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Production of secondary metabolites from callus cultures of *Centella asiatica* (L.) Urban

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Abstract

Centella asiatica (L.) Urban commonly known as Brahmi and Indian Pennywort in English, is a small herbaceous annual plant, belonging to the family, Apiaceae. It is used in traditional medicine for the treatment of various ailments. Present study deals with the quantitative analysis of ethanolic extract of root, stem and leaf and leaf derived callus of this plant. An efficient and reproducible protocol was developed for callus production using leaf explants of *Centella asiatica*. The combination of NAA, 2, 4-D and BAP, was used for the callus induction. NAA (1 mg/l) with BAP (0.5 mg/l) was effective for maximum callus induction from leaf explants. Various secondary metabolites like alkaloids, saponins, terpenoids and flavonoids are quantified using standard protocol.

Key words: *Centella asiatica*, callus, secondary metabolites, quantitative analysis, elicitation

1. Introduction

Plants are endowed with various phytochemical molecules such as vitamins, terpenoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains, and other metabolites which are rich in antioxidant activity (Zheng and Wang , 2001; Cai *et al*., 2003). *Centella asiatica* (L.) Urban (= *Hydrocotyle asiatica* L.) is a polymorphous, creeping plant, rooting at nodes with sometimes significant tap root and cylindrical and stems. It belongs to family Apiaceae (Umbeliferae); common names of *C. asiatica* include, Brahmi, gotukola, Indian pennywort. The plant is found in abundance on moist, sandy or clay soils, often in large clumps, forming a dense green carpet. It is useful cover crop in plantations. This Indian herb has a historical reputation for boosting mental activity and also for leprosy treatment. It is one of the important herbs for treating skin problems, to heal wounds, for revitalizing the nerves and brain cells, hence, primarily known as a "Brain food" in India (Singh *et al*., 2010). The main active constituents of *C. asiatica* (L.) are the triterpenes, 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, centicacid, and centellicacid (Zheng and Qin, 2007), which are known to possess antileprotic, antifilarial, antibacterial, adaptogenic, antifeedant and antiviral properties (Warrier *et al*., 1994). Extract of the whole plant is reported to have anticancerous activity (Yu *et al*., 2006) and the methanol extract of aerial parts of the *C. asiatica* inhibits the growth of human uterine carcinoma, human gastric carcinoma and murine melanoma cells *in vitro* (Yoshida *et al*., 2005). In the search for alternatives to production of desirable medicinal compounds from plants, biotechnological approaches, specifically, plant tissue cultures, are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites (Ravishankar and Ramchandra Rao, 2002; Vanisree *et al*., 2004; Sharma *et al*., 2011). Plant cells *in vitro*, showed physiological and morphological responses to microbial, physical or chemical factors which are known as 'elicitors'. Elicitation is a process of induced or enhanced synthesis of secondary metabolites by the plants to ensure their survival. Treatment with elicitors is reported to enhance secondary metabolites in *C. asiatica* (Kim *et al*., 2004; Mangas *et al*., 2006; Prasad *et al*., 2013). Therefore, in the present investigation, an attempt is made to enhance the secondary metabolites through *in vitro* culture and elicitation with methyl jasmonate and salysylic acid in *C.asiatica*.

2. Materials and Methods

2.1 Collection of plant material

The fresh parts of *C. asiatica* were collected in flowering period from Botanical garden, Gulbarga University, Kalaburgi-585106, Karnataka State, India.

2.2 Sterilization of explants

Plants were washed under running tap water to make them free from dust and soil particles followed by immersing in 2% mild detergent for 10 to 15 min and then dipped in 1% Bavistin (A fungicide) for half an hour, rinsed three to four times with sterile water. It was further surface sterilized using 70% ethanol for 3-5 min followed by 0.1% $HgCl_2$ for a minute and then again washed with sterile distilled water 3 to 4 times to remove the traces of $HgCl_2$. The pH of the medium was adjusted to 5.8 using 0.1 N HCl or 0.1 N NaOH solution and autoclaved for 20 min at 120°C and 15 lb pressure. The cultures were incubated at 26 ± 2°C under 16 h

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photoperiod and light intensity of 3000 lux. For callus induction, sterile explants (leaf and stem) were inoculated aseptically in culture tubes (25x150 mm) containing 20 ml. MS medium supplemented with different phytohormones in various combinations namely; 2-4 D, NAA and BAP. After 5-6 days of culture, calli initiated from the excised leaf and stem explants and were further subcultured in the fresh medium after 30 days for continuous growth.

