension, jaundice, pleuritis, rheumatism, spasms, tuberculosis, ulcers and urethritis (Hausen, 1993). It has also been used as a brain tonic, psycho-physical regenerator and blood purifier (Jorge and Jorge, 2005). In the traditional system of Indian medicine, Centella is a reputed nervine tonic and is used for treatment of asthma, bronchitis, dropsy, elephantiasis, gastric catarrh, kidney troubles, leprosy, leucorrhoea, skin disease and urethritis (Kakkar, 1988) with antibacterial, antifeedant, antifilarial, antileprotic, antistress, antituberculosis activities and wound-healing properties (Chakraborty *et al*., 1996; Srivastava et al., 1997). The plant shows good therapeutic effects on peptic ulcers. It is one of the components of the drug ‘Geriforte’ which is used for senile pruritus (Anonymous, 1992). In 1990, the estimated annual requirement of *C. asiatica* was around 12,700 tonnes of dry biomass valued at Rs.1.5 billion (Ahmad, 1993).

Centella asiatica is widely used for its medicinal properties like sedative, analgesic, antidepressive, antimicrobial, antiviral and immunomodulatory (Brinkhaus and Hahn *et al* 2000). The medicinal properties of the plants are due to the presence of secondary metabolites like alkaloids, cardiac glycosides, tannins, flavonoids, saponins, reducing compounds, minerals and vitamins 2. The triterpenes of Centella are composed of many compounds including asiatic acid, madecassic acid, asiaticosside, madecassoside, brahmoside, brahmic acid, brahminoside, thankiniside, isothankunisode, centelloside,

madasiatic acid, centic acid, and centellic acid (Zheng *et al* 2007). Phytochemicals are plant derived chemicals, which may protect human from a host of numerous diseases. These chemicals are naturally occurring in medicinal plant leaves, vegetables and roots that have defense mechanism and protect from various diseases. Phytochemicals are primary and secondary compounds. Chlorophyll, proteins and common sugars are included in primary constituents and secondary compounds have terpenoids, alkaloids and phenolic compounds. Terpenoids exhibit various important pharmacological activities *i.e*., anti-inflammatory, anticancer, anti-malarial, inhibition of cholesterol synthesis, anti-viral and anti-bacterial activities. Terpenoids are very important in attracting useful mites which would consume the herbivorous insects. Alkaloids are used as anaesthetic agents and are found in medicinal plants. The present study is aimed to assess the phytochemical constituents in methanol, acetone, chloroform and aqueous extracts of the leaf extracts of *C. asiatica* L. Secondary metabolites are chemically and taxonomically diverse compounds with obscure functions. Knowledge of the chemical contents of plants is desirable because such information will be valuable for synthesis of chemical substances (Mojab *et al*, 2003).

Chromatography techniques are extensively used in bioanalysis for the separation, isolation and purification of drugs and their metabolites. Due to its medicinal importance, this plant is overexploited and there is a decline in the population of *Centella asiatica.* In recent years, there has been an increased interest in *in vitro* culture techniques which offer a viable tool for mass multiplication and germplasm conservation of rare, endangered and threatened medicinal plants (Ajithkumar and Seeni, 1998; Sahoo and Chand, 1998; Prakash *et al.*, 1999). Therefore, it is important to develop an efficient micropropagation technique for *C. asiatica.* Hence the present reports showed the rapid method for the *in vitro* multiplication of *C. asiatica* through high-frequency axillary shoot proliferation from nodal explants.

MATERIALS AND METHODS

Plant material & explants collection:

Actively growing, healthy and disease free young shoots of *Centella asiatica* were collected from green house and washed with 2% (v/v) detergent Teepol (Qualigen, India) and rinsed several times with running tap water. The explants were surface sterilized in 0.1% (w/v) aqueous mercuric chloride solution for 3-4min followed by four washing with sterile distilled water. *Centella asiatica* was extremely sensitive to surface sterilizing agent as well was

water, therefore, the surface sterilization procedure was optimized and this helped in preventing blackening of tissues and establishment of clean cultures.

Culture medium, condition & Callus Induction:

The nodal and leaf explants were placed on semi solid basal MS medium (Murashig & Skoog media) supplemented with BAP (0.5mg/l) and various concentration of NAA (0.1, 0.2, 0.3, 0.4, 0.5 mg/l), 2% sucrose (w/v) and agar 0.7% (w/v), for callus induction. The pH of the media was adjusted to 5.8 using 0.1N NaOH or 0.1N HCl before autoclaving. The cultures were maintained at 25±2ºC less than 16h photoperiod light at cool, white fluorescent lamps. The callus were transferred after 4-5 weeks of inoculation to MS medium containing various concentration of BAP (0.5,1.0,2.0,3.0,4.0mg/l) and NAA (0.5mg/l) in combination for shoot regeneration and multiplication. The cultures were maintained by regular subcultures at 1-2 week intervals on fresh medium with the same compositions.

