Research paper

Induction of Hairy Roots in *Cyclobalanopsis glauca* and Production of Catechin in Hairy Roots

Min-Yi Lin,[1,3] Da-Chung Wang[2]

【Summary】

Cyclobalanopsis glauca (Thunb.) Oerst is a tree species with rich polyphenol compounds, including catechins, which increase the oxidation resistance of blood plasma and reduce the risk of cardiovascular disease in humans. Catechin may also have an inhibitory effect on carcinogens, specifically those related to skin and lung cancer. This study investigated hairy root induction in leaves of *C. glauca* infected with 2 strains of *Agrobacterium rhizogenes* (BCRC15010 and BCRC15785) under the influence of α-naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D). After 45 d, explants co-cultivated with the BCRC15785 strain in MS medium with 0.1 mg L^{-1} NAA and 300 mg L^{-1} cefotaxime exhibited 32.5% greater efficiency of hairy root induction. Induction of calli and hairy roots was observed under the influence of NAA, while only calli were induced under the influence of 2,4-D. We observed the direct influence of the infection period on the transformation frequency, and it increased as the infection period increased up to 24 h. Extracts from hairy roots, calli, and natural seedling roots revealed the presence of catechin content, and concentrations were 4.203, 3.413, and 4.668 mg g^{-1}, respectively, in these 3 analytes.

Key words: *Agrobacterium rhizogenes*, catechin, *Cyclobalanopsis glauca*, hairy root induction.

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研究報告

青剛櫟之毛狀根誘導與兒茶素生產

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摘 要

青剛櫟(*Cyclobalanopsis glauca* (Thunb.) Oerst)是富含多酚類化合物之樹種，其中內含之兒茶素(catechins)可增加血漿之抗氧化性，降低罹患心血管疾病的風險，catechins也證實對致癌物質具有抑制作用，特別是可預防皮膚癌和肺癌的發生。本試驗以陽明山之青剛櫟葉片接種購自食品工業研究所二種不同菌株(BCRC15010和BCRC15785)；培養條件中使用α-naphthaleneacetic acid (NAA)與2,4-dichlorophenoxyacetic acid (2,4-D)二種植物生長調節劑。培殖體與BCRC15785菌株進行感染，經45天後，培養於MS培養基(含0.1 mg L^{-1} NAA + 300 mg L^{-1} cefotaxime)中具有較佳之毛狀根(hairy root)誘發率(32.5%)。植物生長調節劑NAA的影響下可誘導出癒合組織和毛狀根，而2,4-D只對癒合組織的誘導有效。研究發現從感染時間1到24小時增加，轉殖率也相對增加。毛狀根、癒合組織和種子苗的根部均有兒茶素的存在，含量濃度分別為4.203、3.413及4.668 mg g^{-1}。

關鍵詞：農桿根群菌、兒茶素、青剛櫟、毛狀根。

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INTRODUCTION

Cyclobalanopsis glauca (Thunb.) Oerst is an important medicinal plant of the Fagaceae family naturally occurring in mid-elevation mountainous regions of Taiwan. Its medicinal properties are related to a variety of chemical constituents, including (+)-catechin, (-)-epicatechin, (+)-gallocatechin, and (-)-epigallocatechin (Sheu et al. 1997). Ko et al. (2009) investigated the effects of epigallocatechin (EGC), gallocatechin (GC), and gallocatechin gallate (GCG) on bone metabolism. Results demonstrated that tea catechins, EGC in particular, had positive effects on bone metabolism through combined processes of promoting osteoblastic activity and inhibiting osteoclast differentiation.

In vitro production of (+)-catechin and (-)-epicatechin was reported in *Fagopyrum esculentum* Moench calli and cultured hairy roots (Trotin et al. 1993), and in cultured cells of *Polygonum hydropiper* L.; the maximal yield of total catechins in suspension-cultured cells was 4.3% dry weight (Ono et al. 1998); and cultured cells of *Taxus cuspidata* Sieb. & Zucc produced 3.4% catechins when stimulated with methyl jasmonate (Bulgakov et al. 2011).