2.3 Preparation of ethanolic extract of root, stem, leaf and callus

For preparation of ethanolic extract, method of Abdulrahman *et al.* (2004) was used. The fresh parts of the plant and callus were air dried and ground to fine powder with mechanical grinder. 100 g of each powder was then extracted in 1000 ml of absolute ethanol (1:10) for 72 h. After 72 h. of extraction, each extract was filtered through Whitman's Filter Paper No.1 separately. The filtrate was evaporated to dryness at room temperature and stored at 4°C in refrigerator until further use.

2.4 Elicitor preparation and treatment

100 mg of salicylic acid dissolved in 10 ml distilled water and was autoclaved at 15 psi for 20 min. 50,100 and 150 µM solutions were used for elicitation of callus cultures. One month old calli pieces (about 250 mg) were cultured on MS medium supplemented with various concentrations (50-150 µM) of MJ or SA for one month. The control treatment was cultured on MS medium without any elicitor being added.

2.5 Quantitative analysis of alkaloids, phenolics, flavonoids and saponins

The phytochemicals which are present in the ethanol extracts *of C. asiatica* were determined and quantified by standard procedures.

2.5.1 *Determination of plant yield*

The percentage yield was obtained using the following formula:

$$w_2, w_1/w_0 \times 100$$

where w_2 is the weight of the extract and the container, w_1 is the weight of the container alone and w_0 is the weight of the initial dried sample.

2.5.2 *Determination of total alkaloids*

Alkaloids were determination using Harborne (1973) method. 100 g of the sample was weighed and taken into a 250 ml beaker and to this powder, 200 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume, then concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue contains the alkaloid, which was dried and weighed.

2.5.3 *Determination of total phenolics*

The amount of total phenolics in extracts was estimated by the Folin- Ciocalteau method. For this, 3 mL aliquots of the diluted extracts were pipetted into different test tubes to which 0.5 mL of Folin- Ciocalteau reagent and 2 mL of 20 % (w/v) Na_2CO_3 solutions were added. The tubes were placed in a boiling water bath for exactly1min and then cooled under running tap water. The absorbance of the solutions was measured at 550 nm against the reagent blank. Calibration curve for gallicacid was plotted and total phenolics content was expressed as mg of gallicacid equivalents

2.5.4 *Determination of flavonoids*

Flavonoids were determined by the method as described by Boham and Kocipai-Abyazan (1994). 100 g of the plant sample was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature, and then the solution was filtered through Whatman Filter Paper No. 42 (125 mm). The filtrate was later transferred into a crucible and evaporated into dryness over a water bath and weighed to a constant weight.

2.5.5 *Determination of saponins*

Saponins were determined as per the method described by Obadoni and Ochuko (2001). 100 g of powder was put into a conical flask and 50 ml of 20% aqueous ethanol was added. The sample was heated over a hot water bath for 4 h with continuous stirring at 55°C. The mixture was filtered and the residue re-extracted with another 100 ml 20% ethanol. The combined extracts were reduced to 40 ml over water bath at 90°C. The concentrate was transferred into a 250 ml separating funnel and 10 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 30 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in the oven to a constant weight and the saponin content was calculated as percentage (Obdoni and Ochuko, 2001). Total terpenoids were determination as described by Ferguson (1956). 100 g of plant powder was taken separately and soaked in alcohol for 24 hours, then filtered, the filtrate was extracted with petroleum ether; the ether extract was treated as total terpinoids.

3. Results and Discussion

3.1 Callus induction

Callogenic response was observed in all the combinations of growth regulators used except on the media free of growth regulators. Leaf explants developed callus at cut surfaces within 6-8 days and subsequently covered the entire surface of explants within 28-30 days. Leaf explants are reported to be the best explants for callus induction when compared to other parts (Nath and Burgohain, 2005; Gandi and Giri, 2013). Media containing low (0.5 mg/l) concentration of NAA or 2, 4-D was not supportive for callus induction. Both auxins supported callus induction and growth between 1-2 mg/l concentration, maximum (78%) frequency and growth (780 mg) being on medium with 2 mg/l 2, 4-D. Supplementing BAP (0.5 mg/l) to 2, 4-D containing medium further enhanced frequency (86%) and the growth of callus (998 mg); however BAP at 1mg/l was not favorable for further growth of callus (Figure 1). The calli were light green on 2, 4-D and BAP supplemented media and greenish yellow on medium supplemented with NAA and BAP. The finding is in accordance with earlier studies on this plant (Banerjee *et al.*, 1999; Deshpande *et al.*, 2010; Bibi *et al*, 2011). Increased callus growth is reported on MS medium supplemented with 2, 4-D and BAP (Mercy *et al.*, 2012; Gandi and Giri, 2013), on the contrary good growth of callus in this species is reported in combination on NAA and BAP (Arekar and Barve, 2005; Joshi *et al.*, 2013), however Purshotham *et al.* (1999) reported that 2, 4-D at 2 mg/l + 0.5 mg/l kinetin was found better for induction and growth of callus.

