Research paper

Establishment of Hairy Root Cultures of *Nothapodytes nimmoniana* to Produce Camptothecin

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【Summary】

Nothapodytes nimmoniana hairy roots were induced by infecting stem and leaf segments with 3 wild-type *Agrobacterium rhizogenes* strains, AR281, AR1600, and ATCC15834. Results showed great variations in the transformation efficiencies among different *A. rhizogenes* strains and *N. nimmoniana* clones in vitro. Strain ATCC15834 had the highest transformation ability. Among all 10 clones, 7 clones were infected, and hairy roots were successfully induced. These hairy roots grew vigorously and were highly branched on phytohormone-free solid medium. Three fast-growing hairy root lines, for which integration of the *rol* B gene into the root genome was verified using PCR and Southern blotting, were used for the growth and alkaloid production tests. After being cultured for 40 d, the growth index of hairy roots was 2.6~3 and camptothecin (CPT) contents were 0.0537~0.1555% dry weight (DW). When cultured in liquid medium, hairy roots grew up to 1.8-times faster than they did in solid medium. After 30 d in liquid culture, the CPT concentration was 9.8 mg/L. Approximately 93.9% (9.2 mg/L) of the total CPT produced was excreted into the culture medium. With continual harvesting of CPT from the liquid medium, this system will benefit the development of large-scale bioreactor culture in the future.

Key words: *Nothapodytes nimmoniana*, *Agrobacterium rhizogenes*, hairy roots, camptothecin.

Chang SH, Chen FH, Tsay JY, Chen J, Huang CY, Lu WL, Ho CK. 2014. Establishment of hairy root cultures of *Nothapodytes nimmoniana* to produce camptothecin. Taiwan J For Sci 29(3):193-204.

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Received March 2014, Accepted July 2014. 2014年3月送審 2014年7月通過。

研究報告

建立青脆枝毛狀根培養以生產喜樹鹼

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摘 要

本研究利用農桿叢根菌AR281、AR1600與ATCC15834建立青脆枝的毛狀根培養系統。農桿菌的轉殖率在不同菌種與單株間有很大的差異，以ATCC15834轉殖率最高，在接種的10個營養系中，有7個營養系可成功誘導毛狀根，以H2營養系的莖段轉殖率在3個菌種都最高，將毛狀根培養於不含有植物生長調節劑的MS培養基中生長快速且有很高的分枝性，具有毛狀根的特性。選3個來自G1-4或H2接種ATCC15834產生之生長快速毛狀根進行鑑定與培養，以PCR與南方雜交法確認農桿叢根菌的rol B基因已轉移至毛狀根。將3個毛狀根在MS固體培養基培養40天，growth index為2.6~3.0，喜樹鹼含量為0.0537~0.1555% (乾重)。將毛狀根移入液體培養基中培養，其生長比固體培養增加1.8倍，培養30天後，喜樹鹼產量為9.8 mg/L，其中93.9% (9.2 mg/L)會從毛狀根系釋出於培養液中，因此可不斷收穫培養液提煉喜樹鹼，對未來利用生物反應器培養是一項有利的生產模式。

關鍵詞：青脆枝、農桿叢根菌、毛狀根、喜樹鹼。

張淑華、陳芬蕙、蔡錦瑩、陳媜、黃芷雲、呂汶玲、何政坤。2014。建立青脆枝毛狀根培養以生產喜樹鹼。台灣林業科學29(3):193-204。

INTRODUCTION

Nothapodytes nimmoniana (Graham) Mablerley, a member of the Icacinaceae family, is a small evergreen tree widely distributed in southern India, Sri Lanka, Cambodia, Okinawa (Japan), and southern China, and Taiwan. In Taiwan, it is only distributed on Green and Orchid Islands (Chang 1993). *Nothapodytes nimmoniana* was reported to contain camptothecin (CPT) (Gvindachari and Viswanathan 1972, Wu et al. 1995).

