Short Communication

Callus induction and *Agrobacterium tumefaciens* mediated transfer of hydroxy methyl glutaryl CoA reductase (*HMGR*) gene in *Centella asiatica* L.

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Abstract

Centella asiatica is an important medicinal plant, possessing a blend of compounds including triterpenes. Among the various secondary metabolites, triterpenes have remarkable pharmaceutical value due to their anti inflammatory, antitumour, neuroprotective, skin care and toning effects. But the quantity of triterpenes in Indian ecotypes is very low. In the present study, an attempt was made to transfer *HMGR* gene, encoding one of the key enzymes involved in triterpene biosynthesis, to *C. asiatica*. Callus cultures of *C. asiatica* were established in MS medium with Kn 4 mgl^{-1} and NAA 2 mgl^{-1} and the regeneration of callus was obtained in MS medium supplemented with Kn 4 mgl^{-1} and NAA 2 mgl^{-1}. *Agrobacterium tumefaciens* strain EHA 105 harbouring the plasmid pBE2113 containing *nptll* and *hmgr* gene was used for transformation of callus and the transformation was confirmed by PCR.

Keywords: *Centella asiatica*, *Agrobacterium tumefaciens*, Asiaticoside, *hmgr* gene, Plasmid pBE 2113.

Centella asiatica, commonly known as Asiatic Pennywort or Gotu Kola, is a herbaceous annual belonging to the family Apiaceae. It is a widely used medicinal herb due to its revitalizing function that strengthens nerves and improves memory. The whole plant is a cardio-depressant, hypotensive, and rejuvenating diuretic herb that clears toxins and digestive disorders, reduces inflammations and fevers, and improves healing and immunity. It is recommended for the treatment of various skin conditions like leprosy, eczema and psoriasis (Giardina et al., 1987), diseases of female genitourinary tract, for relieving anxiety and improving cognition (Gohil et al., 2010). *Centella asiatica* contains a variety of constituents of which the triterpenoids have attracted the most attention. The triterpene content varies with ecotypes, and in Indian ecotypes its content is very low. Conventional breeding methods for improving ecotype as well as cell/tissue culture and elicitation methods using enhancers for enhancing secondary metabolite production have their own limitations.

Metabolic pathway engineering is a comparatively new approach for the qualitative and quantitative improvement of the pharmacologically important metabolites in medicinal plants (Yun et al. 1992, Kim et al., 2005; Mishra et al., 2013). This study was undertaken to insert 3-hydroxy methyl glutaryl CoA reductase gene (*HMGR*), using *Agrobacterium tumefaciens*. 3-hydroxy- 3-methyl glutaryl coenzyme A reductase (HMGR) catalyses the irreversible conversion of 3-hydroxy- 3-methyl glutaryl coenzyme A to mevalonate, which is considered a key regulatory step controlling isoprenoid metabolism (Chappell et al., 1995).

For callus induction leaves and nodes collected from

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naturally grown healthy *Centella asiatica* plants were used. Explants were washed in laboline and surface sterilized using mercuric chloride (0.08%) for five minutes, washed using sterile water 3-4 times and inoculated in Murashige and Skoog (MS) medium containing different concentrations and combinations of 2,4-D, Kinetin (Kn), and NAA. Cultures were incubated at 25 ± 2^0C with 16 hour photoperiod and the relative humidity was maintained between 50 and 60 per cent.

There was considerable variation in the percentage of callus induction and time taken for callus induction in the different treatments tried (Table 1). Observations were recorded on the percentage establishment of explants and percentage of callusing. Highest percentage of callus induction (100% from nodal explant and 92.85% from leaf explant) was obtained in MS medium with Kn 4 mg l^{-1} and NAA 2 mg l^{-1} (Fig.1) within 23 and 25 days respectively. Although callus induction from node occurred in 22 days in C2, percentage induction was less (76.24). Minimum period for callus induction was observed in C3 medium (21 days) from leaf, but percentage induction was also less. Nodal explants produced more callus compared to leaf explants in all the treatments.