Root induction & Acclimatization:

Elongated shoots were transferred to rooting medium, which consisted of MS supplemented with different concentrations of IAA (0.5, 1.0 mg/l), IBA (0.5, 1.0 mg/l) and NAA (0.5, 1.0mg/l) and combination of IAA (2.5mg/l) and NAA(0.5mg/l). All the cultures were incubated in culture room at 25 ± 2°C, light intensity (3000lux) with a photoperiod of 16 hours with 60-70% relative humidity. The cultures were monitored and the data were recorded at every week interval. After 15-20 days of culture, the sufficient rooted plantlets were dipped in bavistin solution for approx 2-3 minutes and planted carefully in the poly bags containing soil mixtures (organic soil mixed with garden soil 1:1). They were maintained at about 70% relative humidity in the greenhouse with 75% shading to produce newer leaves/roots.

Observation of cultures:

33 cultures were used per treatment and each experiment was repeated at least three times. The data pertaining to mean percentage of cultures showing response, number of shoots/culture and mean percentage of rooting were statistically analysed by the Post-Hoc Multiple Comparison test (Selvakumar *et al.* 2012).

Phytochemical Screening:

For phytochemical analysis extract was prepared by dried and powder form of leaf. The powdered sample were dissolved in methanol and kept it on rotary shaker for 48hr at room temperature. The extracts were filtered and kept it for further uses. The presence or absence of phytoconstituents such as alkaloids, glycosides, terpenoids, steroids, flavonoids, tannins, saponins and reducing sugars in leaf (Siddiqui and Ali, 1997; Evans, 2002) and presence of amino acids by Thin layer chromatographic techniques.

Thin Layer Chromatography:

TLC is a chromate graphical method which is employed to separate mixtures. It is performed on a glass sheet, aluminium or plastic foil, which is covered with a slim layer of adsorbent substance, generally silica gel, aluminium oxide, or cellulose (blotter paper). This film of adsorbent is identified as the stationary silica phase. After the sample has been filled on the plate, a solvent or solvent mixture (mobile phase) is drained up the plate via capillary action.

RESULTS & DISCUSSION

Callus Induction:

Callus development was important condition for the micropropagation. The induced calli were green in leaf and creamish green in stem explants. The best callus response observed in the media with MS supplemented with BAP (0.5 mg/l) + NAA (0.2 mg/l) and BAP (0.5 mg/l) + NAA (0.3 mg/l) in both node and leaf explants have callus induction frequency 83.33% and 86.66% respectively.

Table No. 1: Effect of different concentrations of growth regulators on callus induction from nodal and leaf part of the *Centella asiatica*

Explant	Medium	Hormone (mg/l)	Callus induction (%)	Mean no of Callus induction
Node	T0	MS (Control)	16.67	30.6±0.6
	T1	BAP (0.5) + NAA (0.1)	43.33	62.2±0.36
	T2	**BAP (0.5) + NAA (0.2)**	**83.32**	**83.33±1.63**
Leaf	**T3**	**BAP (0.5) + NAA(0.3)**	**86.66**	**86.66±0.05**
	T4	BAP (0.5) + NAA (0.4)	54.43	58.66±1.69
	T5	BAP (0.5) + NAA (0.5)	25.5	36.00±2.10

Organogenesis and Shoot bud regeneration:

Shoot induction occurred within 2 to 3 weeks of culture from callus. Among the various combinations of phytohormones used, MS supplemented with BAP (2.0 mg/l) + NAA (0.5 mg/l) was found to be optimum for leaf explants with 80.88% shoot induction. Shoot length was about 5.3 cm and 5 numbers of shoot induced in 2 weeks. The rate of shoot bud proliferation was maintained up to 6th subculture period on MS medium supplemented with BAP (4.0 mg/l)+NAA (0.5mg/l) in 3 weeks was found to be most conducive 95.77% shoot induction and 6.6 cm shoot length and 7 number of shoots initiated.

