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RESEARCH ARTICLE

High efficiency adventitious indirect organogenesis and plant regeneration from callus of *Centella asiatica* (L.) -An important Antijaundice medicinal plant

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Abbreviations:
BAP: Benzyl amino purine; Kn: 6-Furfuryl amino purine; 2,4- D: 2, 4-Dichloro phenoxy acetic acid; IAA: Indole-3-acetic acid; IBA: Indole-3-butyric acid; NAA: Naphthalene acetic acid.

Abstract

An efficient protocol was designed for successful regeneration of *Centella asiatica* (L.) from *in vitro* derived callus through somatic embryogenesis. Nodal explants were isolated and cultured on MS medium fortified with 0.5 - 1.0 mg/l 2,4- D alone and in combination with BAP. The above combination induced profuse, compact, light green to greenish coloured calli. It was observed that the increased concentration of 2,4- D induced the direct shoot organogenesis occasionally. Some differences in the morphology of callus such as changes in the colour and texture was also observed with increasing the concentration of 2,4- D. Maximum frequency of callus induction was noticed on 2.0 mg/l 2,4- D and 0.5 mg/l BAP which was greenish colour, friable and granular in appearance. The calli were separated and further cultured on fresh media containing BAP alone and in combination with auxins such as NAA and IAA. The maximum regeneration frequencies of shoots were recorded on BAP 2.5 mg/l + 0.5 NAA mg/l. The well regenerated healthy micro shoots were separated and transferred to rooting medium for profuse rooting. MS medium augmented with IBA 1.0 mg/l showed maximum rooting frequency with high mean root number and root length. The well rooted plants were transferred to field conditions. Survival frequency was found to be 80%. This reproducible protocol can be used for further regeneration and genetic transformation studies.

Introduction

Centella asiatica (L.) Urban is a stoloniferous perennial herb belonging to the family *Apiaceae* or *Umbelliferae*. It is a slender creeping plant rooting at the nodes. In India it is commonly known as 'Indian pennywort' 'Jal bramhi' 'Mandookaparni' and 'Gotukola'. The substances of therapeutic interest are the saponin containing triterpene acids, asiatic acid, madecassic acid, asiaticosides as A and B (Kirtikar and Basu, 1975). It acts as a potent memory enhancer (Silviya Rajakumari Jared et al., 2010). Also consists of glycosides as brahmoside, brahminoside, indocentelloside and leaves are rich in carotenoids, Vitamins B and C. It has been used in the treatment of jaundice, measles, hepatitis, syphilus, smallpox and rheumatism (Prajapati et al., 2010). The herb was reported as antidiabetic, antiviral, antiulcer, antibacterial, antitumour and high memory enhancing activity (Chakraborty et al., 1996; Srivasthava et al., 1997; Vasantharuba Seevaratnam et al., 2012). This plant is conventionally propagated by cuttings and seeds. This type of propagation cannot meet the increasing demand. This plant used as the raw material for the preparation of pharmaceutical, dermaceutical and aroma therapeutical products. The *in vitro* culture techniques can be the alternative for the continuous provision of plantlet stocks for the large scale field cultivation and commercial industries (Chan Lai Keng et al., 2009).

Callus is an amorphous tissue consisting of dedifferentiated, unorganized masses of cells. The callus consists of majority of parenchyma tissue cells in nature. Callus will undergo three successive stages of development process

such as induction, dedifferentiation, and finally the cell division (George et al., 2008). In *in vitro* regeneration methods auxins are widely used for callus induction. The auxin commonly used for callus induction is 2, 4- D, but NAA and IAA are also used (Chawla, 2002). Multiplication of *C. asiatica* through shoot tip culture (Nath and Alak, 2003) meristem tip culture (Tiwari et al., 2000) and regeneration from non embryogenic cell lines (Yamin Bibi et al., 2011) has been reported. Although callus culture and regeneration of *C. asiatica* has already been established, the present study assessed to investigate the efficacy of different auxins and cytokines on highly proliferated greenish calli induction and to produce high efficiency adventitious indirect shoot organogenesis of *C. asiatica* from field grown axillary buds as explants.

Materials and methods

Collection of plant material and surface sterilization

Young *C. asiatica* plants were collected from herbal garden, Department of Biotechnology, Dravidian University, Kuppam, Andhra Pradesh, India. Axillary buds were collected from the young sprouts of the stock plants were selected as explants. These explants were washed under running tap water for 10 min, followed by immersing in liquid detergent solution 5 % (v/v) Tween 20, for 15 min and then washed under running tap water. The explants were surface sterilized with 0.4 % (w/v) Bavistin, a systemic fungicide (BASF India Ltd.,) and then with 70 % (v/v) ethanol for 90 seconds.

