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Effect of TDZ on callus induction and estimation of Phenol, Flavoniod and Antioxidant property in *in vivo* and *in vitro* grown *Centella asiatica* (L.) Urban

Shweta Kumari and Maheshwar Prasad Trivedi

Department of Botany, Patna University, Patna-800005 (India)
Email Id- shwetabt21@gmail.com, mptrivedi1956@rediffmail.com
Shweta Kumari ORCID ID – 0000-0001-8823-4431

Abstract

Medicinal plants are enriched in various bioactive molecules such as vitamins, terpenoids, phenolics, lignins, stilbenes, tannins, flavonoids, quinines, coumarins, alkaloids, amines, betalains and other metabolites. The present study was carried out to establish an efficient and reproducible protocol for callus induction in *Centella asiatica* (L.) Urban belonging to family Apiaceae. Combination and composition of different plant growth regulators were used for callus induction. Out of various compositions MS medium fortified with TDZ 1mg/l and NAA 0.1 mg/l showed maximum response in both stem and leaf explants in terms of callus induction. Although MS medium supplemented with BAP 4.5 mg/l and NAA 0.1 mg/l also showed better responses. TDZ was the best for callus induction (91.6%) of callus against BAP respectively (75 %). Stem showed better explants (83.3 %) for callus induction as compared to leaf explants (75 %). Determination of phenolics and flavonoid contents were performed by using Folin-Ciocalteu reagent and aluminium chloride colorimetric method. Free radical scavenging activity was estimated by DPPH (1, 1-diphenyl-2-picrylhydrazyl). Maximum phenolics (6.35) and flavonoid contents (37.5) were obtained from leaf derived callus. Antioxidant property was also highest in leaf derived callus. Micropropagation allows conservation of this plant and can be used to produces large scale of clones in a continuous manner and callus induction enhances production of bioactive molecules.

Key word : Bioactive molecules, BAP, DPPH, Micropropagation, NAA.

Medicinal plants are enriched in various bioactive molecules such as vitamins, terpenoids, phenolic acids, lignins, stilbenes, tannins, flavonoids, quinines, coumarins, alkaloids, amines, betalains and other metabolites[6]. *Centella asiatica* (L.) Urban is a small herbaceous annual plant also known as Gotu Kola belongs to family Apiaceae and is distributed in India, Sri Lanka, China, Japan, Madagaskar and Australia[3,11]. It has various

names such as Thankuni (Bengali), Mandoo-kparni (Hindi), Pegaga (Malay), Kodagam (Malayalam), Gotukola (Sinhalese), Vallari (Tamil) and Bekaparanamu (Telgu). *Centella asiatica* (L.) is a stoloniferous, perennial, prostrate, creeper herb, having height upto 6 inches. Stem is striated, glabrous contain rooting at the node. It has been used for the treatment of leprosy, varicose veins, ulcer, lupus, certain eczemas, and mental retardation[12,20]. *Centella asiatica* possess a wide variety of bioactive molecules i.e. secondary metabolites. The major bioactive molecules of *Centella* are asiatic acid, madecassic acid, asiaticoside, and madecassoside[24]. *Centella* having high antioxidant capacity as compared to grape but low than vitamin C. boiled aqueous extract of *Centella* showed higher antioxidant activities as relative to aqueous extract. Total phenolics and flavonoid content are also higher in boiled aqueous extract than aqueous extract of *Centella asiatica*[25]. It was observed that the callus possess high quantity of phenolic and flavonoid content as compared to field grown plant[27]. Overexploitation of this valuable medicinal plant has led to its depletion and the species is listed under highly threatened species by the International Union of Conservation of Nature (IUCN)[6]. Micropro-pagation has allowed conservation of this plant and can be used to produce large scale of clones in a continuous manner. The objective of present work was to establish a reproducible and efficient protocol for callus induction in *Centella asiatica* and evaluation of TDZ on callus induction over BAP. We report an efficient protocol for callus proliferation and large scale clonal propagation to provide a useful tool for enhancement of secondary metabolites production. Apart from callus culture we also compared phenolics, flavonoids and antioxidant properties of *Centella asiatica*. To production of secondary metabolites callus culture is first step towards either pilot or large scale industrial production.

