

Hairy root cultures of *Chonemorpha fragrans* (Moon) Alston.: A potential plant for camptothecin production

Pradnya Kedari and Nutan P Malpathak*

Department of Botany, University of Pune, Pune 411 007, India

Received 10 December 2012; revised 4 April 2013; accepted 10 June 2013

An efficient transformation system has been developed in *Chonemorpha fragrans* (Moon) Alston., an important medicinal plant. It has been reported from our laboratory that this rare and endangered liana contains camptothecin (CPT), a well known anticancer drug used in the treatments of various types of cancer after derivatization. *In vitro* leaf and callus explants were used for infection with three strains of *Agrobacterium rhizogenes*. Explants infected with *A. rhizogenes* strain A4 showed root initials with an optimal transformation frequency (up to 45%). The confirmation of transformation was done by PCR using *rolB* specific primers. This is the first report regarding hairy root induction in the genus *Chonemorpha*.

Keywords: *Agrobacterium rhizogenes*, Apocynaceae, camptothecin, *Chonemorpha fragrans*

Introduction

Chonemorpha fragrans, (Moon) Alston., previously named *C. macrophylla*, is a woody liana belonging to family Apocynaceae. All the plant parts are widely used in traditional systems of medicines for the treatment of fever and stomach disorder¹. Recent studies have shown that alcoholic extract of *C. fragrans* contain antidiabetic activity² and also muscle relaxant properties³. The plant is a source of wide range of metabolites like camptothecin, chonemorphine and funtumafrine. For its slow growth, the plant takes several years to mature. Therefore, an alternate source like *in vitro* propagation of the plant and increase in the metabolites by developing strategies like transformation are necessary to prevent overexploitation and for continuous production of secondary metabolites.

Camptothecin (CPT) is a plant derived monoterpene alkaloid, first reported from *Camptotheca acuminata*. It is currently used clinically in the treatment of various types of cancers⁴. It targets topoisomerase I and inhibits its activity by binding to the topoisomerase I-DNA binary complex, thereby inducing single strand breaks of cellular DNA. It is known that very few sources have shown the presence of CPT, especially plants belonging to the families Apocynaceae and Icacenaceae⁵. This compound

exhibits a broad spectrum of antitumor activity and used in the treatment of lung, uterine, cervical and ovarian cancers⁶. The demand for CPT and its derivatives is very high⁷. Currently, the global demand of CPT is fulfilled mainly by extraction from *C. acuminata*⁸ and *Nothapodytes foetida*⁹ using bark and roots. CPT was detected in the ethanolic extracts of bark and callus cultures of *C. fragrans* from our laboratory¹. These studies have revealed that *C. fragrans* plant could be a new potential source for production of CPT. Since relatively low levels of CPT were isolated from natural sources, plant cultures have been extensively explored for CPT production. Plants showing presence of CPT are often overexploited and have become endangered. This necessitates the need for alternate candidates as well as a system that can provide continuous production of CPT¹⁰. In this paper, *Agrobacterium rhizogenes* strains ATCC 15834, A4 and NCIM 5140 were assessed for their potential to induce hairy roots *in vitro* in entire leaves, stem segments and callus tissues of *C. fragrans*.

Materials and Methods

Shoot cuttings of *C. fragrans* were collected from Kerala and maintained in the Botanic Garden, Department of Botany, University of Pune, Pune. From them, leaf and nodal segments were used to establish *in vitro* shoot cultures. The explants were surface sterilized with 0.1% Bavistin for 30 min, followed by 75% alcohol for 30 sec and then

*Author for correspondence:

Tel: +91-20-25601438/25601439; Fax: +91-20-25690498

E-mail: mpathak@unipune.ac.in

rinsed with sterile distilled water twice. The explants were further sterilized with 0.1% (w/v) HgCl_2 for 1 min and rinsed 4-5 times with sterile distilled water.

