

## ***In vitro* callus culture of *Centella asiatica* L. (Urb.) and its HPLC analysis**

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(Received: September 20, 2009; Accepted: October 27, 2009)

### **ABSTRACT**

Different explants of *Centella asiatica* were excised and cultured on Murashige & Skoog (MS) media supplemented with different growth hormones at different concentrations. It has been found that the frequency of callus induction was more on MS media supplemented with Naphthalene acetic acid (NAA) (0.5 mg<sup>-1</sup>) + kinetin (2.0 mg<sup>-1</sup>) and NAA (0.5mg<sup>-1</sup>) + Benzyl Amino Purine (BAP) (2.0 mg<sup>-1</sup>). Among the leaf, petiole and root explants, petiole explants showed more frequency of callus induction followed by leaf and root explants. Qualitative chemical tests of the methanolic extracts of callus showed the presence of saponins, triterpenes, flavonoids but the absence of alkaloids and qualitative analysis by thin layer chromatography (TLC), ultra violet (UV) and infra red (IR) analysis confirm the presence of asiaticoside. Quantitative High performance liquid chromatography (HPLC) analysis reveals that the leaf callus contains significantly more amounts of asiaticoside (1.1%) and madecassoside (2.4%) compared to the root and petiole callus on dry weight basis.

**Key words:** *Centella asiatica*, callus culture, asiaticoside, HPLC.

### **INTRODUCTION**

*Centella asiatica* L. (Urb.) belongs to the family Apiaceae is slender, prostrate, creeping perennial herb that flourishes in and around damp-swampy area. It contains triterpene saponins such as asiaticoside, madecassoside, brahmoside, triterpenic acids etc<sup>1,2</sup>. It proved to have antileprotic, antibacterial antiulcer, and wound healing properties and useful in the treatment of skin diseases and mental disorders<sup>3</sup>. Ritu *et al.*, achieved shoot bud regeneration from callus of *Centella asiatica* through organogenesis<sup>4</sup>. Shashikala *et al.*, carried out *in vitro* regeneration of *Centella asiatica*<sup>5</sup>. There were fewer reports regarding the callus culture of *C. asiatica* using different explants but no reports regarding presence and determination of secondary metabolites. So this paper through a light on the callus culture showing the effect of various growth regulators and identification and quantification of presence of asiaticoside and madecassoside in the callus by HPLC, obtained from different explants.

### **MATERIAL AND METHODS**

#### **Surface sterilization and inoculation of explants**

Plant material was collected from the Indian Institute of Horticulture, Bangalore and identified by Regional Research Institute (Ay), Bangalore. Plants were washed under running tap water followed by distilled water. Then soaked in bavistan, a fungicide (BASF India, Ltd., Bombay.) 150 mg<sup>-1</sup> for 25-30 min. and washed with distilled water. The leaf, petiole and root explants (1.0-1.7 cm) were excised and transferred to sterilized conical flask containing 70% (v/v) alcohol and surface sterilized for 1-2 min followed by treatment with 0.1% (w/v) mercuric chloride for 2 min. Then explants were washed thoroughly with sterile distilled water to remove excess of mercuric chloride. The sterilized explants were trimmed from both the ends and inoculated aseptically on Murashige and Skoog (MS, 1962) media with 3% (w/v) sucrose and 0.8 % (w/v) agar. MS media was supplemented with different concentrations of (0.5- 2.0 mg<sup>-1</sup>) of

Naphthalene acetic acid (NAA) and 2,4-Dichloroacetic acid(2,4-D) alone and in combination with different concentration (0.5-2.0mg-l) of kinetin and Benzyl amino purine (BAP) for callus induction<sup>6</sup>. (Table 1 & 2).

The pH of the media was adjusted to 5.7±0.1 with 0.1 N NaOH or 0.1N HCL. The cultures were maintained at 24±0.5°C with 16 hrs photoperiod.

Root and petiole explants were inoculated horizontally on the medium and the leaves were placed so that dorsal side in contact with the medium. All the treatments were replicated thrice with 20 culture tubes in each set. The responses of explants were represented in the form of frequency (% response) of callus induction.