The explants were surface sterilized with 0.1 % (w/v) $HgCl_2$ (Merck India) for 1-3 minutes and thoroughly washed with sterile double distilled water for thrice to remove the traces of $HgCl_2$ before inoculation.

Culture medium and culture conditions

MS medium (Murashige and Skoog, 1962) supplemented with various plant growth regulators auxins such as 2, 4-D, and IAA, cytokinins such as BAP and Kn at different concentrations either alone or in combination were used for callus initiation and shoot proliferation. The P^H of the medium was adjusted to 5.8 and gelled with addition of 0.8 % (w/v) agar. Molten medium was dispensed approximately 15 ml into culture tubes (25 × 150 mm) and closed with non-absorbent cotton plugs. The medium was autoclaved at 15 lbs / Sq inch pressure and 121^0C for 20 min. MS basal medium. All the cultures were incubated in an *in vitro* culture room maintained at 26 ± 2^0C temperature and 55 - 60 % relative humidity with a photo period of 16 hours day light and 8 hours dark with a light intensity of 3000 lux provided by cool white fluorescent tubes (Phillips India Ltd.,). All the cultures were transferred to fresh culture medium after 21 days of culture duration for the development of *in vitro* rooting.

The frequency of callus induction was calculated by applying the formula

$$\text{Callus induction frequency (\%)} = \frac{\text{No. of nodal explants produced calli}}{\text{No. of nodal explants cultured}} \times 100$$

Data analysis

Visual observations were recorded on the frequency in terms of number of cultures responding for axillary shoot proliferation, shoot development, number of shoots per explant, average length of the regenerated shoots, number of roots per shoot and average root length.

Statistical analysis

All the experiments were conducted with a minimum of 20 explants. All assays were repeated at least three times. The experimental data were statistically analyzed by one-way ANOVA using the DMRT (Duncan's Multiple Range Test) ($P < 0.05$) and were presented as the mean ± standard error (SE).

RESULTS

In the present study the results showed that the nodal explants cultured on MS medium supplemented with combination of 2,4- D and BAP have regenerated and gave best response in terms of callus induction and multiple shoot production but different in callus characteristic features as colour and morphology.

Effect of different auxins such as 2,4- D and BAP on callus induction and proliferation from nodal explants of *C. asiatica.*

A perusal of the obtained data reveals that different auxins at various concentrations were showed potential consequence on callus induction. Initially the nodal explants were cultured on MS medium which is devoid of growth hormones. Then the medium augmented with 2,4- D (0.5 - 2.5 mg/l). Callus induction was observed after 3 weeks of inoculation of nodal explants. The addition of 2,4- D (0.5 mg/l) in the medium favoured callus induction (**Fig.1a & 1b**). Callusing formation was noticed on centre portion of many explants, whereas at the cut ends of only some nodal buds. The frequency of callus proliferation was continued up to 2.0 mg/l. Frequency of callus induction (60%) was observed at 2,4- D (2.0 mg/l) (**Fig.1b**). By further increase in the 2,4- D concentration the callus induction frequency becomes decreased (< 2.5 mg/l). Surprisingly when the explants were cultured on MS medium containing both 2,4- D (2.0 mg/l) and BAP (0.5 mg/l) which induced maximum callus induction frequency (95%) when compare to 2,4- D alone (**Fig.1c**). By further increasing the BAP concentration the dark brownish colour callus induction was noticed. The results were tabulated in **Table 1**.
The morphology of regenerated callus in terms of colour and texture were appeared from greenish to greenish yellow in colour. The combination of both 2,4- D and BAP was exhibited high quality colour and profuse organogenic in texture (**Fig.1c**).

Impact of IAA/NAA with BAP on callus induction from nodal explants of *C. asiatica*.

Nodal explants when cultured on MS basal media supplemented with other auxin such as NAA alone or in combination with BAP was also employed to trace their effect on callus induction. But the callus induction frequency was too low when it was used alone or with the combination of BAP. Callus induction was failed was not found after 5 weeks of culture on MS media containing NAA (2.0 mg/l) along with BAP (0.5 mg/l). In the same way MS media containing IAA along with BAP was also reported to induce fewer calluses frequency. Further increase in the concentrations of either these auxins results in poor callus or no callus and colour of the explants were distorted to dark and compact in nature (Data not shown).