In all of the experiments, B5 medium¹¹ supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar was used. Prior to autoclaving at 121°C for 15 min, pH of the medium was adjusted to 5.5. For shoot development, surface sterilized nodal segments were cultured on B5 medium supplemented with 8.7 μM 1,6-benzylaminopurine (BAP) for 4 wk. While surface sterilized leaves and internodes were cultured on B5 medium supplemented with 10 μM BAP+3 μM NAA for the development of callus. These cultures were maintained at 26±2°C and 50-60% relative humidity under a 16/8 h (light/dark) photoperiod with light provided by cool-white fluorescent tubes at an intensity of 48 $\text{mol m}^{-2} \text{s}^{-1}$. Entire leaves (1-2 cm) and stem segments (nodes or internodes, 2-3 cm) excised from 4 to 6-wk-old shoots and 3-wk-old callus were used for transformation. Explants pre-cultured for 8 d on B5 medium were used for further experiments. Three wild-type agropine strains of *A. rhizogenes*: A4 (pRiA4), NICM 5140 and ATCC 15834, were used for transformation. Single colony was inoculated in 5 mL YMB medium (pH 7.0) supplemented with 50 mg/L rifampicin. After 12-16 h of growth, 1 mL of this culture was inoculated in 100 mL YMB supplemented with 50 mg/L rifampicin. The cultures were placed on shaker with 180 rpm for 14 h at 26°C. To these bacterial cultures acetosyringone (100 μM) was added and the cultures were kept for further growth till 0.55 optical density (OD) was achieved at 600 nm.

Bacterial culture with OD ~0.55 (wavelength 600 nm) was centrifuged at 6,000 rpm for 8 min at 26-28°C temperature. Supernatant was discarded and bacterial pellet was resuspended in liquid B5 medium so as to adjust OD 0.55 at the wavelength 600 nm. *In vitro* cultured leaves, nodes, internodes (8 d) and callus were used for transformation experiment. Explants were wounded using hypodermic needle and placed in sterile conical flask containing above mentioned bacterial suspension with 100 μM acetosyringone. The flask was vacuum-infiltrated for 90 min (ChemHEMKER 300 Vacuum Pump with max pressure-600 mm Hg) and then kept on shaker at 180 rpm for 60 min. Explants were then washed once with sterile distilled water. They were blotted well using sterile blotting

papers and then cultured in Petri plates containing solid B5 medium supplemented with 50 μM acetosyringone. These were sub-cultured at 4 d interval and plates were incubated in dark at 26±2°C for 7 d. On 8th d, all the explants were washed with sterile distilled water, followed by 500 mg/L cefotaxime. Explants were blotted well and placed on B5 medium supplemented with 400 mg/L cefotaxime. Dark condition was maintained throughout the experiment. The experiment was repeated twice. After every 4 d, the explants were regularly transferred onto fresh medium. The cefotaxime concentration was gradually reduced if no bacterial growth was seen around explants after 4th d.

Root initials were seen after 4-5 wk. Each root formed at the wound sites of the infected explants was treated as a separate clone. These were later excised and cultured under the dark conditions in Petri plates containing B5 medium supplemented with 300 mg/L cefotaxime to establish transformed root cultures. Each excised primary root was propagated as a separate clone and sub-cultured at 4 d interval. The amount of cefotaxime in the culture medium was gradually reduced to 50 mg/L.

DNA from all putative transformants and non-transformed (control) roots was extracted using Quigene DNA extraction kit. The plasmid isolated from A4 was used as a positive control. The fragments of *rolB* gene was amplified by using forward primer 5'-ATGGATCCCAAATTGCTATTCCTTCCACGA-3' and reverse primer 3'-TTAGGCTTCTTTCTT CAGGTTTACTGCAGC-5'. The PCR was performed in 25 μL reaction volume containing 50 ng DNA and 25 μmol of each primer, 0.4 mM mixture of dNTP, 2.5 μL of 10× Taq DNA polymerase buffer containing 2.5 mM MgCl_2 and 2 U Taq DNA polymerase (Promega, Madison, Wis.). The PCR reaction was carried out at 94°C for 5 min, followed by 30 cycles of amplifications (94°C for 1 min, 56°C for 30 sec, 72°C for 1 min) and final extension at 72°C for 4 min. The final product was analyzed on 1% of (w/v) agarose gel.

Methanolic extracts of transformed as well as non-transformed roots were prepared and were subjected to HPTLC analysis. Chromatographic analyses of the extracts were performed on TLC silica gel 60 F254 plates (10×10 cm^2 ; Merck, Darmstadt, Germany). Aliquots (10 μL) of each extract were applied on the plates as bands using a Linomat V automatic sample applicator (Camag, Muttenz, Switzerland). Each band

(5 mm-wide) was separated from its neighbouring bands by a distance of 11.4 mm. Plates were developed in a glass twin trough chamber (20×10×4 cm³) (Camag), previously saturated (20 min) using ethyl acetate:chloroform (1:1 v/v) as the mobile phase; the development length was 80 mm and the development time approx 10 min. After development, plates were dried for 10 min. Plates containing methanol extracts and standard CPT (Sigma) were scanned using Camag TLC scanner operated by winCATS software (Camag) running on a computer under the following conditions: scanning mode, re-emission-fluorescence (mercury lamp); measurement wavelength, 366 nm; slit dimension 4.00×0.03 mm².