The growth study of leaf, petiole and root callus were carried out by inoculating one month old callus on MS media supplemented with NAA (0.5 mg<sup>-1</sup>) +kinetin (2.0 mg<sup>-1</sup>) by fresh weight method. The culture tubes were incubated under the same conditions as mentioned above. The weights of callus were taken in the intervals of 8 days for 2 months<sup>7</sup>.

#### Qualitative analysis

Callus dried at 60<sup>o</sup> C in an incubator was macerated with methanol for 10 hrs and filtered under vacuum and concentrated. The residue was subjected to standard chemical tests for saponins, triterpenins, alkaloids and flavonoids, and also to thin layer chromatography (TLC), ultra violet (UV) and infra red (IR) analysis<sup>8, 9</sup>.

#### TLC

The residue obtained above was dissolved in methanol and spotted on precoated silica gel GF<sub>254</sub> TLC plate (E. Merck) and developed using n-Butanol: Glacial Acetic Acid :Water(5:1:4) as a mobile phase. The spots were visualized by spraying with Anisaldehyde-Sulphuric acid reagent and RF values were compared with reference standard. (Fig. 3).

#### UV and IR analysis

Asiaticoside was isolated from the residue obtained above, by two major steps, viz., fractional separation of saponins and preparative TLC.

The residue was suspended in the warm water and extracted with n-Butanol. The n-Butanol layer was concentrated and dissolved in methanol and poured into excess of acetone to precipitate saponins. The precipitate is dissolved in methanol to carry out preparative TLC.

Preparative TLC was carried out according to the method mentioned in TLC of callus using reference standard asiaticoside. The band corresponding to asiaticoside was scraped off and extracted with methanol and filtered. The filtrate was subjected to UV and IR analysis.

For UV analysis methanolic solution was scanned at 200-400 nm using UV spectrometer (Shimadzu UV-1601). For IR the solution was concentrated and evaporated to dryness. The residue was subjected to IR spectral studies using KBR pellet technique (Shimadzu-FTIR, 8900).

#### High performance liquid chromatographic (HPLC) analysis

5 g of dried leaf, petiole and root callus were macerated with methanol for 10 hrs, filtered and evaporated to dryness. The residue obtained were dissolved in HPLC grade methanol individually and analysed using HPLC model Shimadzu LC-2010A with RP8 lichrosphere column length of 30 cm, internal diameter of 4 mm and particle size of 5 µ. The mobile phase was Acetonitrile: Water (3:7) with a flow rate of 1 ml/min detected at 210 nm<sup>10</sup>.

## RESULTS AND DISCUSSION

#### Callus induction

Variable responses were observed when the leaf, petiole and root explants were cultured on MS media supplemented with various concentrations (0.5, 1.0, 2.0 mg<sup>-1</sup>) of NAA and 2-4-D alone and in combination with various concentrations (0.5, 1.0, 2.0 mg<sup>-1</sup>) of BAP and kinetin for the callus induction. In all cultures the formation of callus initiated after 15 days of incubation.

When auxins used alone the highest (50-59%) frequency of callus induction of all the explants was observed in the media with 2.0 mg<sup>-1</sup> of NAA and 2, 4-D at the end of fourth wk. The nature of



**A) Rooting of callus on MS media with NAA (0.5 mg l<sup>-1</sup>) +Kn (2.0 mg l<sup>-1</sup>)**



**a) Callus on MS media with NAA (0.5mg l<sup>-1</sup>) + kinetin (2.0 mg l<sup>-1</sup>)**



**B) Callus on MS media with NAA (0.5 mg l<sup>-1</sup>) + BAP (2.0 mg l<sup>-1</sup>)**



**b) Callus on MS media with NAA (0.5 mg l<sup>-1</sup>) + BAP (2.0 mg l<sup>-1</sup>)**



**C) Browning of callus on MS with 2, 4-D (0.5 mg l<sup>-1</sup>) + Kn (2.0 mg l<sup>-1</sup>)**



**c) Browning of callus on MS media with 2, 4-D (0.5 mg l<sup>-1</sup>) + kinetin (2.0 mg l<sup>-1</sup>)**

**Fig. 1A -C: Effect of auxins in combination with cytokinins on LEAF callus induction**

**Fig. 2a-c: Effect of auxins in combination with cytokinins on PETIOLE callus induction**

leaf and petiole callus was pale green and spongy where as root callus was creamy and compact. But the callus on media with 2, 4- D was become brown after 40 days of incubation showing no further growth. (Table1).