Collection of plant material and surface sterilization :

The leaf (without petiole) and stem (5-7 cm) of 1-1.5 year old plants of *Centella asiatica* were collected from Botanical garden of Patna University, Patna-5. The explants were first washed thoroughly under running tap water for 15 minutes then submerged in 70% ethanol for 10 minute. Final step of surface sterilization was carried out by 0.1 % $HgCl_2$ for 4 min followed by five times washing thoroughly with autoclaved double distilled water under laminar air flow hood.

Implantation of explants and culture condition :

Leaf and stem were excised aseptically into 1-2 cm and were implanted in MS medium[19] with different concentration and combination of TDZ, BAP, Kn, and NAA. The pH of media was adjusted at 5.6 applying 1 N NaOH and 1 N HCL prior to autoclaving. All the culture was maintained at 25±2 ºC under a 16-h photoperiod with illumination of 35-40 $\mu mol\ m^{-2}\ s^{-1}$. Uniform culture conditions were maintained throughout the experiment. The calluses were subcultured at regular interval of 4 weeks and data were collected after 6 weeks of inoculation of explants.

In vivo and in vitro extraction :

Centella asiatica leaves collected from Patna Science College campus allowed drying for 12 days. Thenafter leaf was grounded into powder with the help of motor pestle. Leaf and stem derived callus was also allowed to drying for 12 days and grounded

into powder with the help of motor pestle. Now 10 mg of powdered samples were soaked in 10 ml of 90 % ethyl alcohol and 10 ml of methanol respectively for preparation of ethanolic and methanolic extract. Allowed kept on shaker with shaking speed 120 rev/min for overnight. Then extract was filtered through whattman filter paper no. 1 and transferred into fresh prepared 90 % 10 ml of ethyl alcohol and methanol. When the extract became transparent, filtered and concentrated at 70 ^{0}C temperature by using rota evaporator[16].

Estimation of Phenolic contents :

We investigated differences of total phenolic content of *in vitro* grown leaf and stem callus, and field grown leaf by slightly modification[12]. The folin-ciocalteu's (FC) reagent was used for determination of total phenolic. One ml of methanolic extract and nine ml of double distilled water was added in 25 ml of conical flask. One ml of folin - ciocalteu reagent was added in the mixture with constant stirring. After 5 min 10 ml of 7 % (Na_2CO_3) was added in the mixture and volume was made up to 25 ml with sterile distilled water. The reaction was incubated for 90 min on room temperature and absorbance was determined at 550 nm with the help of an ultraviolet (UV) visible spectrophotometer. Total phenolics content was expressed as mg gallic acid equivalents (GAE)/gm of *C. asiatica* extract.

Estimation of flavonoid content :

Determination of flavonoid contents was estimated by aluminium chloride colorimetric method[4]. 50 µl of ethanolic extract (1 mg/ml ethanol) of *in vivo* leaf, *in vitro* grown leaf and stem derived callus and regenerated leaf were added in 950 µl of methanol to made the volume 1 ml. 1 ml of methanolic extract dissolved in 4 ml of double distilled water. 0.3 ml of 5 % $NaNO_2$ solution. Allowed to incubate for 5 min then add 0.3 ml of 10 % of $AlCl_3$ solution. Mixture left for 6 min. Thenafter 2 ml of 1 M solution were mix and final volume made up to 10 ml by addition of double distilled water. The solution was left for 15 min, and absorbance was determined at 510 nm. Total flavonoid content estimated by calibration curve of mg rutin per gm dry weight.

Estimation of antioxidant properties :

The antioxidant properties of extract were determined by DPPH method (1,1-Diphenyl-2-picryl-hydrazyl) by slightly modification[18]. 2 ml of freshly prepare methanolic solution of concentration 0.1mmol was mixed with different doses of 25-85 µl of methanolic extract of sample (4 mg/ml) *in vivo* leaf, *in vitro* grown leaf and stem derived callus and regenerated leaf of plant were mixed in different test tube and made up final to 3 ml by addition of methanol. 2 ml of DPPH and 1 ml of Methanol were mixed and evaluated as positive control. Methanol was evaluted as blank against sample solution. Ascorbic acid was determined as standard graph. The absorbance of all sample were determined after 40 min at 517 nm. Each sample was measured in triplicate and average was determined. Sample capacity to scavenge DPPH radical was as followed, DPPH scavenging capacity: $[(Ac-At)/ Ac-As)] \times 100$ Ac= Absorbance of control, At= Absorbance of test sample whose DPPH capacity to determined, As= Absorbance of standard solution.