Results and Discussion

So far genetic transformation has not been reported in the genus *Chonemorpha* and thus efforts have been made to establish a protocol for genetic transformation by using various explants and bacterial strains. Among the various explants and bacterial strains used, wounded leaves and callus masses were found suitable to infection by strain A4, with more than 45% of hairy root induction, as compared to nodes and internodes. In contrast, other strains NICM 5140 and ATCC15834 did not show any infection to the exposed tissues. Explant and strain specificity observed in the present study agrees with the hypothesis that ability for infection of different *A. rhizogenes* strains in a given species is variable^{13,14}. As the plant is a rich source of alkaloids, injuries led to decaying of the plant material because of leaching out of the metabolites. To overcome this problem, methods like sonication, vacuum infiltration, pricking of explants with hypodermic needles and injecting the explants with bacterial suspension were tried to injure the plants for the entry of *Agrobacterium*. Further, bacterial suspension with an OD of 0.5, 0.55, 0.6, 0.65 at 600 nm were considered during initial experiments (Table 1).

The problem of excessive bacterial growth as well as explant recovery was solved by changing the method of injury to the plant prior to infection. Variations used for preculture of explants, strains used, infection time, OD, co-cultivation period have been mentioned in Table 1. Various combinations of above mentioned parameters were tried, out of which pricking of explants and vacuum infiltration proved to be more effective. All these combinations were repeated for bacterial

suspension with OD ranging from 0.5 to 0.65. The survival rate of the infected explants was increased by washing the explants with distilled water after co-cultivation. Three different strains of *A. rhizogenes* were evaluated for their ability to induce the formation of hairy roots on *C. fragrans* explants. The sensitivity of *C. fragrans* explants to cefotaxime was tested using different concentrations. The plant cells were unaffected at 400 mg/L cefotaxime, while the antibiotic was effective in eliminating *Agrobacterium* in hairy root cultures at a concentration of 300 mg/L.

After 7 d of co-cultivation with the *A. rhizogenes* strains, explants were transferred to B5 hormone-free medium supplemented with 300 mg/L cefotaxime. Root initials were seen after 4-5 wk of infection (Fig. 1). The roots were branched, showing negatively geotropic growth. After attaining 2-3 cm length, they started desiccating from the tip region. Phytohormones strongly influence secondary metabolism, affecting growth rate of culture, its organization and alkaloid production. There are reports of few hairy roots, which require external auxin supply for proper elongation¹⁵. Elongation of

Table 1—To establish transformation in *C. fragrans* following parameters were considered

	Variations used
Explants used	Nodes, Internodes, Leaves, Callus
Preculture of explants (d)	0, 1, 2, 3, 4, 5, 6, 7, 8 , 9, 10
Strains used	ATCC 15834, NICM 5140, A4
Optical density (OD)	0.5, 0.55 , 0.6, 0.65
Co-cultivation period (d)	1, 2, 3, 4, 5, 6, 7, 8
<i>Methods of injury</i>	<i>Infection time</i>
Pricking	-
Pricking+Shaker (min)	20-60 min
Pricking+Sonication (sec)	10, 15, 20, 30, 60, 90, 120, 150, 180, 240
Pricking+Vaccum infiltration (min)	5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90
Pricking+Vaccum infiltration (min)+Shaker (min)	-(5-90)+(20-60)
Sonication (sec)	10, 15, 20, 30, 60, 90, 120, 150, 180, 240
Sonication (sec)+Shaker (min)	(10-240)+(20-60)
Sonication (sec)+Vaccum infiltration (min)	(10-240)+(5-90)
Sonication (sec)+Vaccum infiltration (min)+Shaker (min)	(10-240)+(5-90)+(20-60)