CPT, a specific inhibitor of DNA topoisomerase I, can cause cell death by effectively inhibiting the DNA replication process. In 1966, Wall et al. successfully isolated CPT from the wood of the *Camptotheca acuminata*. In 1971~1972, CPT was first proven to be effective at curing digestive system tumors and melanomas. Further clinical trials with CPT were abandoned during the 1970s due to its severe side effects. Since the early 1990s, 2 semisynthetic CPT derivatives, i.e., irinotecan and topothecan, with decreased cytotoxicity, better anticancer activities, and water solubility were approved and are now used in cancer chemotherapy including for colorectal, ovarian, lung, stomach, and small cell lung cancers, as well as in inhibiting HIV replication (Priel et al. 1991). Currently, those derivatives are synthesized from natural CPT obtained by extraction from cultivated *C. acuminata* and *N. nimmoniana*, although CPT is also found in several other plant species, e.g., *Ophiorrhiza* spp., *Ervatamia heyneana*, and *Chonemorpha* spp. (Gunasekera et al. 1980, Saito et al. 2001, Kulkarni et al. 2010, Kedari and Malpathak 2013). The amount of CTP used for medicine is estimated to be at least 200 kg annually.

To establish a sustainable CPT production system, several attempts have been made to produce CPT by callus and cell cultures, mostly with *C. acuminata* and *N. nimmoniana*. The CPT production ability of *N. nimmoniana*, however, has generally not been acceptable, as the contents of CPT were only 1/100~1/1000 of those from soil-grown plants (Roja and Heble 1994, Ciddi and Shuler 2000. Fulzele et al. 2003). Only 2 studies reported they produced comparatively adequate amounts of CPT, i.e., Fulzele et al. (2001) from cell culture (35 mg/l) and Thengane et al. (2003) from callus culture (1.3% dry weight, DW). In the case of *C. acuminata*, CPT produced from callus and cell cultures was 0.0002~0.2% DW (Wiedenfeld et al. 1997, Chang et al. 2006), which was also lower than the whole plant which contained 0.0.011~0.5% DW (López-Meyer et al. 1994, Wiedenfeld et al. 1997). Moreover, only 16.7% of calluses continuously produce CPT after being cultivated on medium with plant hormones for 3 yr (Chang et al. 2006). Calluses of *Ophiorrhiza pumila* and *Chonemorpha grandiflora* contained undetectable or very low amounts of CPT (Kitajima et al. 1998, Sudo et al. 2002, Kulkarni et al. 2010).

Roots are another part of higher plants that may contain or synthesize chemicals for potential medical use. In *N. nimmoniana*, roots contain more CPT than other parts of the plant (Padmanabha et al. 2006). Nonetheless, in an untransformed root culture system of *N. nimmoniana* established by Fulzele et al. (2002), concentrations of CPT (0.00017% DW) and 9-methoxy CPT (0.000058% DW) were about 12 times less than those of normal roots of intact plants.

Hairy roots and crown galls, induced by transformation with *Agrobacterium rhizogenes*, were found to be a potentially favorable system of producing high-value pharmaceuticals because of their stable and high productivity in hormone-free culture conditions with growth rates comparable to those of the fastest growing cell suspension culture (Hu and Du 2006). It was reported that hairy roots of several *Ophiorrhiza* spp. can produce CPT (Saito et al. 2001, Asano et al. 2004, Lorence et al. 2004). Moreover, hairy roots of *O. pumila* (Sudo et al. 2002) can be successfully cultivated on a large scale in a bioreactor and maintain CPT production even after 5 yr. On the other hand, although crown gall culture of *C. acuminata* is able to produce CPT (0~0.039%), large cell aggregates occur in the liquid culture and limit the development of scaling-up to bioreactor culture (Chang et al. 2007).

In the present study, we established hairy root cultures of *N. nimmoniana* by infection with *A. rhizogenes* and investigated the production of CPT. This method may provide an important sustainable source for CPT, as well as facilitate the development of scaled-up bioreactor culture of hairy roots in the future.

MATERIALS AND METHODS

Plant material

Ten clones of *N. nimmoniana* seeds, obtained from Green Island, Taiwan, were surface disinfected by immersion in 70% (v/v) ethanol for 1 min, followed by 1.2% NaClO containing a drop of Tween 20 in an ultrsonicator for 15 min. After being rinsed 4 times with sterile deionized water, seeds were grown on 0.75% (w/v) agar solidified MS medium (Murashige and Skoog 1962) containing 30 g/L sucrose and incubated at 25℃ under a 16-h photoperiod (60 μEs^{-1} m^{-2}) for germination. Leaves and stems aseptically excised from 6-mo-old seedlings were used as explants.