Statistical analysis :

A random block was designed for callus culture. Data were recorded on the basis of number of explants used. Twenty five replicates applied per treatment were repeated three times and statistical analysis was carried out by one way analysis of variance (ANOVA). The significantly difference of means was determined by Post Hoc tukeys comparison test ($P < 0.05$) using Graphd Prism software 8 (Graph Pad Software, Inc., USA) values represented by mean ± SE twelve explants per culture vessels. For estimation of phenol, flavonoid and antioxidant properties were calculated as mean±SE and the number of experiments (n=3). Means were significantly different at ($P < 0.05$).

Effect of different concentrations and combinations of cytokinin and auxin on callus induction :

Surface sterilization treatment with 0.1 % $HgCl_2$ for 4 min was found to be compatible and only 6-7 % contamination was found in primary culture. The initiation of callus induction commenced within 10 days after inoculation of explants. Diverse effect of callus proliferation was obtained from different concentration and combination of BAP and NAA (Figs. 1). Highest callus response was obtained (83.3 % in stem and 62.5 % in leaf Table-1) from BAP 4.5 mg/l and NAA0.1 mg/l respectively. Increase in concentration of BAP led to callus proliferation upto the 4.5 mg/l, onwards callus proliferation was decreased. Callus obtained from BAP and NAA was green (g), compact and non friable (nf) and globular (g) in both the explants. TDZ (0.11-1.5 mg/l) along with NAA showed maximum callusing (Table-2 Figs. 2). The callus proliferation was (91.6 % in stem and 75 % in leaf). Callus obtained from TDZ and NAA was white, green, whitish and friable (f) (Figs. 2) in both the explants.

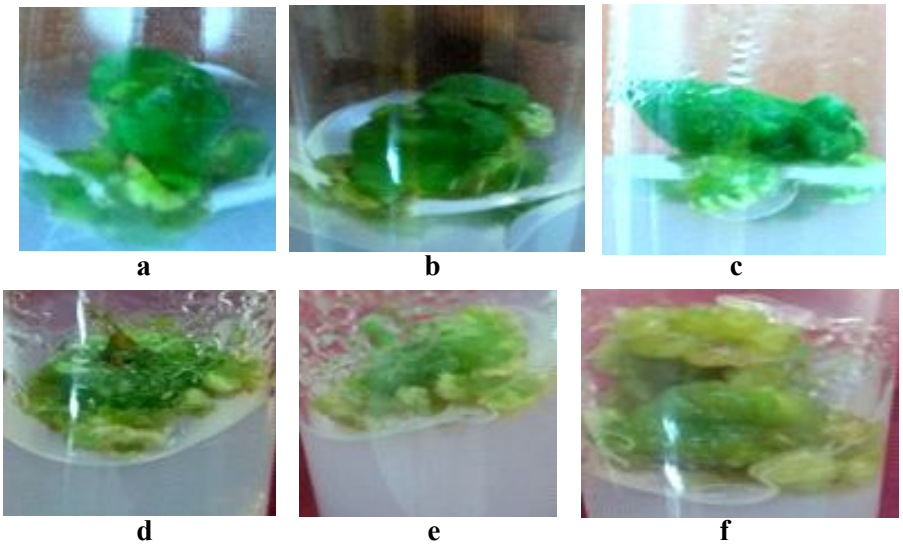

Fig. 1. Callus inductions in leaf and stem explants MS supplemented with BAP (a) 4 mg/l and NAA 0.1 mg/l (b) 4.5 mg/l and NAA 0.1 mg/l (c) 5 mg/l and NAA 0.1 mg/l. Callus inductions in stem explants MS supplemented with BAP (a) 2 mg/l and NAA 0.1 mg/l (b) 2.5 mg/l and NAA 0.1 mg/l (c) 3.5 mg/l and NAA 0.1 mg/l

Table-1. Represents MS media supplemented with BAP (1-1.5) mg/l and NAA (0.1) mg/l