Indirect shoot organogenesis from *in vitro* derived callus
Effect of BAP on shoot regeneration

Well profused induced calli were separated and sub cultured on fresh media for shoot induction and proliferation. Calli were cultured on BAP (0.5 - 2.0 mg/l), NAA (0.5 mg/l), Kn (0.5 - 1.5 mg/l) respectively. Shoot induction fallowed by gradual elongation was observed after 2 weeks of culture. Callus cultured on BAP (2.0 mg/l) alone reported maximum regeneration frequency (80%), mean shoot number (4.4 ± 0.62) and shoot length (3.98 ± 0.05 cm) (**Fig 1d**). Kinetin at 1.5 mg/l was produced a least regeneration frequency (55%) and decreased mean shoot number (3.34 ± 0.73) and shoot length (2.87 ± 0.86 cm). Data collection was performed after 4 weeks of inoculation. Of all the combinations employed maximum shoot regeneration frequency (95%) was found in BAP (2.0 mg/l) along with combination of NAA (0.5 mg/l) with mean shoot number (11.4 ± 0.46) and shoot length (8.43 ± 0.22 cm) (**Fig 1e & 1f**).

Effect of different auxins on rooting of *in vitro* regenerated plantlets from callus

In vitro derived shoots (3.0 – 6.0 cm) were removed from culture tubes and separated from shoot clumps and sub cultured on the MS medium augmented with different auxins as IBA and NAA (0.2 - 1.0 mg/l). Among all concentrations tested, root proliferation rate (95%) was noticed on MS medium with IBA (1.0 mg/l) with maximum mean number of roots (18.4 ± 0.48) and mean root length (5.6 ± 0.91cm) (**Table 3**). Micro shoots regenerated from medium containing IAA was reported to produce least mean number of root (12.6 ± 0.18) and mean root length (5.0 ± 0.64 cm) when compare to IBA containing rooting medium (**Fig 2a**).

Acclimatization and hardening
Well developed rooted plants were carefully removed from culture tubes and washed to remove the remnants of agar (**Fig 2b**). These healthy shoots were transferred to tray containing soil and vermiculate in 1:1 ratio for acclimatization. For a period of week the plants were kept in polythene membrane. After that the surviving plants were transferred to pots and maintained under greenhouse for hardening (**Figs 2c & 2d**).

Figure 1. Callus induction from nodal explants and regeneration form the calli.

1a) Nodal explants cultured on MS + 2,4- D medium alone (0.5 mg/l).
1b) Callus induction after 3 weeks of culture.
1c) Induction of greenish colour, high quality texture profuse organogenic calli on MS + 2,4-D (2.0 mg/l) in combination with BAP (0.5 mg/l).
1d) Indirect shoot regeneration from BAP (2.0 mg/l) alone medium.
1e) Indirect shoot proliferation on MS+BAP (2.0 mg/l) along with NAA (0.5 mg/l).
1f) Maximum frequency of adventitious shoot regeneration after sub culture.