Infection and culture of hairy roots

Three wild-type *A. rhizogenes* strains, AR281, AR1600, and ATCC15834, were used for infection. Prior to inoculation, a bacterial suspension was cultured on YEB nutrient medium (Van Larebeke et al. 1977) incubated on a shaker (200 rpm) at 28℃ in the dark for 12 h. For initiation of callus cultures, 20~25 small segments of leaf (0.5 x 0.5 cm^2) and stem (0.5 cm) explants were immersed in the *A. rhizogenes* culture suspension for 5 min, and excess bacterial suspension was blotted off using sterile filter paper. Explants were then cultivated on 0.8% agar solidified MS basal medium supplemented with sugar (3%). After 48 h of incubation, explants were rinsed 4 times with sterile deionized water and immersed in 1 g/L timetin for 1 h to eliminate bacteria. Infected tissues were then transferred to MS liquid medium containing an antibiotic (200 mg/L timetin), and plates were incubated at 25℃ in the dark. Explants were made bacteria-free by transferring to fresh medium weekly 4 times. Established hairy root cultures were then subcultured in antibiotic-free MS medium with 2% sucrose.

Hairy roots began to emerge 2 wk after infection. After 40 d, the top 3 fastest growing hairy root lines (named HR1 to 3) were selected for future growth and alkaloid production tests. HR1 to 3 alone with a control group of untransformed root cultures derived from the root segments of seedlings in vitro were weighed using 0.5 g of root explants as the initial fresh weight (FW), and they were then cultured in MS medium with 2% sucrose with or without 1 g/L activated charcoal in 3 replications. All cultures were placed in continuous darkness at 25℃. The weights of the initial and 40-day-old cultured hairy roots were investigated. The growth index was calculated as:

$$\frac{\text{Final FW - initial FW}}{\text{Initial FW}}.$$

To evaluate the potential of scaled-up culture, 0.2 g of HR2 hairy roots was cultured in MS liquid medium with 2% sucrose (20 ml per 100-ml flask) at 25℃ on a rotary shaker (60 rpm) in the dark with 3 replications. Cultured hairy roots were harvested after 30 d, and the FW and DW were measured.

DNA extraction, polymerase china reaction (PCR), and Southern blot analysis

Nothapodytes nimmoniana DNA was isolated from hairy roots and untransformed roots in liquid nitrogen using a DNeasy plant mini kit (Qiagen, Valencia, CA, USA). To amplify the inserted *rol*B gene homologous sequences, the primers used were *rol*B1: 5'-ATGGATCCCAAATTGCTATTCCCCCAC-GA-3' and *rol*B2: 5'-TTAGGCTTCTTTCAT-TCGGTTTACTGCAGC-3' (Hamill et al. 1991). PCR amplification was performed with a program of initial denaturation at 95℃ for 5 min, 40 cycles of denaturation at 94℃ for 15 s, annealing at 50℃ for 10 s, and extension at 72℃ for 1 min, with a final extension at 72℃ for 5 min and storage at 4℃. PCR-amplified products were subjected to electrophoresis in 1% agarose gels followed by staining with ethidium bromide and UV detection.

For the Southern blot analysis, DNA samples (10 μg) digested by HindIII were subjected to electrophoresis on 1.0% agarose gels. Separated DNA was then blotted from the gel onto a nylon membrane (Roche Molecular Biochemicals, Mannheim, Germany), and immobilized with a UV crosslinker (Stratagene UV Stratalinker 1800, La Jolla, CA, USA). The probe was obtained from the PCR using *A. rhizogenes* 15834 DNA as a template and gene-specific primers for *rol*B genens. Procedures for Southern hybridization and chemiluminescent detection followed instructions of

the DIG kit (Roche Molecular Biochemicals) and the method of McCabe et al. (1997).