Patra et al. (1998) have reported the *in vitro* induction of callus from leaf explants of *C. asiatica* in MS medium supplemented with similar combination of growth regulators, i.e. 2 mg l^{-1} Kn and 4 mg l^{-1} NAA. The study on the influence of auxin and cytokinins on the production of callus in

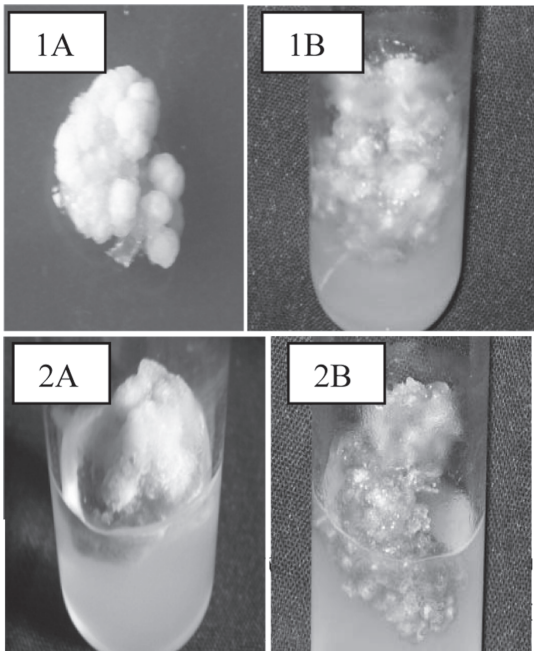


Figure 1. Callus induction from node (A) and leaf (B). (1): MS + Kn 4 mg l^{-1} and NAA 2 mg l^{-1} and (2): MS + NAA 1 mg l^{-1} and 2, 4-D 0.8 mg l^{-1}

C. asiatica by Rao et al. (1999) also support this result. According to them, kinetin supplementation (0.25 and 0.5 mg l^{-1}) along with auxin (2, 4-D, 2 mgl^{-1}) proved to be beneficial for the growth of callus and the best combination of growth regulators for maximum callus induction reported was 2 mg l^{-1} NAA along with + 0.5 mg l^{-1} Kn. Bibi et al. (2011) have reported that 6-benzyladenine either alone or in combination with 1-naphthalene acetic acid supplemented in MS medium at different concentrations produced good quality callus from leaf explants of *C. asiatica*.

Table 1. Effect of growth regulators on *in vitro* callus induction in *Centella asiatica*

Medium	Growth regulators (mg l^{-1})	Time taken for callus induction (days)		Callus induction (%)	
		Leaf	Node	Leaf	Node
C1	Kn 4.0 + NAA 2.0	25.00	23.00	92.85	100.00
C2	NAA 1.0 + 2,4-D 0.8	25.00	22.00	26.08	76.24
C3	2,4-D 2.0	21.00	24.00	66.07	80.35

Basal Medium: MS (full strength) + sucrose 30 g l^{-1} + agar 5 g l^{-1}

Table 2. Response of callus in different regeneration media

Regeneration medium	Composition (mg l^{-1})	Response of callus
RI	BA 3.0 + Kn 2.0	Callus turned green
R2	BA 3.0 + Kn 1.0	Callus turned green
R3	BA 3.0 + Kn 0.5	Callus turned green
R4	BA 3.0 + Kn 0.5 + IAA 1.0	Callus turned green
R5	BA 2.0 + Kn 1.0	Callus turned green
R6	BA 3.0 + Kn 2.0 + NAA 0.25 + ADS 20.0	Callus turned green
R7	Half MS + BA 4.0 + Kn 2.0 + NAA 0.25 + ADS 20.0	Callus turned green
R8	BA 4.0 + Kn 2.0 + NAA 0.25 + ADS 20.0	Shoot induction
R9	BA 4.0 + NAA 1.0	Callus turned green
R10	Half MS + BA 2.0 + 2,4-D 0.5	Callus turned green
R11	BA 4.0 + Kn 3.0	Callus turned green
R12	R6 + Proline 40.0	Callus turned green
R13	R6 + Proline 20.0	Callus turned green
R14	R6 + CH 20.0	Callus turned cream
R15	(R6 + CH 20.0) – ADS	Callus turned cream
R16	BA 8.0 + NAA 1.0	Callus turned green
R17	BA 6.0 + NAA 1.0	Callus turned green
R18	BA 4.0 + NAA 1.0	Callus turned green
R19	BA 2.0 + NAA 1.0	Callus turned green
R20	BA 8.0 + IAA 1.0	Callus turned cream
R21	BA 6.0 + IAA 1.0	Callus turned cream
R22	BA 4.0 + IAA 1.0	Callus turned cream
R23	BA 2.0 + IAA 1.0	Callus turned cream
R24	NAA 2.0 + Kn 4.0	Shoot induction
R25	NAA 1.0 + Kn 4.0	Callus turned green
R26	NAA 0.5 + Kn 4.0	Callus turned green
R27	NAA 0.25 + Kn 4.0	Callus turned green
R28	Kn 4.0	Callus turned green
R29	R26 + Proline 20.0	Callus turned pale green
R30	R27 + Proline 20.0	Callus turned pale green
R31	R28 + Proline 20.0	Callus turned pale green
R32	R29 + Proline 20.0	Callus turned pale green
R33	R30 + Proline 20.0	Callus turned pale green
R34	BAP 3.0 + NAA 1.0	Callus turned dark green
R35	BAP 3.0 + NAA 0.5	Callus turned dark green
R36	BAP 3.0 + NAA 0.25	Callus turned dark green
R37	BA 1.0 + Kn 3.0 + NAA 0.25 + ADS 20.0	Callus turned green
R38	BA 2.0 + Kn 2.0 + NAA 0.25 + ADS 20.0	Callus turned green
R39	BA 5.0 + Kn 2.0 + NAA 0.25 + ADS 20.0	Callus turned green
R40	BA 6.0 + Kn 1.0 + NAA 0.25 + ADS 20.0	Callus turned green