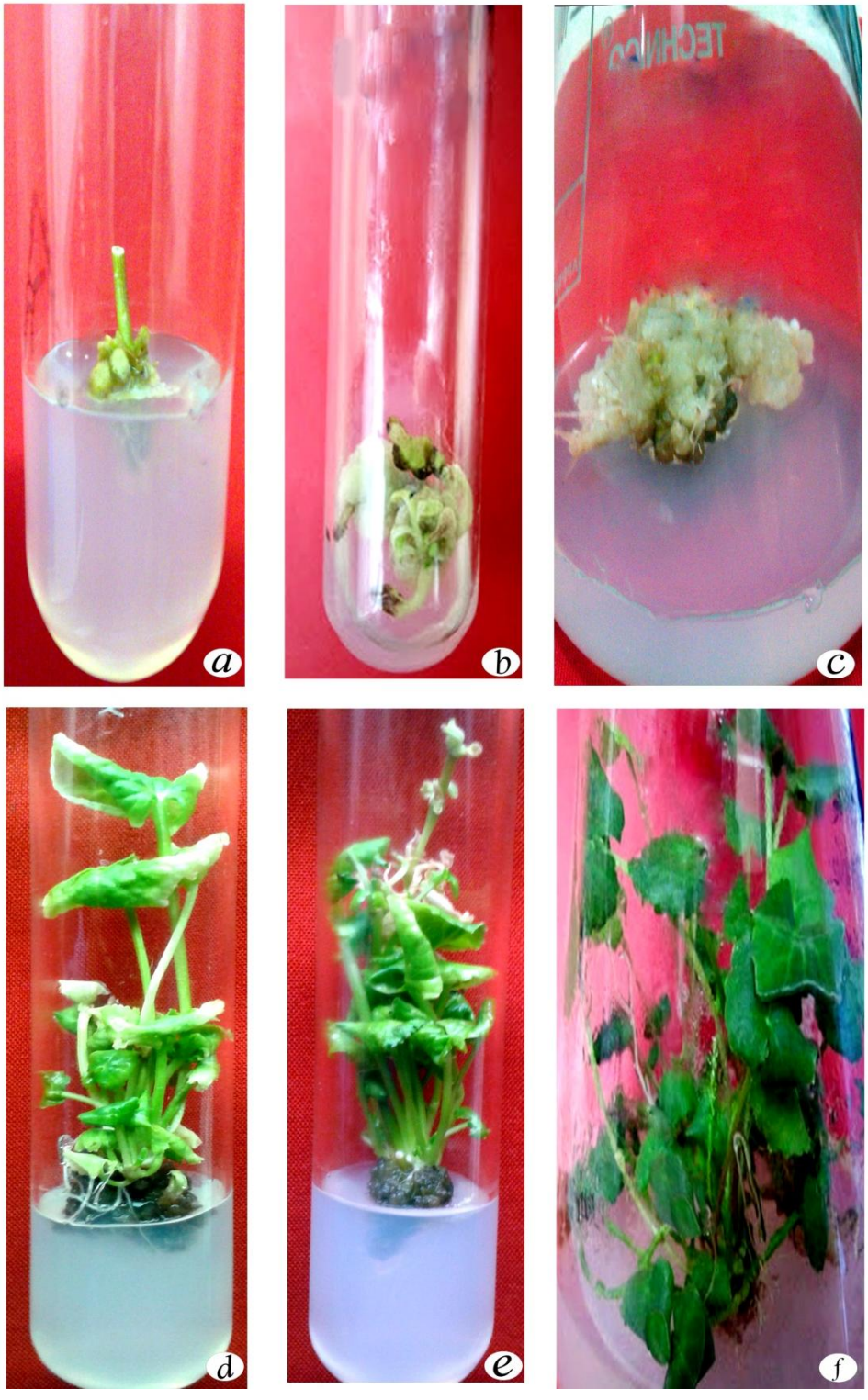
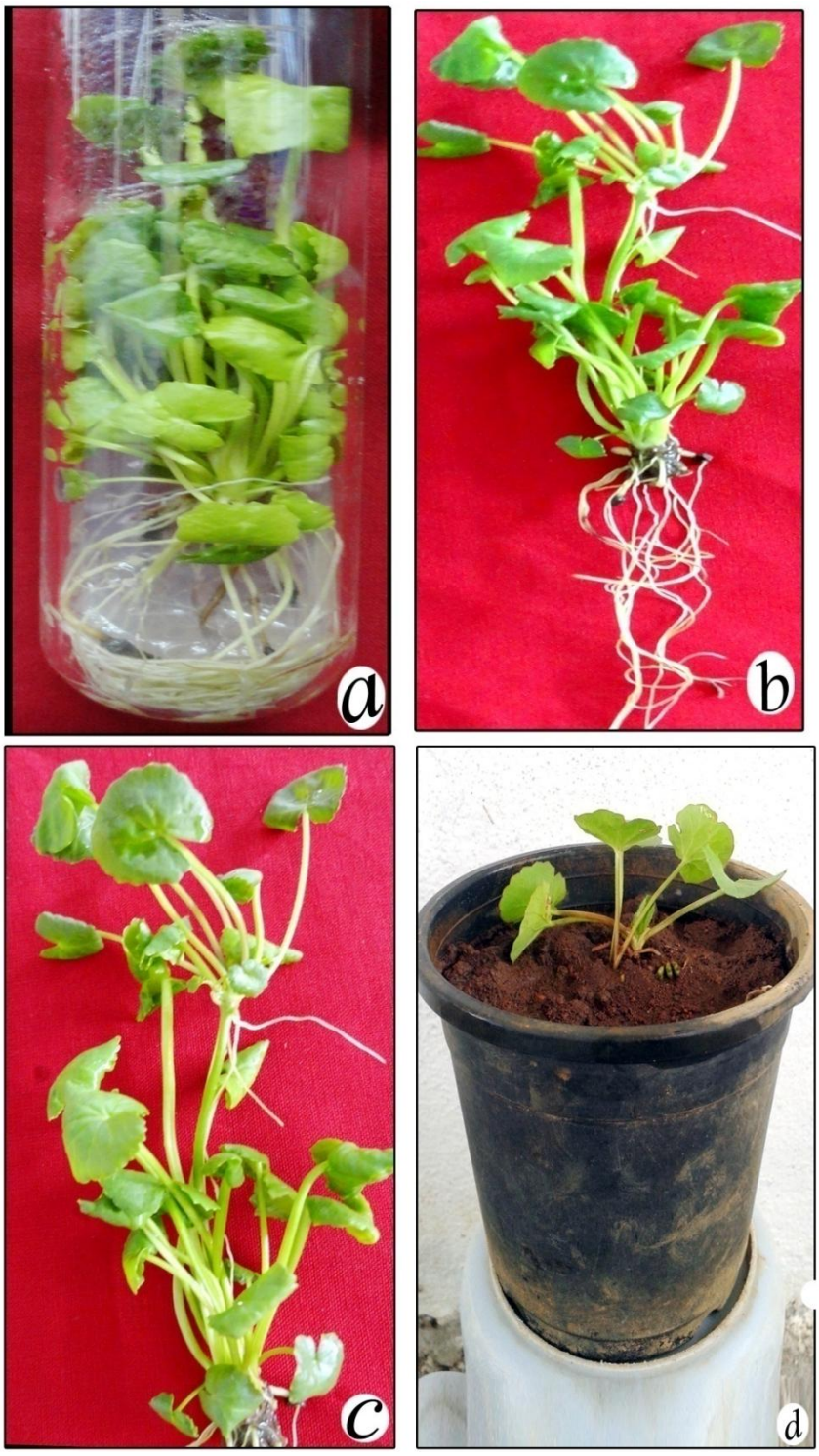