Extraction and determination of CPT

Both roots and culture medium were used to determine the CPT content in 3 replications. Each root sample (1~2 g FW) was freeze-dried and ground to a fine powder with a mortar and pestle. The powder was lyophilized and extracted with 10 ml methanol by sonication for 30 min. The methanolic extract was centrifuged for 5 min at 1000 x*g*. The residue was re-extracted with 10 ml methanol. Clear supernatants and the re-extract were combined and passed through a 0.2-μm filter (Acrodisc^{R} Lc 13 PVDF, Gelman, Ann Arbor, MI, USA). Culture medium was extracted twice with 20 ml chloroform in a sonicator for 1 h, then concentrated, diluted in methanol, and passed through a filter. The high-performance liquid chromatographic (HPLC) analyses were carried out on a Waters 600 system (Milford, MA, USA) equipped with a 717 plus 10-μl loop autosampler injector and a 2996 system photodiode-array detector using a Chromolith Performance RP-18e monolithic column (150 x 4.6 mm, 5-μm particle size) (Supelco Discovery, Bellefonte, PA, USA). The mobile phase for alkaloid elution was 0.03 M ammonium acetate: acetonitrile (60: 40), at a flow rate of 1 ml/min and UV detection at 254 nm. A standard curve was obtained using an authentic CPT sample (Sigma-Aldrich, St. Louis, MO, USA). The CPT concentration in extracts was expressed as a percentage of DW of roots or milligrams per liter for culture medium. For DW measurements, residues were dried at 60℃ for 48 h after being extracted.

Statistical analysis

Means of the growth index of 5 hairy root lines in different media were calculated and compared by Duncan's multiple-range test at the 5% level of significance using an analysis of variance (ANOVA) in SAS (vers. 8.2, SAS Institute, Cary, NC, USA).

RESULTS

Induction and culture of *N. nimmoniana* hairy roots

After about 14 d of co-cultivation with *A. rhizogenes*, hairy roots initially emerged (Fig. 1). There are 2 forms of hairy roots: emerging directly from an incision of plant segments, and emerging from a nodule which develops from an incision of plant segments. Over 70% of roots were from the second form.

Normally, explants of *N. nimmoniana* leaves and stems became swollen after a week on MS medium, but were gradually moribund without forming any callus at week 4. Therefore, development of nodules or hairy roots is proof of successful transformation.

Results showed great variations in transformation efficiencies among different *A. rhizogenes* strains, plant parts, and *N. nimmoniana* clones (Table 1). Strain ATCC15834

Fig. 1. Development of hairy roots induced from *Nothapodytes nimmoniana* stem explants after co-inoculation with ATCC 15834.

Table 1. Percentages of explants forming hairy roots after being infected with *Agrobacterium rhizogenes* strains AR208, AR1600, and ATCC15834 with explants from different clones of *Nothapodytes nimmoniana*

Clone	Hair root (%)					
	AR281		AR1600		ATCC15834	
	Leaves	Stems	Leaves	Stems	Leaves	Stems
487	4.0	33.3	4.0	50.0	8.0	75.0
G1-1	0	0	0	0	0	0
G1-2	0	0	0	0	0	0
G1-4	0	8.3	4.0	22.7	4.0	21.7
G3-1	0	0	0	0	0	0
G3-3	0	4.3	0	29.2	0	33.3
G3-4	0	16.0	0	40.0	0	40.0
H1	0	20.8	0	48.0	0	58.3
H2	4.0	24.0	4.0	82.6	4.8	100.0
T2	0	8.0	0	20.8	0	28.0
Average	0.8	11.5	1.7	29.3	1.7	35.6

had the highest transformation ability, followed by AR1600 and AR281. Stems were more susceptible to infection then leaves in this study. Stems from 7 clones were infected, and hairy roots were successfully induced, although there were variations within a clone among strains. Among *N. nimmoniana* clones, stems of clone H2 formed the most hairy roots regardless of which strain was used for infection. Strain ATCC15834 was even able to infect all H2 stem explants. Only 3 clones could establish hairy roots from leaves with the highest transformation efficiency of 8%. No hairy roots were induced by the 3 clones under any condition.

Fast-growing hairy root lines RH 1-3 (2 from clone G1-4 and 1 from clone H2), used for the growth and alkaloid production tests, were infected with strain ATCC15834.

Verification of the transformed status of hairy root culture

The transformed statuses of hairy root lines RH 1-3 were analyzed by a PCR and the Southern blot analysis. An expected 780-bp DNA fragment of the gene was amplified from the DNA of all 3 hairy root lines (Fig. 2), but was not detected in untransformed roots. The primer of the *rol*B gene was used as a probe for the Southern blot analysis of transgenic plants. Various bands were observed for all hairy root lines but not for untransformed roots (Fig. 3). Both results confirmed that the foreign *rol*B gene was transferred into *N. nimmoniana* hairy root cultures.