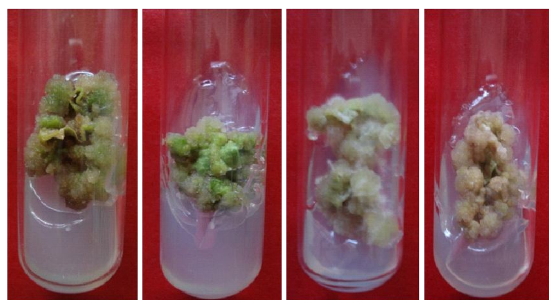

Figure 1: Callus induction from leaf explant on MS medium supplemented with **(a).** 2 mg/l 2, 4-D+ 1.0 mg/l BAP (Maximum amount of callus), **(b)**. 2 mg/l 2, 4-D+ 1.0 mg/ l BAP, **(c)**. 2 mg/l 2, 4-D and **(d).** 2 mg/l NAA after 30 days of culture.

3.2 Estimation of alkaloids

The percentage yield of ethanol extract was 11.86 for plant material and 20.60 for callus culture. Subsequent analysis of the secondary metabolites was carried out in 40 days old callus cultures. Total alkaloids content was estimated from powdered tissue of root, stem, leaf and callus raised from leaf explants and the data is presented in Table 1.The total alkaloids content was 3.2, 1.8, and 1.2, mg per gram dry weight of the powder, in leaves, stem and roots, respectively. Alkaloid content increased in the callus cultures initiated from leaf explants it was 4.2 mg/gm dry weight in callus raised on medium supplemented with 2 mg/l 2, 4-D + 0.5 mg/l BAP. The alkaloid content decreased to 3.2 mg/ gm dry weight, however when the concentration of BAP was raised to 1 mg/l the alkaloid content decreased (5.26 mg) In general, leaf of *Centella asiatica* was found to contain the highest flavonoids content compared to either root or stem.

Table 1: Effect of different phytohormones on callus induction from leaf explant of *C. asiatica*

Hormone(mg /l)	% of callus induction	Fresh weight(mg)	Dry weight(mg)	Colour
MS (control)	NC	NC	NC	NC
1.0 mg/l 2,4-D	72	486	43.6	Whitish green, friable
2.0 mg/l 2,4-D	78	780	70.0	Whitish green, friable
1.0 mg/l NAA	46	320	28.7	yellowish green friable
2.0 mg/l NAA	52	510	40.6	yellowish green friable
2 mg/l 2, 4-D +0.5 mg/l BAP	80	998	80.4	Greenish, white, friable
2 mg/l 2, 4-D + 1.0 mg/l BAP	86	876	70.2	Greenish, white, friable

Each value represents the mean of three replicates.

Table 2: Phytochemical constituents of different parts and leaf derived callus of *C. asiatica*

Secondary metabolites/tissue	Phenolics mg/g.dw	Alkaloids mg/g.dw	Flavanoids mg/g.dw	Saponins mg/g.dw	Terpenoids mg/g.dw
Leaf	0.62 ±0.08	3.22 ± 0.21	3.40 ± 0.14	4.0 ± 0.18	6.2 ± 0.21
Stem	0.58±0.06	1.80 ± 0.17	2.80 ± 0.20	3.6 ± 0.12	2.3 ± 0.15
Root	0.60±0.06	1.26 ± 0.16	2.32 ± 0.37	2.2 ± 0.10	3.6 ± 0.16
2 mg/l 2, 4-D + 0.5 mg/l BAP	0.82±0.08	4.28 ± 0.18	6.20 ± 0.26	4.8 ± 0.24	8.6 ± 0.28
2 mg/l 2, 4-D + 1.0 mg/l BAP	0.68±0.06	3.86 ± 0.12	5.56 ± 0.10	4.2 ± 0.18	6.8 ± 0.28

Each value represents the mean of three replicates.