Table No. 2: Effects of various auxins in combination with cytokinin on shoot bud induction:

Hormone (mg/1)	Days to shoot initiation	% of callus response	Number of shoots explants (M ± SE)	Shoot length (cm) (M ± SE)
BAP(0.5)+NAA(0.5)	22	22.24	22.24±0.12	1.1±0.12
BAP(1.0)+NAA(0.5)	25	55.53	55.53±0.16	2.7±0.16
BAP(2.0)+NAA(0.5)	16	80.88	80.88±0.16	5.3±0.05
BAP(3.0)+NAA(0.5)	20	66.66	66.66±0.05	3.1±0.16
BAP(4.0)+NAA(0.5)	14	95.77	95.77±0.33	6.6±0.33

Table No. 3: Effect of growth regulators on root induction in regenerated shoots

Root Induction:

Serial No.	MS + concentrations of PGR (mg/l)		Days for root induction	Percent of rooting (Mean ±SE)*	Number of roots	Root length (cm)
1	IAA	0.5	16	33.33 ± 0.02	19±0.46	1.6±0.10
		1.0	19	44.44 ± 0.06	16±0.93	1.4±0.03
2	IBA	0.5	12	77.77 ± 0.01	28±0.96	6.0±0.07
		1.0	14	55.55 ± 0.06	23±0.22	5.8±0.10
3	NAA	0.5	14	55.55 ± 0.06	24±0.22	5.7±0.12
		1.0	17	33.33 ± 0.02	21±0.23	5.6±0.10
4	IAA + NAA	2.5	22	87.77±0.01	33±0.06	6.1±0.04
		0.5				

Elongated shoots were excised from parent culture and transfer onto full strength basal MS medium with growth regulators. Inclusion of NAA, IAA and IBA (0.5–25 mg/l) with 2 % sucrose induced the rooting within 3 weeks of culture. The highest percentage of rooting was observed 87.77% with MS medium supplemented with IAA (2.5 mg/L) in combination with NAA (0.5) and 33 roots observed with length 6.1 cm in 22 days.

Acclimatization & Hardening:

In vitro plantlets were transferred to soil rite for 15 to 17 days and subsequently transferred to polypots containing soil, sand and cow-dung manure at the ratio of 1:1:1 (v/v). All the regenerated plantlets were kept in the greenhouse with 65% relative humidity to produce newer leaves/roots. About 80 % of the plantlets established within 15 days of transfer. The plants grew well and attained 4–5 cm height within 1 months of transfer.