Figure 2. *In vitro* rooting of regenerated shoots and hardening of *Centella asiatica*

2a) Initiation and elongation of roots of micro shoots on MS + IBA (1.0 mg/l).
2b) Adventitious shoot let with elongated rooting system.
2c) Acclimatization of regenerated shoot.
2d) Hardening of a tissue cultured plant in earthen pot containing soil and vermiculate mixture (1:1) under green house condition.



Discussion

Plant biotechnology is well thought-out in a wide sense which comprises the various culture methods of plant organs and explants. In order to establish the most optimum condition for induction of greenish, well textured organogenic calli, we reported a few concentrations of growth hormones such as the combination of 2,4- D and BAP. This combination was found to induce maximum callus induction frequency as compare to any other treatments. Callus is undifferentiated and unorganized mass of parenchyma cells formed by the proliferation of parent tissue of explants. In general callus tissue is considered as good source of genetic variability and adventitious shoot induction and regeneration (Dodds and Roberts, 1982). In general low auxin and high cytokinin concentration in the media results in the formation of shoot regeneration. Our results are accordance with the findings of *Bacopa monniera* in which maximum callus formation was observed from nodal explants when cultured on MS medium containing 2, 4- D (Tiwari et al., 1998; Vijayakumar et al., 2010). Addition of 2,4- D in to the culture media that facilitates the greenish colour for the calli. Our results are accordance with reports of *Biophytum sensitivum* (Shivanna et al., 2009), *Solanum nigrum* (Sridhar and Naidu, 2011). Light green and creamish colour calli were induced in the auxin **:** cytokinin combination. The reason may due to the effect of auxin on chlorophyll pigment development. (Viegas et al., 2007). Similar to our findings medium containing BAP and 2,4- D was showed high frequently of callus in *Anthurium andreanum* (Jahan et al., 2009). But in contrast to these results 2, 4- D with combination of NAA were found to induce maximum calli in *Stevia rebaudiana* (Preethi et al., 2011). This may be due to their role in DNA synthesis and mitosis (Skoog and Miller, 1957). The further increase in the auxin concentration was results in change in the colour of callus from greenish to dark brownish, compact and hard. This is in concurrence with prior reports in *Ipomea aqualtica* (Nagendra Prasad et al., 2006). If the quantity of phenolic compounds increases they will accumulate in the cytoplasm of cell, it undergoes oxidation and polymerization and oxidized derivate products appear in brownish/black colour (Lukas et al., 2000). High concentrations of 2, 4-D was able to inhibit callusing of basal segments and it had effect as the herbicide (Wernicke and Mikovits, 1984). Callus induction on MS basal media devoid of growth hormones was also reported in *Ocimum tenuiflorum* (Singh et al., 1999). But in contrast to these findings *Ocimum sanctum* cultures fail to induce callus on growth hormones free medium (Lim et al., 2009)

In the present study *in vitro* regeneration of micro shoots was achieved via callus as explants through the process of indirect shoot organogenesis. BAP favoured for multiple shoot induction and proliferation from calli. In general, BAP is the most efficient growth regulator for stimulation and shoot proliferation. BAP mimics as an inhibitor agent and functions against apical dominance of shoot induction and shoot bud formation (Wang and Charle, 1991). Superiority of BAP over any cytokinin in induction and proliferation of shoots has been very well documented in *Dalbergia sisso* (Arya et al., 2013), *D. latifoloia* (Raghavaswamy et al., 1992), *Quercus rober* (Puddephat et al., 1997) and *Lens culinaris* (Sharma Richa et al., 2014).