**Table 1: Effect of auxins on the frequency of callus induction**

Growth regulators (mg l <sup>-1</sup> )	Frequency (% response) & the nature of the callus		
	Leaf	Petiole	Root
NAA			
0.5	30 PG, S, a	34 PG, S, c	28 Cr, C, c
1.0	52 PG, S, b	55 PG, C, c	50 Cr, C, c
2.0	55 PG, S, b	59 PG, C, c	53 Cr, C, c
2, 4-D			
0.5	20 PG, S, c	25 PG, S, c	19 Cr, S, c
1.0	49 PG, S, c	56 PG, S, c	43 Cr, S, c
2.0	52 PG, S, c	55 PG, S, c	50 Cr, S, c

PG: pale green; S: spongy; C: compact; Cr: creamy;<sup>a</sup> rooting to small extent; <sup>b</sup> average rooting; <sup>c</sup> no rooting 20 explants were maintained in each treatment data were recorded up to 6 wks

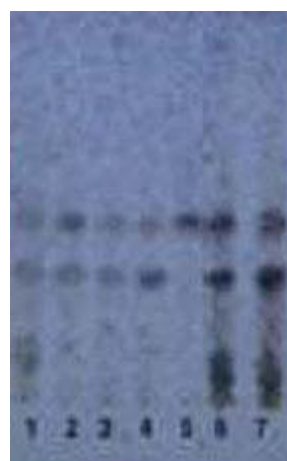
Amongst the different concentrations of auxins in combination with different concentrations of cytokinins tested, NAA (0.5mg<sup>-1</sup>) + kinetin (2.0 mg<sup>-1</sup>) and NAA (0.5 mg<sup>-1</sup>) + BAP (2.0 mg<sup>-1</sup>) found to be effective. In case of leaf explants the callus formed at the edges initially followed by more callus formation near the vascular tissue due to the availability of more nutrients around the conducting tissue<sup>11</sup> and in petiole and root explants the callus initiated at only one end. The nature of leaf callus was pale green and spongy on the media with NAA (0.5 mg<sup>-1</sup>) + BAP (2.0 mg<sup>-1</sup>). Even though the media with NAA (0.5mg<sup>-1</sup>) + kinetin (2.0mg<sup>-1</sup>) showed similar response but rooting of the callus was observed after 25 days of incubation. The frequency of callus induction was 75- 88% at the end of fourth

week. Even media with 2,4-D (0.5 mg<sup>-1</sup>) + kinetin (2.0 mg<sup>-1</sup>) found to be effective (80-84%) but the callus become brown after 40 days of incubation showing no further growth as in case of 2,4- D alone. The browning of the callus was influenced by the accumulation of phenolics in the cytoplasm which undergo oxidation and polymerization and oxidized products appears dark brown<sup>12</sup>. (Table 2) and (Fig 1, A- C).

The callus produced from petiole, root explants was light green and compact without rooting. (Table2) and (Fig 2, a-c).

From this it has been concluded that if only auxins are used, media requires high concentrations of auxins (up to 2.0mg<sup>-1</sup>) to get high frequency of callus induction where as if it is supplemented with cytokinins, the media requires less concentrations of auxins. Further, the media supplemented with auxins in combination with cytokinins is effective for the induction of the callus.

The callus obtained above was proliferated on media with NAA (0.5 mg<sup>-1</sup>) + BAP (2.0 mg<sup>-1</sup>). The callus was light green spongy without rooting after 6 weeks of incubation and it was used for the qualitative and quantitative analysis.