Table 3: Effect of elicitors on the secondary metabolites content in leaf derived callus cultures of *C. asiatica.*

Concentration Of SA & MeJ (µM)	Phenolics mg/g.dw	Alkaloids mg/g.dw	Flavanoids mg/g.dw	Saponins mg/g.dw	Terpinoids mg/g.dw
SA-0	0.82±0.08	4.2±0.18	6.20±0.26	4.8±0.34	8.6±0.28
50	1.20±0.26	6.8±0.20	8.28±0.26	8.6±0.14	12.5±0.21
100	1.87±0.23	8.6±0.24	10.80±0.32	10.8±0.43	16.6±0.21
150	0.94±009	4.0±0.26	5.27±0.29	4.3±0.28	8.5±0.21
MeJ-0	0.68±0.06	4.2±0.18	6.20±0.26	4.8±0.34	8.6±0.28
50	1.32±0.24	6.4±0.26	6.00±0.20	8.8±0.24	8.8±0.38
100	1.86±0.24	8.2±0.24	10.30±0.36	11.6±0.46	12.8±0.26
150	0.90±0.36	4.0±0.22	5.28±0.22	4.2±0.32	8.2±0.26

Each value represents the mean ± SE of three replicates.

3.3 Estimation of flavonoids

Total flavonoids were estimated from powdered tissue of leaf, stem, and callus raised from leaf explants and the data are presented in Table 1. The flavonoids content was 3.40, 2.80 and 2.32 mg/gm dry weight in leaf, stem and root tissue, respectively. An increase in the content of flavonoids was noticed in leaf derived callus tissue, raised on MS medium supplement with 2,4-D and BAP. Maximum amount (6.20 mg) of flavonoids was obtained on MS medium supplemented with 2 mg/l 2, 4-D + 0.5 mg/l BAP, however, its content decreased (5.26 mg) when the concentration of BAP was raised to 1 mg/l. (Table 2).

3.4 Effect of elicitors on secondary metabolites

Plant tissue culture techniques employing elicitors has been widely used to enhance the production of secondary metabolites (Table 3). Biotic elicitors such as methyl jasmonate (MeJA) and salicylic acid (SA) are used in the culture medium for the highest level of production of secondary metabolites. In the present investigation, it was noticed that when callus cultures of *C. asiatica* were subjected to 50 and 100 µM of MeJA secondary metabolites (Alkaloids, phenolics, flavonoids and Saponins) increased considerably. MeJA induced increase in the secondary metabolites is reported earlier in *C. asiatica* ((Mangas *et al*., 2006; Kim *et al*., 2004; 2007; Bonfill *et al*., 2011; Ruslan; *et al*., 2012). Increased secondary metabolites due to elicitation with MeJA, are also reported in several other medicinal plants. Chaichana and Dheeranupattana (2012) reported increased production of *Stemona* alkaloids in *Stemona* species. After treatment with various concentrations of MeJA, Goyal and Ramawat (2008) reported that 20 µM concentration of MeJA was most effective in isoflavonoid production in the cell cultures *of Pueraria tuberose* , and further noted that MeJA was more effective compared to SA . Deng (2005) showed that tropane alkaloids were induced by MeJA in jimsonweed (*Datura stramonium*). Jasmonates are molecules known to be efficient elicitors for a wide range of secondary metabolites from different plant origins (Gundlach *et al*., 1992; Memelink *et al*., 2001) and it is reported that the biosynthetic activity of cultured cells can be enhanced by MeJA (Suzuki *et al*., 2005; Yoon *et al*., 2000).

Similarly salicylic acid (50 and 100 µM) treatment also resulted in increased production of secondary metabolites (alkaloids, phenolics, flavonoids and saponins) in callus cultures of *C. asiatica*. The plant growth regulator, salicylic acid (SA), when applied to plants, affects diverse physiological processes (Malabadi *et al*., 2008a, 2008b). The alkaloid production in hairy root cultures of *Brugmansia candida* is reported to be enhanced after 24 h. of treatment with 0.01 mM of SA (Pitta, *et al.* 2000). It is reported that when SA is applied to the cell culture of *Salvia miltiorrhiza* (Dong *et al*., 2010) and grape cell cultures (Obinata *et al*., 2003). Taguchi *et al.* (2001) have reported that salicylic acid (SA) induces gene regulation related to the biosynthesis of secondary metabolites in plants.

4. Conclusion

From the present investigation, it is concluded that methyl jasmonate and salisylic acid can be used as elictors to enhance the secondary metabolites in *C. asiatica* and the optimum concentrations of these elicitors is 100 µM and concentration beyond this is inhibitory for the production of secondary metabolites in this species.

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Conflict of interest

We declare that we have no conflict of interest.

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