Among the auxins employed for rhizogenesis IBA facilitates maximum rooting efficiency. The results on rooting experiments coincide with other observations of others that NAA and IAA were less effective when compared to IBA. These results are accordance with *Gossypium hirsutum* (Markkandan Ganesan et al., 2005). An increased concentration of auxins facilitates callus formation (Chawla, 2002). The slow movement and slow degradation of IBA facilitates its localization near the site of application and thus it functions better in inducing roots (Nickel, 1982).

Table-1: Effect of different concentrations of auxins and cytokinins on callus induction from *in vitro* cultured nodal explants.

Plant growth regulators (mg/l)			Callus proliferation rate	Frequency of callus induction (%)	Callus characteristics
2,4-D	BAP	NAA			
-	-	-	-	-	No callus
0.5	-	-	C^{+}	40	Yellowish green, fragile, very
1.0	-	-	C^{+}	55	small
1.5	-	-	C^{++}	60	Brown, compact
2.0	-		C^{++}	60	Brown, fragile, compact
1.0	0.5		C^{++}	85	Greenish white, fragile
1.5	0.5		C^{+++}	80	Light brown, fragile
2.0	0.5		C^{++++}	95	Green, fragile, Granular Lush
2.5	0.5		C^{+}	55	green, fragile
-	0.5	0.5	C^{++}	45	Dark green ,compact
-	1.0	1.0	C^{++}	50	Whitish green, fragile
-	1.0	1.5	C^{+}	55	Light green, fragile
	2.0	2.0	-	-	-

Table-2: Effect of different concentrations of cytokinins and auxins on indirect shoot organogenesis of *in vitro* derived callus cultures of *C. asiatica* (L.). Data represent treatment means ± SE fallowed by different letter(s) within column indicate significant differences according to ANOVA and DMRT test (P < 0.05).

Plant growth regulators (mg/l)			Regeneration frequency (%)	Mean no. of shoots/callus	Mean shoot length (cm)
BAP	Kn	NAA			
0.5	-	-	60	2.4 ± 0.35^{b}	1.54 ± 0.23^{b}
1.0	-	-	65	3.1 ± 0.60^{c}	2.62 ± 0.15^{e}
1.5	-	-	75	3.9 ± 0.55^{d}	3.42 ± 0.13^{f}
2.0	-	-	80	4.4 ± 0.62^{e}	$3.98 \pm 0.05^{g\,h}$
-	0.5	-	60	2.2 ± 0.45^{a}	1.0 ± 0.09^{a}
-	1.0	-	60	3.0 ± 0.20^{c}	2.1 ± 0.75^{c}
-	1.5	-	55	3.34 ± 0.73^{c}	2.87 ± 0.86^{e}
0.5	-	0.5	50	5.2 ± 0.54^{f}	$3.71 \pm 0.53^{f\,g}$
1.0	-	0.5	70	6.8 ± 0.66^{i}	4.22 ± 0.23^{i}
1.5	-	0.5	75	8.2 ± 0.39^{j}	5.64 ± 0.31^{j}
2.0	-	0.5	95	11.4 ± 0.46^{k}	8.43 ± 0.22^{k}
2.5	-	1.0	60	7.32 ± 0.28^{l}	5.42 ± 0.42^{l}
	0.5	0.5	65	4.32 ± 0.76^{d}	2.56 ± 0.11^{d}
	1.0	0.5	55	$4.98 \pm 0.22^{e\,f}$	3.10 ± 0.24
	1.5	0.5	50	5.63 ± 0.18^{g}	$4.14 \pm 0.65^{h\,i}$
	2.0	0.5	45	5.32 ± 0.54^{f}	$3.59 \pm 0.24^{g\,h\,i}$

Table-3: Effect of different concentrations of auxins on rooting of *in vitro* derived micro shoots of *C. asiatica* (L.) on half strength MS medium. Data represent treatment means ± SE fallowed by different letter(s) within column indicate significant differences according to ANOVA and DMRT test (P < 0.05).