Basal Medium: MS (full strength) + sucrose 30 g l^{-1} + agar 5 g l^{-1}

For regeneration, the calli were transferred to MS medium with varying concentrations and combinations of Kn, NAA, BA, and adenine sulphate (ADS) (Table 2). Shoot regeneration was obtained only in R8 and R24 (MS medium supplemented with NAA 2 mg l^{-1} and Kn 4 mg l^{-1} and MS+NAA 2.0 mg l^{-1} + Kn 4.0 mg l^{-1}) (Fig.1). Percentage shoot regeneration was 0.052 in R24 and time taken for regeneration was 24 days, while in R8 it was only 0.018 per cent in 32 days. There was proliferation of callus in the other treatments but the callus remained green showing the regeneration potential, but shoot induction was not observed (Treatments except R8 and R24). Patra et al. (1998) have successfully regenerated callus derived from stem and leaf explants of *C. asiatica* on the same medium; reproducibility of the result was very less. Multiple shoot regeneration from *C. asiatica* callus using Indole-3-butyric acid was reported by Singh et al. (2010). In their study callus production from node was maximum on basal medium supplemented with 3 mg l^{-1} IBA + 3 mg l^{-1} Kinetin and the maximum shoots were obtained on MS + 1 mg l^{-1} IBA + 3 mg l^{-1} Kinetin media, but regeneration percentage was less.

As a pre requisite for transformation, sensitivity of *Centella* cultures to antibiotics Kanamycin and Cefotaxime was evaluated to utilize it as a marker system for selection of transformed callus (Cucu et al., 2002). *Agrobacterium tumefaciens* strain

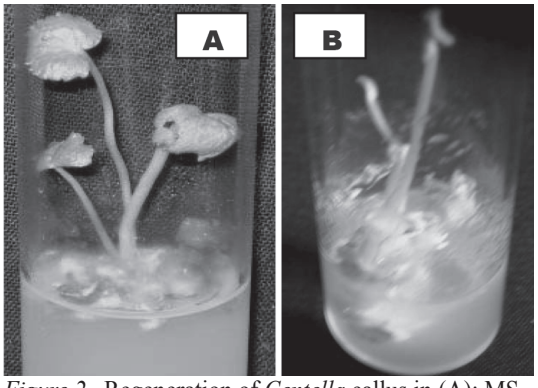


Figure 2. Regeneration of *Centella* callus in (A): MS + NAA 2 mg l^{-1} and Kn 4 mgl^{-1} and (B): MS + Kn 2 mg l^{-1}, BA 4 mg l^{-1}, NAA 0.25 $mgl^{-1}$and ADS 20 mg l^{-1}

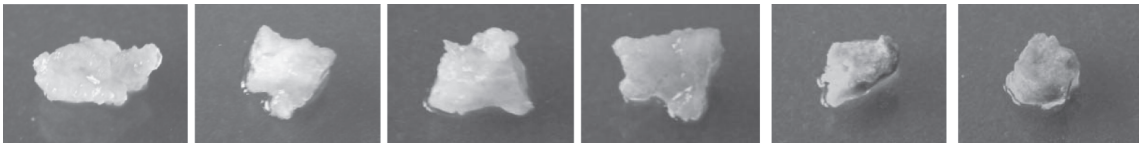
EHA105 with the plasmid pBE2113 containing the *HMGR* and the *npt II* (*Neomycin phosphotransferase II*) genes under the control of CaMV 35S promoter was used for transformation. The sensitivity of callus to different doses of Kanamycin (5-350 mg l^{l}) and Cefotaxime (5 - 100 mg l^{-1}) was recorded (Fig.3, 4).