Subculture of hairy roots

Compared to untransformed roots, hairy root cultures grew rapidly, showed plagiotropic root growth, and were highly branched on phytohormone-free medium (Fig. 4). Growth rates of hairy roots were 7~8 times higher than untransformed roots (growth index = 0.3~0.5) (Table 2). After being cultured in MS medium for 40 d, hairy roots had increased 2.6~3-times over the initial FW and 3.5~4-times when cultured with activated charcoal, with no differences among the hairy root lines. In spite of the increasing growth rate, the medium with activated charcoal did not

improve the CPT content of hairy roots (Table 3). On the contrary, hairy root CPT contents were more closely related to the original clone. For example, more CPT accumulated in untransformed clone G1-4 roots then in induced clone H2 hairy roots; more CPT

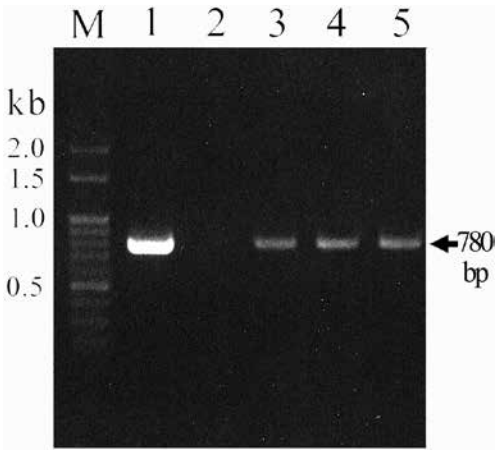

Fig. 2. Detection of the *rol*B gene in transgenic *Nothapodytes nimmoniana* by a PCR. Lane M, size marker DNA; lane 1, positive control (plasmid); lane 2, control (untransformed root); lanes 3~5, hairy root lines HR1~3, derived from stem explants inoculated with *Agrobacterium rhizogenes* ATCC15834.

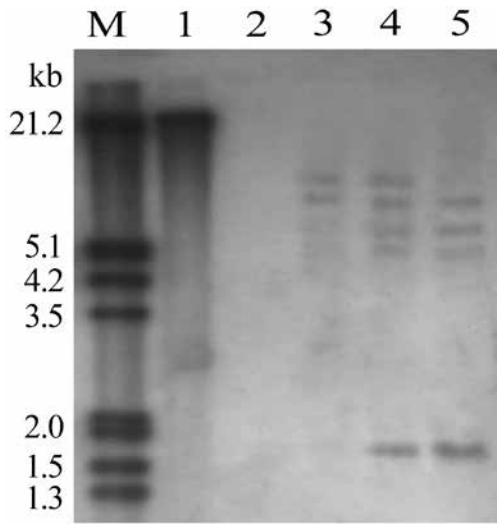

Fig. 3. Southern blot analysis of hairy root cultures. HandIII-digested DNA samples were hybridized with a 780-bp *rol*B gene fragment. Lane M, DIG-labeled DNA maker; lane 1, ATCC15834 plasmid DNA; lane 2: control (untransformed root); lanes 3~5, hairy root lines HR1~3 carrying the *rol*B gene.

Fig. 4. Hairy roots of *Nothapodytes nimmoniana* cultured on MS medium containing 1 g/L activated charcoal.

Table 2. Effect of MS medium with or without 1 g/L activated charcoal (AC) on fresh weight (FW), dry weight (DW) and growth index after 40 d of culture

Roots	Final FW (g)		Final DW (g)		Growth index[1)]	
	MS	MS+AC	MS	MS+AC	MS	MS+AC
Untransformed roots (G1-4)	0.71±0.04	0.75±0.06	0.068±0.007	0.069±0.011	0.4[c]	0.5[c]
Untransformed roots (H2)	0.65±0.05	0.73±0.06	0.061±0.003	0.070±0.005	0.3[c]	0.5[c]
Hairy roots 1 (form G1-4)	1.82±0.16	2.52±0.14	0.158±0.026	0.211±0.027	2.6[b]	4.0[a]
Hairy roots 2 (form G1-4)	2.00±0.17	2.41±0.18	0.157±0.017	0.191±0.029	3.0[b]	3.8[a]
Hairy roots 3 (form H2)	1.80±0.17	2.25±0.10	0.144±0.013	0.183±0.013	2.6[b]	3.5[a]

[1)] Values of the growth index defined as ((final FW - initial FW)/initial FW) followed by the same letter do not significantly differ at the 5% level by Duncan's test.