		Stem			Leaf		
BAP (mg/l)	NAA (mg/l)	Callusing Percentage	Degree of Callogenic response	nature of callus	Callusing percentage	Degree of callogenic response	Nature of callus
1	0.1	37.5 ± 0.57^{a}	+	Green, nf	29.1 ± 1.20^{b}	+	Green, nf
1.5	0.1	45.8 ± 1.85^{b}	+	Green, nf	25 ± 0.57^{f}	+	Green, nf
2	0.1	50 ± 1.15^{b}	++	Green , nf	20.8 ± 1.20^{b}	+	Green ,nf
2.5	0.1	41.6 ± 0.33^{c}	+	Green, nf	33.3 ± 0.33^{ce}	+	Green, nf
3	0.1	66.6 ± 1.2^{d}	++	Green, g	41.6 ± 0.88^{c}	+	Light green, nf
3.5	0.1	54.1 ± 0.13^{e}	++	Whitish green, nf	20.8 ± 0.11^{g}	+	Whitish green, nf
4	0.1	75 ± 0.57^{f}	+++	Light green, nf	50 ± 0.57^{f}	++	Green,nf
4.5	0.1	83.3 ± 0.33^{c}	+++	Light green, g	62.5 ± 2.51^{h}	++	green,nf
5	0.1	33.3 ± 0.13^{e}	+	Green, nf	33.3 ± 0.66^{ce}	+	Brownish green, nf

Values represents Mean±SE of 25 replicate repeated three times. Significantly differences of means were determined at ($P<0.05$) comparison by Post Hoc tukeys test. Mean values of different letters from each other represents significant diffenences in mean. (-) Represents no callus induction, while (+) indicates status of callus induction, + =average, ++ = good, +++ = best.

Table-2. Represents MS media supplemented with TDZ (0.1- 1.5) mg/l and NAA (0.1) mg/l

		Stems			Leaf		
TDZ (mg/l)	NAA (mg/l)	Callusing Percentage	Degree of callogenic response	Nature of callus	Callusing percentage	Degree of callogenic response	Nature of callus
0.11	0.1	37.5 ± 1.73^{a}	+	White, f	29.1 ± 0.33^{b}	+	Green, f
0.22	0.1	50 ± 1.15^{a}	++	Whitish green, f	21 ± 1.20^{a}	+	Green, f
0.33	0.1	66.6 ± 0.33^{b}	++	Brownish white, f	33.3 ± 1.33^{ab}	+	Green, f
0.44	0.1	79.1 ± 0.57^{c}	+++	Greenish white, g	41.6 ± 0.88^{g}	+	Green, f
0.55	0.1	83.3 ± 0.66^{d}	+++	Brownish white, f	75 ± 1.15^{c}	++	Green, f
1	0.1	91.6 ± 0.57^{c}	+++	Green, f	50 ± 1.15^{a}	+	Green, f
1.5	0.1	25 ± 0.88^{e}	+	Green, g	16.6 ± 0.33^{h}	+	Green, f

Values represents Mean±SE of 25 replicate repeated three times. Significantly differences of means were determined at ($P<0.05$) comparison by Post Hoc tukeys test. Mean values of different letters from each other represents significant diffenences in mean. (-) Represents no callus induction, while (+) indicates status of callus induction, + = average, ++ = good, +++ = best.