The following scoring method was used for evaluation.

Score	Culture response
'++++'	Fully green
'+++'	Partially discoloured
'++'	Bleached tissues
'+'	Tissues turning brown and dead

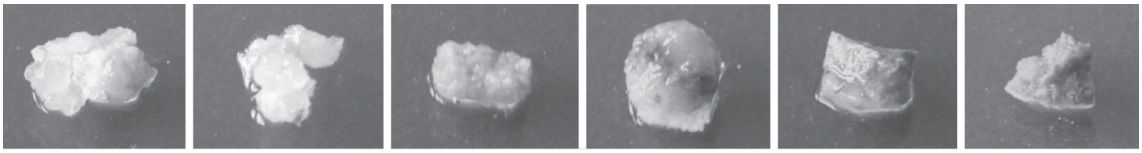
Based on the response of the tissues, Kanamycin at concentrations of 300 mg l^{-1} was used for the selection of the transformants. Cefotaxime was found to be toxic at 100 mg l^{-1}. Hence for the elimination of *Agrobacterium* after co-cultivation, 50 mg l^{-1} cefotaxime was used. Cefotaxime has been used successfully for the elimination of *Agrobacterium* in transformation in a number of crops. Vergauwe et al. (1996) during the transformation of *Artemisia annua* could effectively control *Agrobacterium* by using cefotaxime at 50 mg l^{-1}. It was successfully used at a strength of 200 mg l^{-1} in eliminating *Agrobacterium* from the inoculated leaf explant during transformation in *Datura* (Curtis et al., 1999). In *Atropa belladona*, cefotaxime 500 mg l^{-1} was used (Negoianu et al., 2002).

The *Agrobacterium was* grown on AB minimal medium and Luria-Bertani (LB) medium supplemented with Kanamycin 50 mg l^{-1} and Rifampicin 20 mg l^{-1}. *A. tumefaciens* EHA 105 was grown overnight on petriplates containing AB minimal medium with Kanamycin 50 mg l^{-1} at 28^{0}C. For co-cultivation, *Agrobacterium* suspension was prepared in AB broth with Kanamycin 50 mg l^{-1}. It was kept in a shaker overnight at 28^{0}C at 10 rpm. The culture was centrifuged at 5000 rpm at 4^{0}C for 5 min. The pellet obtained was



5 mg l^{-1} 50 mg l^{-1} 100 mg l^{-1} 150 mg l^{-1} 250 mg l^{-1} 350 mg l^{-1}

Figure 3. Sensitivity of *Centella* callus to different doses of kanamycin



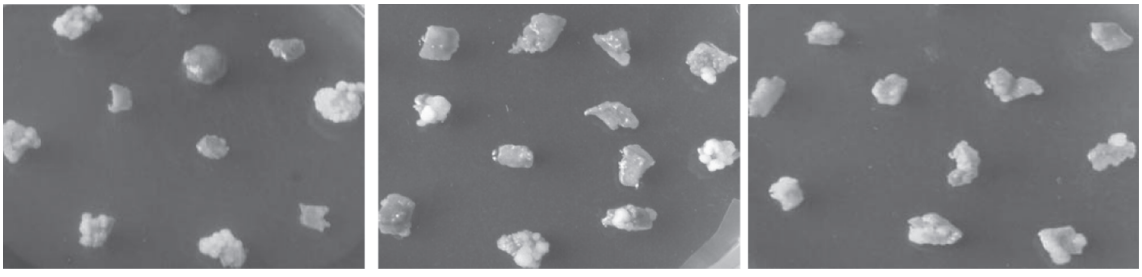
Control 5 mg l^{-1} 25 mg l^{-1} 50 mg l^{-1} 75 mg l^{-1} 100 mg l^{-1}

Figure 4. Sensitivity of *Centella* callus to different doses of cefotaxime.

resuspended in 1 ml of half strength MS broth. Before transformation, calli were pre-cultured on MS medium containing 4 mg l^{-1} Kn and 2 mg l^{-1} NAA for 15 days to maintain cells in active cell division stage. An infection time of 20 minutes was given in half strength MS broth. Co-cultivation was done in suspension containing 100 mM acetosyringone for four days in dark. The explants were transferred to solidified half MS medium and kept in dark for four days at 28^0C (Rathnavijaya et al., 2011). Elimination of bacteria from the transformed tissues was done in half MS medium supplemented with Cefotaxime, 50 mg l^{-1}.