Table 3. Effect of MS medium with or without 1 g/L activated charcoal (AC) on the camptothecin (CPT) content after 40 d of culture

Roots	CPT content (% dry weight)	
	MS	MS + AC
Untransformed roots (G1-4)	0.08055±0.00247	0.08195±0.00495
Untransformed roots (H2)	0.05395±0.00191	0.05138±0.00103
Hairy roots 1 (from G1-4)	0.08255±0.00148	0.08379±0.00175
Hairy roots 2 (from G1-4)	0.14965±0.00163	0.15551±0.00665
Hairy roots 3 (from H2)	0.05522±0.00193	0.05365±0.00120

accumulated in HR1 and HR2 (from clone G1-4) then in HR3 (induced from clone H2). However, different hairy root lines had variable CPT contents even from the same clone (i.e., HR1 and HR2).

Liquid culture of hairy roots and CPT production

After 30 d of cultivation in MS liquid medium, the FW of HR2 hairy roots had increased from 0.2 to 1.26 g. The 5.3-fold growth rate indicates fairly rapid growth (Table 4, Fig. 5). The CPT content in hairy roots was 0.6 mg/L (volume of the medium), or 0.00691% DW, while the CPT in the medium was 9.2 mg/L. The total CPT production was 9.8 mg/L; however, the majority of CPT (93.9%) was excreted to the liquid medium. Chromatographic results of both hairy roots and media are shown in Fig. 6. Compounds in the medium were lower and the CPT peak was greater at the same level compared to those in hairy roots. When comparing CPT yields based on DW, the CPT content in liquid roots was 0.10478%, a little lower than that in solid roots. However, due to a much higher growth rate, the total CPT production in liquid culture was still greater.

DISCUSSION

Hairy roots infected with *A. rhizogenes* carrying a hormone gene were able to grow on hormone-free medium. However, in some cases, hairy roots grew poorly, and plant growth regulators were added to the medium to promote more-rapid growth, such as with *C. acuminata* hairy root cultures (Lorence 2004). This increased the chance of the formation of undesirable calluses and untransformed roots when cultivated in hormone-supplemented medium for a period of time.

Table 4. Growth and camptothecin (CPT) production of hairy root line HR2 after 30 d of culture in liquid medium (initial fresh weight (FW) 200 mg in 100-ml wide-mouth flasks with 10 ml MS medium)

Hairy root line HR2	Growth/CPT content
Final FW (g)	1.26±0.055
Final dry weight (DW) (g)	0.0958±0.0033
Growth index ((final FW-initial FW)/initial FW)	5.3±0.35
CPT production in hairy roots (% of DW)	0.00691±0.00045
= CPT production in hairy roots (mg/L)	0.6±0.06
CPT in medium (mg/L)	9.2±0.57
Total CPT production (mg/L)	9.8±0.51
= Total CPT production (% of DW)	0.10478±0.00852
% CPT in medium	93.9±0.90

Fig. 5. Hairy roots line HR2 of *Nothapodytes nimmoniana* cultured on MS liquid medium for 30 d. Left: initial culture (0.2 g fresh weight (FW) of hairy roots/10 ml medium/100-ml flask). Right: final culture (1.26 g FW of hairy roots).

This is the first report on successfully establishing hairy root cultures of *N. nimmoniana.* We established a system of hairy roots induced from stems or leaves of *N. nimmoniana* which grew rapidly on hormone-free medium and produced 0.1555% CPT which was the highest among all previous studies for producing CPT from regular roots or hairy roots. For example, hairy roots from *C. acuminata* (Lorence 2004) and *O. pumila* (Saito et al. 2001) produced 0.1% CPT, while only 0.0083 and 0.022% CPT was exuded from hairy roots of *O. liukiuensis* and *O. kuroiwai* (Asano et al. 2004). Fulzele (2002) reported 0.01% CPT from untransformed adventitious root cultures of *N. nimmoniana*.