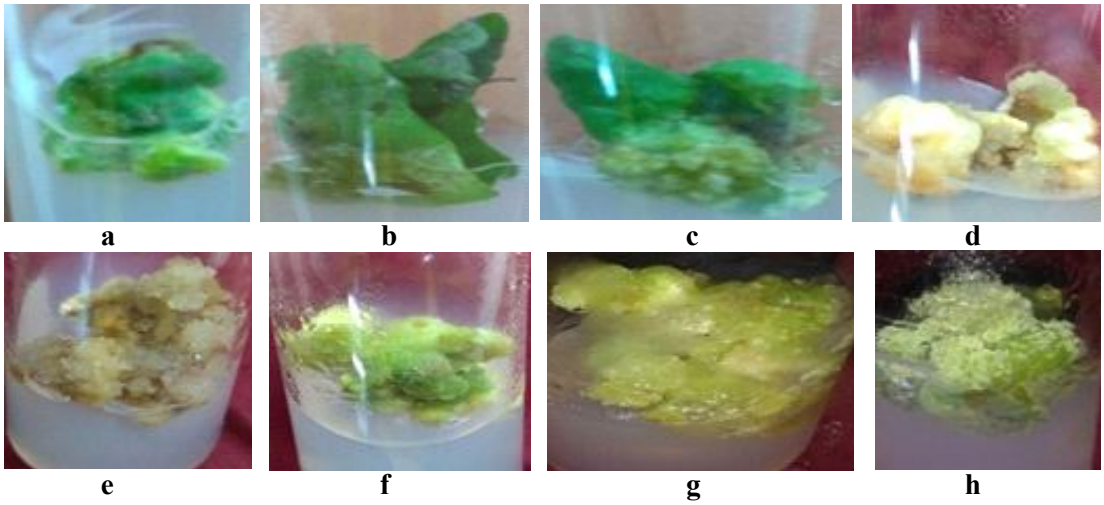

Fig. 2. Callus inductions in leaf explnts and stem MS supplemented with TDZ (a) 0.44 mg/l and NAA 0.1 mg/l (b) 0.55 mg/l and NAA 0.1 mg/l (c) 1mg/l and NAA 0.1 mg/l (d) 0.11 mg/l and NAA 0.1 mg/l (e) 0.33 mg/l and NAA 0.1 mg/l (f) 1 mg/l and NAA 0.1 mg/l (g) 0.44 mg/l and NAA 0.1 mg/l (h) 1.5 mg/l and NAA 0.1 mg//.

Effect of different combinations and compositions of cytokinine :

There was no callus formation observed in MS media augmented with only combination of cytokinin. It reflects that for formation of callus combination of cytokinin and auxin are required.

Phenolic and flavonoid contents :

The total phenolic content of *in vivo* leaf and *in vitro* grown callus leaf and stem callus were obtained. Phenolic concentration were determined from the gallic acid equivalents/gm. Maximum phenolic content was obtained in leaf derived callus (6.5±0.4) while minium in *in vivo* leaf (4.2±1.1) shown in (Table-3). Total flavonoid content was determined from rutin equivant/gm (Table-3). Maximum flavonoid content obtained in leaf derived callus and minimum in field grown leaf.

Table-3. Represents phytochemical constituents of *Centella asiatica* from *in vivo* and *in vitro* grown

Phytochemicals	*In vivo* leaf	*In vitro* leaf callus	*In vitro* stem callus
Total flavonoid mg rutin equivalent/g Dw	23 ± 0.1^{a}	37.5 ± 0.21^{g}	36.1 ± 1.12^{b}
Total phenol mg GAE/g Dw	4.2 ± 1.1^{b}	6.6 ± 0.4^{c}	5.35 ± 0.6^{c}
%DPPH activity (25 µl)	43.21 ± 1.25^{b}	45.68 ± 0.31^{g}	44.31 ± 0.51^{f}
(45 µl)	65.32 ± 0.66^{c}	68.25 ± 0.53^{f}	65.89 ± 0.11^{ac}
(65 µl)	84.28 ± 0.33^{d}	87.91 ± 0.15^{e}	86.27 ± 1.09^{b}
(85 µl)	94.87 ± 0.13^{e}	96.51 ± 1^{h}	95.02 ± 0.19^{h}

Results are mean±SE of three replication. Mean values of different letters from each other represents significant diffenences in mean.

Antioxidant properties :

The antioxidant properties based on DPPH assay was estimated and results is depicted in (Fig. 3 and Table-3). Increasing the concentration of extracts gradually increases the free radical scavenging activity. The percentage free radical scavenging activity was varied from 44 % to 96 % according to the concentration of extract. The maximum antioxidant activity was observed in leaf derived callus followed by stem derived callus (Fig. 3). *In vivo* grown leaf showed less activity. The high antioxidant activity of in vitro callus may be attributing to high concentration of bioactive molecules such as phenols and flavonoids. High concentration of phenol and flavonoid results favoured in increased antioxidant activity.