In *Bacopa monniera,* Nisha et al. (2003) reported that the incubation of leaf segments in bacterial suspension EHA105 (pBE2113) for 15 minutes and then co-cultivation for 48 hours, resulted in efficient transformation. Callus tissues were wounded with sterile disposable syringe to facilitate efficient infection of explants with *Agrobacterium tumefaciens*. Wounded cells release polyphenolic compounds like acetosyringone, which activate the *Agrobacterium vir* genes (Zambryski, 1992). Krishnan (2006) observed that the transformation efficiency in *C. asiatica* could be increased by adding acetosyringone (100 µM) to infection and co-cultivation medium. Hence acetosyringone (100 µM) was added to the infection and co-cultivation medium to increase the transformation efficiency in the present study.

The transformed tissues were selected on MS medium containing Kanamycin (300 mg l^{-1}) and Cefotaxime (50 mg l^{-1}). The bacterial strain EHA105 harbouring pBE2113 survived in the presence of Kanamycin up to a concentration of 300 mg l^{-1}. The tissues were maintained by sub-culturing once in

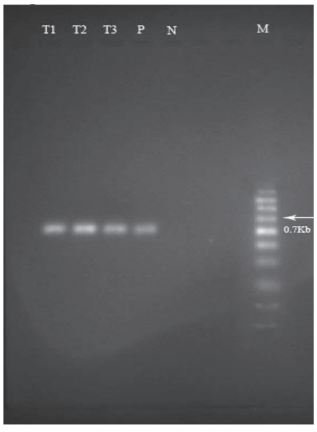


200 mg l^{-1} (18.75%) 250 mg l^{-1} (19.35%) 300 mg l^{-1} (13.63%)

Figure 5. Survival of tissues in selection media containing kanamycin

seven days in the same medium. After four rounds of sub-culture, the transformed and non-transformed tissues were scored based on their response in the medium. A survival percentage of 13.63 was observed in the presence of 300 mg l^{-1} Kanamycin (Fig.5). Nisha et al. (2003) observed that the selection medium comprised of shoot regeneration medium supplemented with 15 mg l^{-1} Kanamycin in Brahmi. Sugimura et al. (2005) obtained putative transformants in patchouli by selection on medium containing 100 mg l^{-1} Kanamycin.

The transgenes were confirmed by PCR using *nptII* gene specific primers. Genomic DNA was isolated from the transformed tissues using modified protocol of Murray and Thompson (1980) and quantified using UV-Visible spectrophotometer (Spectronic Genesys 5). The PCR analysis was performed with the specific primers for *nptII* gene. Plasmid DNA pBE2113 (Sambrook et al., 1989) was kept as positive control. Agarose gel electrophoresis was carried out for the analysis of PCR products and documented using a gel documenting system (BIO-RAD). All the tissues selected on kanamycin yielded an appreciable quantity of the product of size 700 bp (Fig.6). The PCR product yielded by positive control was of the same size. PCR amplification of the selectable maker genes have been carried out for the confirmation of transgenes by several workers (Dronne et al., 1999; Sales et al., 2003; Nisha et al. 2003). Li et al. (2014) confirmed the transformation of *Digitalis purpurea* L. by conducting PCR of *nptII* and *gus* genes. PCR analysis of transgenic *Withania coagulans* plants showed the presence of *gus A* and *npt II* genes confirming the transgenic event.



Lane 1- 3: npt II gene amplified from transformed plants
Lane 4- Positive control
Lane 5- Non-transformed control
Lane 6- 100bp DNA marker

Figure 6. Confirmation of transgenes by PCR using *nptII* gene specific primers

Reports on *A. tumefaciens* mediated genetic transformation in *C. asiatica* are scarce. Gandi et al. (2012) successfully induced hairy roots in *Centella* using *Agrobacterium rhizogenes*. In another study conducted by Kim et al. (2010) in *Centella*, upregulation of phytosterol and triterpene biosynthesis could be obtained in hairy roots. Successful transformation in *C. asiatica* in the present study suggests the possibility of metabolic engineering for manipulating the yield of triterpenes in this medicinal plant.

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