**Fig 3: TLC studies of methanolic extracts of callus on MS medium**

1, 3, 6 root, petiole and leaf callus on media with BAP (2.0 mg l<sup>-1</sup>) + NAA (0.5 mg l<sup>-1</sup>) respectively. 2, 4, 7, root, petiole and leaf callus on media with kinetin (2.0 mg l<sup>-1</sup>) + NAA (0.5 mg l<sup>-1</sup>) respectively. 5 Standard asiaticoside

**Growth studies of callus**

Leaf and petiole callus show same long lag phase, whereas the root shows a shorter lag phase during 8 days of incubation. The exponential phase of the petiole deviates from the leaf and root calli and it was long during 16, 24 and 32 days of incubation. The nature of the callus was light green and compact. The petiole, leaf and root show the stationary phase turning to light yellow and then to the brown colour.

The weight of petiole callus was 1.64±0.03 g, leaf callus, 1.36±0.10 g and root callus, 1.34±0.91 g.

**Qualitative analysis**

Qualitative chemical tests of all three callus confirm the presence of carbohydrates, proteins, sterols, saponins, triterpenes, flavanoids but the absence of alkaloids. The results obtained were compared with methanolic extract of the natural plant.

**Table 2: Effect of auxins + cytokinins on the frequency of callus induction**

Growth regulators (mg l <sup>-1</sup> )	Frequency (% response) and nature of the callus		
	Leaf	Petiole	Root
NAA + kinetin			
0.5 + 0.5	35 PG, S, a	40 PG, C, c	32 Cr, C, c
0.5 + 1.0	60 PG, S, b	63 PG, C, c	60 Cr, C, c
<u>0.5 + 2.0</u>	<u>86</u> PG, S, b	<u>88</u> PG, C, c	<u>78</u> Cr, C, c
1.0 + 0.5	30 PG, S, a	34 PG, C, c	30 Cr, C, c
1.0 + 1.0	40 PG, S, a	30 PG, C, c	32 Cr, C, c
2, 4-D + kinetin			
0.5 + 0.5	30 PG, C, c	35 PG, C, c	32 PG, C, c
0.5 + 1.0	50 PG, S, c	55 PG, C, c	60 Cr, C, c
0.5 + 2.0	80 PG, C, c	84 PG, C, c	80 Cr, C, c
1.0 + 0.5	40 PG, C, c	45 PG, C, c	60Cr, C, c
1.0 + 1.0	50 PG, C, c	55 PG, C, c	23 Cr, C, c
NAA +BAP			
0.5+0.5	35 PG, S, c	42 PG, C, c	35 PG, C, c
0.5 + 1.0	60 PG, S, c	64 PG, C, c	58 PG, C, c
<u>0.5 + 2.0</u>	<u>82</u> PG, S, c	84 PG, C, c	<u>75</u> PG, C, c
1.0 + 0.5	40 PG, S, c	45 PG, C, c	35 PG, C, c
1.0 + 1.0	55 PG, S, c	52 PG, C, c	45 PG, C, c

PG: pale green; S: spongy; C: compact; Cr: creamy; <sup>a</sup> rooting to small extent,; <sup>b</sup> average rooting; <sup>c</sup> no rooting 20 explants were maintained in each treatment, data were recorded up to 6 wks.

The TLC studies of the petiole, leaf and root callus showed two major spots of bluish violet having the Rf values of 0.42 and 0.33. The Rf value, 0.42 was similar to that of reference standard, asiaticoside. Thus the callus was found to contain asiaticoside as major active constituent. (Fig 3).

UV spectra of isolated asiaticoside showed  $\lambda_{\max}$  at 204.4 which was similar to that of reference standard and IR spectra was also similar to that of reference standard and reported data. The major peaks observed were 2923.9 (C-H stretching), 1651.0 (C=C stretching), 1720.4 (C=O stretching), and 3409.9  $\text{cm}^{-1}$  (-OH stretching)<sup>13</sup>.

#### HPLC analysis

The % of asiaticoside and madecassoside in the methanolic extract of leaf callus was found to be 1.1 and 2.4, in petiole callus 0.68 and 1.31 and in root callus 0.93 and 1.65 respectively. It showed that the leaf callus contains significantly more amount of asiaticoside and madecassoside as the leaves are the major sources. Increase in the secondary metabolites through tissue culture work will be the criteria for further study.

#### ACKNOWLEDGMENTS

Authors are thankful to Dr. Deepak. Manager, Phytochemistry, Natural Remedies, R & D center, Bangalore for providing reference standards and HPLC facilities.

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