Plant growth regulators (mg/l)		Root regeneration frequency (%)	Mean no. of roots/shoot	Mean no. of root length (cm)
IAA	IBA			
0.2	-	70.00	6.4 ± 0.21[a]	2.6 ± 0.42[a]
0.4	-	72.00	7.8 ± 0.16[b]	3.2 ± 0.59[b]
0.6	-	75.00	9.0 ± 0.41[c]	3.7 ± 0.61[d]
0.8	-	80.00	10.5 ± 0.72[d]	4.3 ± 0.72[f]
1.0	-	85.00	12.6 ± 0.18[e]	5.0 ± 0.64[h]
-	0.2	78.00	7.0 ± 0.24[a]	3.4 ± 0.29[c]
-	0.4	85.00	9.2 ± 0.36[c]	3. 9± 0.38[e]
-	0.6	90.00	12.5 ± 0.29[e]	4.4± 0.45[g]
-	0.8	94.00	15.4 ± 0.61[f]	4.9± 0.74[h]
-	1.0	95.00	18.4 ± 0.48 [g]	5.6± 0.91[j]

Conclusion

In the present investigation we introduced improved explants, growth hormones and optimized combinations for superior and better callus induction and growth. Also describes a detailed protocol for *in vitro* regeneration of *C. asiatica* from callus tissue through indirect organogenesis. The single node culture method reported here offers an effective method of conservation and multiplication of important medicinal plant *C. asiatica.*

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References

Arya, I.D., Nautiyal, S. and Arya, S. (2013). Tissue culture studies on clonal variations in micropropagation of *Dalbergia sissoo*. Int J Biot Res., 1(4): 58-70.

Chakraborty, T., Sinha B.S. and Sukul, N.C.(1996). Preliminary evidence of antifilarial effect of *Centella asiatica* on canine dirofilariasis. Fitoterapia, 67: 110-112.

Chan Lai Keng, Lim Su Yee. and Pan Lay Pin. (2009) Micropropogation of *Gynura procumbens* (Lour.) Merr. An important medicinal plant. J Med Plants Res., 3: 105-111.

Chawla, H.S. (2002) Introduction to plant biotechnology, 2nd Edition, Science Publishers INC, New Hampshire, United States of America. pp.528.

Dodds, J.H. and Roberts, L.W. (1982). Experiments in plant tissue culture. Cambridge University Press, Cambridge, London, New York.

Jahan, M.T., Islam, M.R., Khan, R., Mamun, A.N.K., Ahmed, G. and Hakim, H. (2009). *In vitro* clonal propagation of Anthurium (*Anthurium andraeanum* Lind) using callus culture. Plant Tiss Cult Biotech., 19 (1): 61-69.

Kirtikar, K.R. and Basu B.D. (1975). Indian medicinal plants. II Edition. M/s Bishen sing Mahendra Pal sing, New Delhi.

Lim, Z.X., Ling, A.P.K. and Hussein, S. (2009). Callus induction of *Ocimum sanctum* and estimation of its total flavonoids content. Asian J Agri Sci., 1: 55-61.

Lukas, A.M., Christoper, D.G., Rebecca, A.S. and Virgnia, W. (2000). AN9-a petunia glutathione s-transferase required for anthocyanin sequestration, is a flavonoid binding protein. Plant Physiol., 123: 1561-1570.

Markkandan Ganesan. and Narayanasamy Jayabalan. (2005). *In vitro* plant regeneration from the callus of shoot tips in cotton (*Gossypium hirsutum* L. cv. SVPR 2). Iranian J Biotech., 3(3): 144-151.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol., 15: 473-479.

Nagendra Prasad, K., Siva Prasad, M., Shivamurthy, G.R. and Aradhya, S.M. 2006. Callus induction from *Ipomoea aquatica* Forsk. Leaf and its antioxidant activity. Ind J Biotech., 5:107 -111.

Nath, S. and Alak, K.B. (2003). *In vitro* method for propagation of *Centella asiatica* (L) Ur Lucas ban by shoot tip culture. J Plant Biochem Biotechnol., 12: 167-169.

Nickell, G.L. and Kirk-Othmer. (1982). Encyclopaedia of chemical technology. Vol. 18. (John Wiley & Sons). New York.

Prajapati, N.D., Purohit S.S., Sharma, A.K. and Kumar, T. (2006). A hand book of medicinal plants, Hindustan press, agro bios, Jodhpur.