It appears that production of CPT from tissue cultures is highly dependent on the original plant or clone used in the study. Using original materials with higher CPT contents should increase the production of CPT (Wiedenfild et al. 1997, Padmanabha et al. 2006). The CPT content of *N. nimmoniana* was reported to be 0.01~0.7% (Chang et al. 2006, Padmanabha et al. 2006), while the contents of *C. acuminate* and *O. pumila*

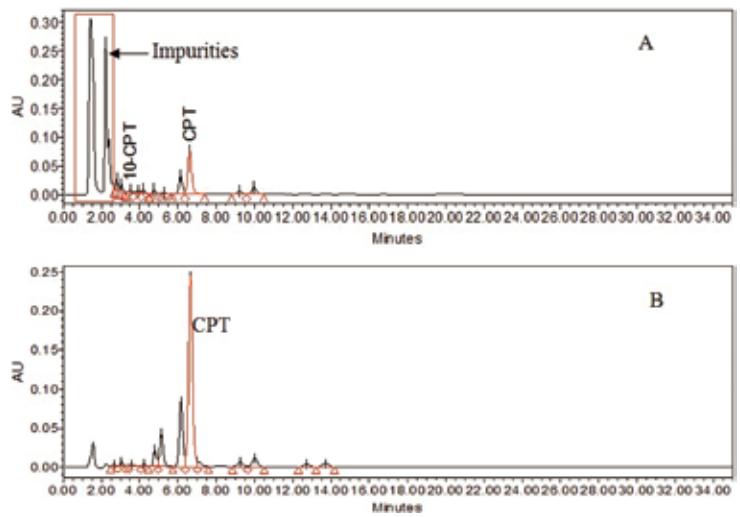

Fig. 6. Chromatograms of hairy root (A) and medium (B) samples from *Nothapodytes nimmoniana*. A had more compounds (impurities) than B, which indicated that camptothecin (CPT) in the medium could easily be purified.

were 0.01~0.5% and 0.07~0.1%, respectively (López-Meyer et al. 1994, Wiedenfeld et al. 1997, Yamazaki et al. 2003). This should be one of the reasons why hairy roots of *N. nimmoniana* in our results produced higher CPT than those of the other 2 species in previous studies, since *N. nimmoniana* contains higher CPT.

In the present paper, it was found *N. nimmoniana* hairy root lines had higher CPT contents, if the original untransformed clone had higher CPT contents. In a study by Thengane et al. (2003), they produced CPT in callus cultures derived from cotyledons of *N. nimmoniana* seeds instead of stem parts, as seeds have higher CPT concentrations than stems. Among various tissues (wood, stem bark, root, and leaf) of *N. nimmoniana* analyzed, the root bark yielded the highest CPT content followed by stem bark (Padmanabha et al. 2006). Therefore, hairy roots are a favorable material to produce CPT. However, the results may vary by species. For example, young leaves of *C. acuminata* yielded the highest CPT (López-Meyer et al. 1994), while roots of *O. pumila* contained the highest levels (Yamazaki et al. 2003).

It was reported that a substantial amount of CPT (> 23%) is excreted into the culture medium from various species or treatments (Saito et al. 2001, Asano et al. 2004, Lorence et al. 2004). In this study, about 94% of CPT was excreted from hairy roots into the liquid medium. This characteristic of CPT being excreted into the culture medium is beneficial for developing a large-scale and continuous culture system. Sudo et al. (2002) also reported about 16.5% of the total 8.7 mg/L CPT produced from hairy roots of *O. pumila* was excreted into 3-L bioreactor culture medium. The CPT excreted in the medium can be absorbed and removed by polystyrene resin, which in turn can increase the total CPT accumulation in the medium (Saito et al. 2001).

CONCLUSIONS

We successfully transformed the *A. rhizogenes* strain ATCC15834 gene into hairy roots of *N. nimmoniana*, which was verified

by a PCR and Southern blot analysis. After culturing hairy roots for several weeks, CPT contents were quantified by HPLC, and CPT was proven to be excreted into the liquid medium. This stable and sustainable production system is able to produce CPT for clinical applications. For scaled-up culture, we suggest that more research on high-CPT hairy root lines selection, suitable induction, culture methods and conditions, and CPT-producing related gene studies in the future are required.

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