Agrobacterium rhizogenes is a gram-negative soil bacterium containing a single copy of a large Ri plasmid. Researchers recently demonstrated that Ri plasmids, which induce growth in hairy root culture systems, can be used to produce secondary metabolites (Merkli et al. 1997, Kittipongpatana et al. 1998, Sevon and Oksman-caldentey 2002). John et al. (2009) reported the induction of hairy roots from *Camellia sinensis* (L.) O. Kuntze leaf explants and confirmed that quantities of polyphenols and catechins in hairy roots were higher than in untransformed leaf

tissues. *Agrobacterium rhizogenes*-mediated transformation offers a promising approach to the production of secondary metabolites, in which transformed roots can be excised and grown in vitro as hairy root cultures (Hamill et al. 1987). Hairy roots have several properties which enhance their use in plant biotechnological applications. Rapid growth, genetic and biosynthetic stability, a low doubling time, and ease of maintenance enable the synthesis of a range of chemical compounds and make them a suitable system for the in vitro production of secondary metabolites (Giri and Narasu 2000). This study describes the Ri plasmid-mediated transformation of *C. glauca*, the establishment of hairy root culture systems, and catechin production in cultured products.

MATERIALS AND METHODS

Plant materials

Seeds of *C. glauca* were collected from Yangming Mt., northern Taiwan. Seeds were removed from the cupula, and the surface was sterilized using a 0.2% antiseptic solution containing 0.02% Tween 20, followed by 3 washes with sterile distilled water. Seeds were further sterilized using 70% ethanol for 10 min, followed by 3 washes with sterile distilled water. Seeds were then treated with 2% mercuric chloride for 15 min followed by 3 washes with sterile distilled water to remove traces of mercuric chloride. Sterilized seeds were transferred aseptically onto sterilized filter paper to remove moisture from the surface of the seeds, and they were sown in germination medium (soil: pearl stone: vermiculite of 2: 1: 1). Following germination, seedlings were treated each week with 1000 mg L^{-1} Benlate.

Agrobacterium strains and culture conditions

Agrobacterium rhizogenes strains BCRC 15010 and BCRC 15785 were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The strains were maintained on agar-solidified nutrient medium (3 g L^{-1} beef extract, 5 g L^{-1} peptone, and 15 g L^{-1} agar; Becton Dickinson, Franklin Lakes, NJ, USA) at 28℃. A single colony was inoculated into 20 mL of liquid nutrient medium in a 125-mL culture flask and incubated at 28℃ and 170 rpm until the cell density (OD_{600} reading) reached 0.7~1.0. Cells were collected through centrifugation and resuspended in Murashige and Skoog (MS) liquid medium (Murashige and Skoog 1962) containing 100 μmol L^{-1} acetosyringone (Sigma-Aldrich, St. Louis, MO, USA).

Hairy root induction

Cyclobalonopis glauca leaf explants were transferred to the aforementioned *A. rhizogenes* suspended in MS liquid media and incubated for various durations (1, 6, 12, 24, and 30 h) before being blotted dry on sterilized filter paper. Infested explants were transferred to MS solid medium (with 100 μmol L^{-1} acetosyringone; Sigma-Aldrich) for cocultivation for 2 d in the dark. Explants were then transferred to MS medium containing various concentrations of α-naphthaleneacetic acid (NAA: 0, 0.01, 0.05, 0.1, 0.5, and 1 ppm; Sigma-Aldrich) and 2,4-dichlorophenoxyacetic acid (2,4-D: 0, 0.01, 0.05, 0.1, 0.5, and 1 ppm; Sigma-Aldrich) with 300 mg L^{-1} cefotaxime (Sigma-Aldrich). Infested explants were subcultured every second week until hairy root induction began. Hairy roots were individually excised and transferred to MS medium containing 300 mg L^{-1} cefotaxime. The induced roots were successfully cleared of bacteria following several passages through medium containing decreasing concentrations of cefotaxime. A single root tip (approximately 3~5cm long) was excised and

cultured in MS medium with plant growth regulator until hairy roots had been induced from the explant. Negative controls consisted of explants treated similarly, except that they were not infected or co-cultivated with *A. rhizogenes*.

Induction of calli (not infected with *A. rhizogenes*)

Cyclobalanopsis glauca leaf explants were transferred to MS medium containing various concentrations of plant growth regulator, except they were not co-cultivated with *A. rhizogenes*. Finally, we analyzed contents of secondary metabolites produced in calli under the same conditions as those of hairy roots.