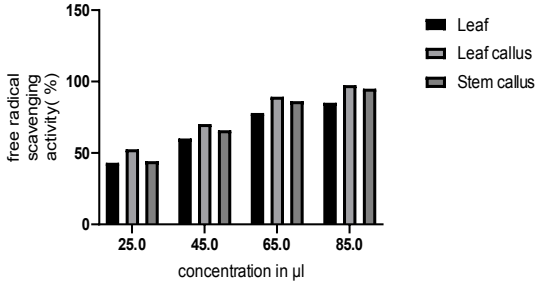

Fig. 3. Free radical scavenging activity of *Centella asiatica*. Error bar represents Standard error of means.

The current study was focused on to develop a reproducible protocol for callus induction and growth of callus in *C. asiatica*. Studies were conducted to standardize suitable media for callus induction. In the present study, it was observed that TDZ significantly influenced the growth of explants[1,9,10,13,14] which was similar with previous finding. Combination of TDZ with NAA showed best responses as compared to BAP along with NAA[17]. Similar response has been observed in various plant species[14]. Higher percentage of callus formation and growth in TDZ suggested that it enhanced biomass production. Among all four combinations of cytokinin and auxin treatments, TDZ was found to be most effective for callus induction[14]. Although MS media supplemented with BAP and NAA showed better response for callus growth. Similar results were reported in *C. asiatica*[7,11,16,21,22]. TDZ might be trigger de novo synthesis of auxins, high level of auxins would responsible for stunting and necrosis of cell[27]. Previously it was found that combination of TDZ and IAA was more effective in callus induction in shrubaceous plant. Similar result was found in present study but contradiction to previous report *Centella asiatica* is a herbaceous plant. The differences in morphology of callus depend on explants type, concentration of growth regulators and plant species[8].

Phenolic and flavonid contents :

Plant phenolic contain hydroxyl group which is a key factor show free radical scavenging activity. Phenolic compounds are directly involved in antioxidant activity[15,26]. Among the two plant parts (leaf, stem) of *Centella* leaf callus showed maximum amount of phenolic compound. Plant tissue culture is a very useful tool for production of secondary metabolites. Callus culture has been prominently applicable for secondary metabolites production over *in vivo* or *in vitro* grown plants. Various authors have been reported secondary metabolites production through plant tissue

culture[5,18,23]. A group of bioactive molecules included in flavonoid such as flavones, flavanols and condensed tannins are secondary metabolites. Flavonoid possesses a 3 free OH groups, which is responsible for antioxidant activity. Plant flavonoid showed both *in vivo* and *in vitro* antioxidant activity. Difference in bioactive molecules was observed in in vitro grown callus and *in vivo* plants. It may be attributed to environmental conditions and nutrient supply[12].

Antioxidant properties :

The antioxidant activity was determined by DPPH assay and result shown in (Table-3 and Fig. 5). An increase in concentration of extract (25-85 µl), a gradual increase in DPPH free radical scavenging activity[12]. Medicinal plants are rich in secondary metabolites including phenolics, flavonoids encompasses antioxidant activity attributable to their chemical structure and redox potentials. The DPPH radical is mainly used in accessment of free radical scavenging activity. Because they facilitate ease of the reaction[4]. The results are compatible with previous findings reporting the significant differences in secondary metabolites in *C. asiatica* growing in *in vitro* and *in vivo* conditions. The differences in secondary metabolite contents were observed between *in vitro* and *in vivo* propagated explants. The similar results were observed in other medicinal plant[12,26].

In this investigation results obtained depicted the influence of combination of TDZ and NAA on callus induction using leaf and stem explants. The advantage of callus culture through in vitro propagation that to get significant concentration of bioactive molecules. This protocol may be useful for higher biomass production in *Centella asiatica*, which leads to production of high amount of bioactive molecules. Based on results reported, the total flavonoid, phenolic contents and the percentage of antioxidant activity of the plant parts (leaf, leaf derived callus and stem derived callus) extracts of *Centella asiatica* are significantly different. Among all the three extracts the total flavonoid and phenolics and the highest free radical scavenging activity were notably maximum in leaf derived callus. This protocol is reproducible and reliable, may be useful for higher biomass production in *Centella asiatica*, which leads to production of high amount of phenolics and flavonoid with potential free radical antioxidant activity through callus culture in *Centella.*

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