Treatments giving positive result are shown in bold letters

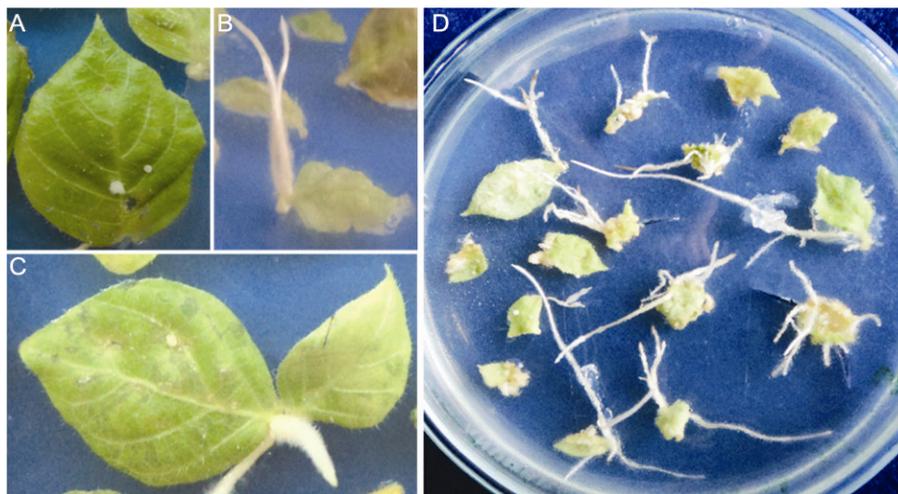


Fig. 1 (A-D)—Hairy root induction in *C. fragrans*: A. Initiation of root; B & C. A4 induced hairy roots of *C. fragrans*; D. Elongation of roots.

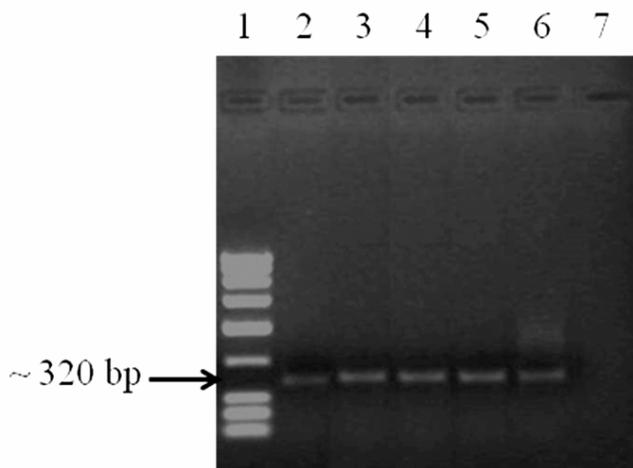


Fig 2—PCR amplified DNA fragments of *rolB* gene: Lane 1, 5 Kb ladder; Lanes 2-5, PCR product of DNA from *C. fragrans* transgenic hairy roots; Lane 6, PCR product of DNA from the plasmid as a positive control; Lane 7, PCR product of DNA from the intact roots as a negative control.

in vitro roots by addition of 3 μM NAA was reported in our laboratory¹⁶. Hence, 3 μM NAA was added to culture medium to prevent desiccation and promote growth of hairy roots. This resulted in proper elongation of roots without drying, confirming that external auxin supply is needed for sustainable hairy root growth in case of *C. fragrans*.

PCR amplification using *rolB* primers showed the amplification of around 320 bp fragment of DNA. All the putative transformed roots showed amplification for the *rolB* genes. The PCR products were of the expected size (about 320 bp) and identical with those of the positive control. The non-transformed roots (negative control) did not

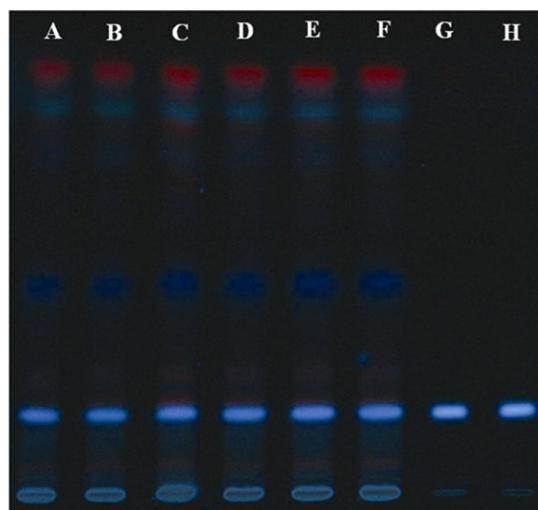


Fig 3—HPTLC chromatogram of hairy roots: A & B, Clone 1; C & D, Clone 2; E & F, Non-transformant; G & H, Camptothecin (Standard)

show amplification for the *rolB* genes (Fig. 2). In the work presented here, a protocol has been established for obtaining hairy roots from *C. fragrans* with a transformation frequency of $45.43 \pm 1.75\%$, producing 2.76 ± 0.035 hairy root initials per explant. We have also assessed the methanolic extracts of hairy roots for CPT contents (Fig. 3). Clones with fastest growth rate accumulated 0.024-0.030% DW of CPT, which was comparable with the normal roots showing accumulation of 0.033% DW of CPT.