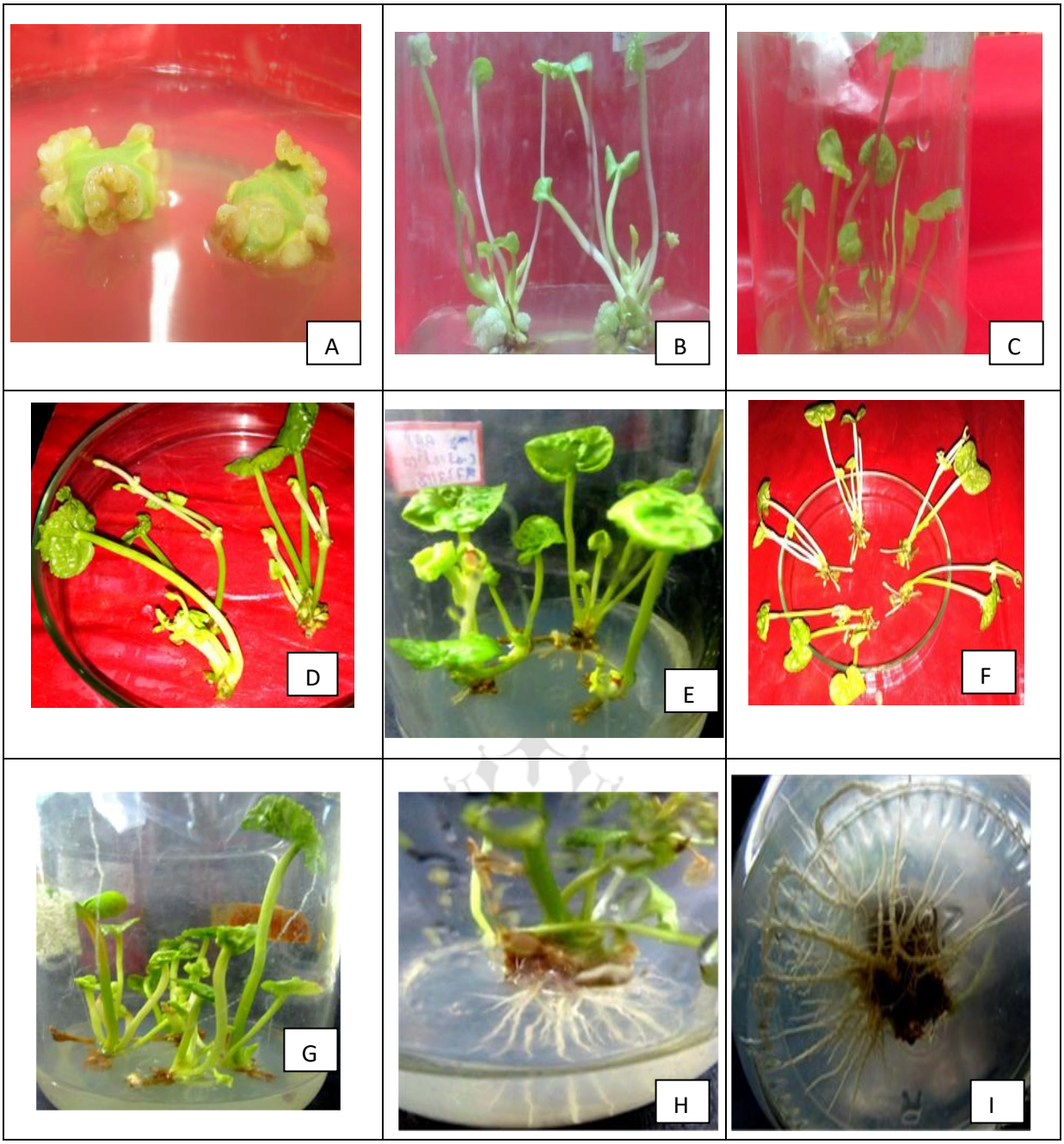

Figure No. 1: (A) Callus induction from leaf explants of *Centella asiatica, (B)* Initiation from leaf explants after 2 to 3 weeks of culture (C) Shoot proliferation after 4 to 5 weeks of culture, (D) Shoot bud initiation (E) Shoot bud after 4 weeks, (F) Root response, (G) Micro roots, (H) Root proliferation after 2 weeks, (I) Root proliferation after 4 weeks

Phytochemical Screening:

Phytochemical screening of the leaf extracts of *C. asiatica* revealed the presence of alkaloids, glycosides, terpenoids, steroids, flavonoids, tannins and reducing sugars. Carbohydrate is not detected in the methanolic extraction method**.**

Table No. 4: Observation of phytochemicals in *C. asiatica* methanolic leaf Extracts

Tests	Observations	Result
Alkaloids	Formation of reddish brown precipitate	+
Carbohydrates	No colour change	-
Glycosides	Appearance of blue or brown colour	+
Flavonoids	Formation of intense yellow colour which becomes colourless	+
Steroids	Brown ring observed at the junction of two layers	+
Phenolic Compounds	Appearance of dark green and black colour	+
Saponins	Formation of persistent foam	+
Terpenoids	Formation of reddish brown colour	+
Tannins	Formation of blue or green colour	+
Quinines	Formation of yellow precipitate	+
Proteins	Formation of yellow precipitate	+
'+' = positive, '-'= negative		

TLC Analysis:

TLC analysis for leaf extract of *Centella asiatica* revealed the presence of amino acids, alkaloids, flavonoids, terpenoids and saponins having R*f* value 0.90, 0.78, 0.82, 0.73 and 0.08 respectively where for amino acid solvent took ethyl acetate : methanol: water (10:2:1), Alkaloids solvent took Chloroform: glacial acetic acid: methanol: water (6:2:1:1) , for flavonoids solvent use Chloroform : Methanol (18:2) , for terpenoids solvent use Benzene: Ethyl acetate (1:1) and for saponins solvent use Chloroform: glacial acetic acid: methanol: water (6:2:1:1) .

Table No. 5: Phytochemical Analysis for Amino acids By Using TLC Techniques

Phyto compound	Solvent System	Plant part	Rf Value	Spray Reagent
Amino Acid	Ea:M:W (10:2:1)	Leaf	0.90	Iodine vapours
Alkaloids	Ch:Ga:M:W (6:2:1:1)	Leaf	0.78	Mayer's reagent
Flavonoids	Ch : M (18:12)	Leaf	0.82	UV light
Terpenoids	B:Ea (1:1)	Leaf	0.73	10% H2SO4
Saponins	Ch:Ga:M:W(6:2:1:1)	Leaf	0.08	Iodine vapours

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