Preethi, D., Sridhar, T.M. and Naidu, C.V. (2011). Efficient protocol for indirect shoot regeneration from leaf explants of *Stevia rebaudiana* (Bert.) – An important calorie free biosweetner. Journal of Phytology, 3(5): 56-60.

Puddephat, I.J., Alderson, P.G. and Wright, N.A. (1997). Influence of explant source, plant growth regulators and culture environment on culture initiation and establishment of *Quercus robur* L. *in vitro*. J Exp Bot., 48 (309): 951-962.

Raghavaswamy, B.V., Himabindu, K. and Lakshmisita, G. (1992). *In vitro* micropropagation of elite rosewood *Dalbergia latifolia* Roxb. Plant Cell Rep., 11: 126-131.

Sharma Richa, Acharjee Sumita. and Sarmah Bidyut Kumar. (2014). An efficient *In Vitro* regeneration system in lentil (*Lens culinaris*) using cotyledons with half embryonic axes. Res J Biotech., 9(1): 9-15.

Shivanna, M.B., Vasantha kumara, M.M. and Mangala, C. (2009). Regeneration of *Biophytum sensitivum* (Linn.) DC. through organogenesis and somatic embryogenesis. Ind J of Biotech., 8: 127-131.

Silviya Rajakumari Jared. (2010). Enhancement of memory in rats with *Centella asiatica*, Biomed Res., 21(4): 429-432.

Singh, N.K. and Sehgal, C.B. (1999). Micropropagation of holy basil (*Ocimum tenuiflorum* Linn.) from young inflorescence of mature plants. Plant Growth Regul., 29: 161-166.

Skoog, F. and Miller, C.O. (1957). Chemical regulation of growth and organ formation in plant tissue cultures *in vitro*. Sym. Soc. Exp. Biol., 11: 118 - 131.

Sridhar, T.M. and Naidu, C.V. (2011). An efficient callus induction and plant regeneration of *Solanum nigrum* (L.) - an important antiulcer medicinal plant. Journal of Phytology, 3(5): 23-28.

Srivastava, R., Shukla, Y.N. and Kumar, S. (1997). Chemistry and pharmacology of *Centella asiatica*: a review. J Med Aromatic Plant Sci., 19: 1049-1056.

Tavakkol Afshari, R., Angoshtari, R. and Kalantari, S. (2011) Effects of light and different plant growth regulators on induction of callus growth in rapeseed (*Brassica napus L.*) genotypes. Journal of Plant Omics, 4(2): 60-67.

Tiwari, K.N., Sharma, N.C., Tiwari, V. and Singh, B.D. (2000) Micropropagation of *Centella asiatica* (L.), a valuable medicinal herb. Plant Cell Tiss Org., 63:179-185.

Tiwari, V., Singh, B.D. and Tiwari, K.N. (1998) Shoot regeneration and somatic embryogenesis from different explants of Brahmi (*Bacopa monniera* (L.) Wettst.). Plant Cell Rep., 17: 538-543.

Vasantharuba Seevaratnam, Banumathi, P., Premalatha, M.R., Sundaram, S.P. and Arumugam, T. (2012). Functional properties of *Centella asiatica* (L.): A review. Int J Pharm Pharm Sci., 4: 8-14.

Viegas, J., Da Rocha, M.T.R., Ferreira-Moura, I., Da Rosa, D.L., De Souza, J.A., Correa, M.G.S. and Da Silva, J.A.T. (2007). *Anthurium andreanum* (Linden ex andre) culture. *In vitro* and *Ex vitro*. Floriculture Ornamental Biotechnology, 1 (1): 61-65.

Vijayakumar, M., Vijayakumar, R. and Stephen, R. (2010). *In vitro* propagation of *Bacopa monnieri* L. - a multipurpose medicinal plant. I J of Sci and Tech., 3(7): 781-786.

Wang, P.J. and Charle, A. (1991). Micropropagation through meristem culture, In Biotechnology in agriculture and forestry. In: Bajaj YPS. (eds). Springer Verlag, New York; pp. 41-44.

Wernicke, W. and Mikovits, I. (1984). Developmental gradients in wheat leaves-response of leaf segments in different genotype cultured *in vitro*. J Plant Physiol., 115: 49- 58.

Yamin Bibi, Muhammad Zia, Sobia Nisa, Darima Habib, Abdul Waheed. and Fayyaz M Chaudhary. (2011). Regeneration of *Centella asiatica* plants from non- embryogenic cell lines and evaluation of antibacterial and antifungal properties of regenerated calli and plants. J Biol Engg., 5(13): 1-8.