In conclusion, we have established the hairy root culture of *C. fragrans* with transformation frequency of 45%. Thus the present protocol could provide a sustainable alternative source of CPT for both commercial and scientific applications.

Acknowledgement

Authors would like to acknowledge the University Grants Commission, Government of India, New Delhi for financial assistance. Authors would also like to thank Dr Sumita Zha, Department of Botany, University of Calcutta, Kolkata for providing *Agrobacterium* strain (A4) and Dr Sr Tessy Joseph, Department of Botany, Nirmala College, Muvattupuzha, Kerala for providing plant material.

References

- 1 Kulkarni A V, Patwardhan A A, Lele U & Malpathak N P, Production of camptothecin in cultures of *Chonemorpha grandiflora*, *Pharmacognosy Res*, 2 (2010) 296-299.
- 2 Shende V S, Sawant V A, Turuskar A O, Chatap V K & Vijaya C, Evaluation of hypoglycemic and antihyperglycemic effects of alcoholic extract of *Chonemorpha fragrans* root in normal and alloxan induced diabetic rats, *Pharmacognosy Magazine*, 5 (2009) 36-41.
- 3 Roy R K, Ray N M & Das A K, Skeletal muscle relaxant effect of *Chonemorpha macrophylla* in experimental animals, *Indian J Pharmacol*, 37 (2005) 116-119.
- 4 Wall M E, Wani M C, Cook C E, Palmer K H, McPhail A T *et al*, Plant antitumor agents. I. The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumor inhibitor from *Camptotheca acuminata*, *J Am Chem Soc*, 88 (1966) 3888-3890.
- 5 Potterat O & Hamburger M, Drug discovery and development with plant-derived compounds, in *Progress in drug research: Drug discovery and development with plant-derived compounds*, vol 65, edited by F Petersen & R Amstutz (Birkhäuser Verlag AG, Basel, Switzerland) 2008, 46-118.
- 6 Lorence A & Nessler C L, Camptothecin, over four decades of surprising findings, *Phytochemistry*, 65 (2004) 2735-2749.
- 7 Watase I, Sudo H, Yamazaki M & Saito K, Regeneration of transformed *Ophiorrhiza pumila* plants producing camptothecin, *Plant Biotechnol*, 21 (2004) 337-342.
- 8 Vincent R M, López-Meyer M, McKnight T D & Nessler C L, Sustained harvest of camptothecin from the leaves of *Camptotheca acuminata*, *J Nat Prod*, 60 (1997) 618-619.
- 9 Aiyama R, Hisako N, Nokata K, Shinohara C & Sawada S, A camptothecin derivative from *Nothapodytes nimmoniana*, *Phytochemistry*, 27 (1988) 3663-3664.
- 10 Gharpure G, Chavan B, Lele U, Hastak A, Bhawe A *et al*, Camptothecin accumulation in *Ophiorrhiza rugosa* var. *prostrata* from northern Western Ghats, *Curr Sci*, 98 (2010) 302-304.
- 11 Gamborg O L, Miller R A & Ojima K, Nutrient requirements of suspension cultures of soybean root cells, *Exp Cell Res*, 50 (1968) 151-158.
- 12 Triplett B, Moss S, Bland J & Dowd M, Induction of hairy root cultures from *Gossypium hirsutum* and *Gossypium barbadense* to produce gossypol and related compounds, *In Vitro Cell Dev Biol-Plant*, 44 (2008) 508-517.
- 13 Maldonado-Mendoza I E, Ayora-Talavera T R & Loyola-Vergas V M, Tropane alkaloid production in *Datura stramonium* root cultures, *In Vitro Cell Dev Biol*, 28 (1992) 67-72.
- 14 Tepfer D, Genetic transformation using *Agrobacterium rhizogenes*, *Physiol Plant*, 79 (1990) 140-146.
- 15 Alpizar E, Dechamp F, Lapeyre-Montes F, Guilhaumon C, Bertrand B *et al*, *Agrobacterium rhizogenes*-transformed roots of coffee (*Coffea arabica*): Conditions for long-term proliferation, and morphological and molecular characterization, *Ann Bot*, 101 (2008) 929-940.
- 16 Kulkarni A V & Malpathak N P, Micropropagation of *Chonemorpha fragrans* (Moon) Alston.: An endangered medicinal plant, *Indian Drugs*, 43 (2006) 1001-1003.