Polymerase chain reaction (PCR) amplification of the *rol* B and *rol* C genes

Total DNA was isolated from hairy root cultures according to the manufacturer's instructions for the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The primers, *rol* B1: 5'-ATGGATCCCAAATTGCTATTCCCCCACGA-3'; *rol* B2: 5'-TTAGGCTTCTTTCATTCGGTTTACTGCAGC-3'; *rol* C1: 5'-ATGGCTGAAGACGACCTGTGTT-3'; and *rol* C2: 5'-TTAGCCGATTGCAAACTTGCAC-3' were used for the PCR amplification of a 780-bp fragment of the *rol* B gene and a 540-bp fragment of the *rol* C gene (Yang and Choi 2000, Subroto et al. 2001). The PCR analysis was carried out in an Eppendorf mastercycler gradient in 25-µl reaction mixtures containing 10 ng μL^{-1} of DNA, 0.2 mM of primer, and 12.5 µL of EconoTaq PLUS Green 2X master mix (Lucigen, Middleton, WI, USA). The PCR profile included predenaturation at 92℃ for 5 min, then 35 cycles of denaturation at 92℃ for 45 s, annealing at 52℃ for 45 s, and extension at 72℃ for 45 s, followed by a final extension for 7 min at 72℃ and storage at 4℃. The amplified products were analyzed on 1% agarose-ethidium bromide gels. The PCR products were sent to Tri-I Biotech (New Taipei City, Taiwan) for sequencing.

Extraction and estimation of catechin

Hairy roots, non-transgenic *A. rhizogenes* seedling roots, and non-transgenic *A. rhizogenes* calli (callus induction used MS medium with 0.1 mg L^{-1} NAA) of *C. glauca* were collected, and their fresh weights were determined. Samples were then freeze-dried for 48 h to determine their dry weights. Approximately 40 mg of dried samples was crushed into a fine powder and dissolved in 30 ml of methanol. This was agitated ultrasonically for 30 min and filtered through Whatman no. 1 filter paper (GE Healthcare life sciences, Piscataway, NJ, USA). This process was repeated 3 times for each sample. Following filtration, the combined methanol extracts were evaporated to dryness using a rotary evaporator. The residue was dissolved in 1 ml methanol and filtered through a 0.45-µm (Millipore, Billerica, MA, USA) membrane prior to analysis. High-performance liquid chromatographic (HPLC) conditions included Inertsil ODS-3 columns (4.6×250 mm; GL Sciences, Torrance, CA, USA), detection at a UV wavelength of 280 nm, migration in acetonitrile with 50 mM KH_2PO_4 in H_2O (30: 70), and a flow rate of 1.0 mL min^{-1}. The peak corresponding to catechin was confirmed by spiking the crude extract samples with standard catechin (Sigma-Aldrich). Catechin contents in the crude extracts were determined by comparing peaks from samples with those of standard catechins.

Statistical analysis

Statistical analysis of hairy root formation consisted of 3 repeated trials with at least 40 explants per treatment. Generalized linear

model (GLM) factor analysis patterns were determined using SPSS software (SPSS, Chicago, IL, USA). Analysis of variance (ANOVA) was then used to determine significant differences among treatments. Where significant differences occurred, Duncan's multiple-range test was used to compare the effects of each treatment.

RESULTS

Hairy root induction

Hairy root induction was observed 45 d after infection (Fig. 1a) from the cut surface of leaves. The growth of excised hairy root tips increased from 3 to 6 cm in the in vitro culture, and hairy roots showed differentiation after 60 d (Fig. 1b). Two different *A. rhizogenes* strains (BCRC 15010 and BCRC 15785) were used for root induction under the influence of NAA and 2,4-D. Only calli were induced under the influence of 2,4-D. We observed 75, 87.5, 100, 100, and 100% callus formation from leaves infected with BCRC15010 under the influence of 0.01, 0.05, 0.1, 0.5, and 1.0 mg L^{-1} 2,4-D, respectively. In contrast, 80, 87.5, 100, 100, and 100% leaves infected with BCRC15785 formed calli under the influence of 0.01, 0.05, 0.1, 0.5, and 1.0 mg L^{-1}, 2,4-D, respectively. No hairy root induction was observed with 2,4-D, while NAA showed significant influence on hairy root growth; thus, NAA was used for hairy root induction and proliferation for the remaining experiments.

Infection of *C. glauca* leaf explants with BCRC15010 in culture medium containing 0.1 and 0.5 mg L^{-1} NAA respectively induced hairy root production in 17.5% and 10.0% of the explants tested. The BCRC 15785 strain, in the same culture medium with 0.1 and 0.5 mg L^{-1} NAA, respectively produced induction in 22.5% and 20.0% of explants. However, decreasing the NAA concentration to 0.01 and 0.05 mg L^{-1} for 3 mo resulted in negligible production of hairy roots. Results showed that low concentrations of NAA (0.01~0.05 mg L^{-1}) only gradually induced hairy roots, while concentrations of 0 and 1 mg L^{-1} NAA did not induce the growth of hairy roots at all. A concentration of 0.1 mg L^{-1} NAA was found to be optimal for inducing hairy root growth. At concentrations of 0.05~0.5 mg L^{-1} NAA, the induction percentages using BCRC 15785 were significantly higher than those using BCRC 15010 (Fig. 2).

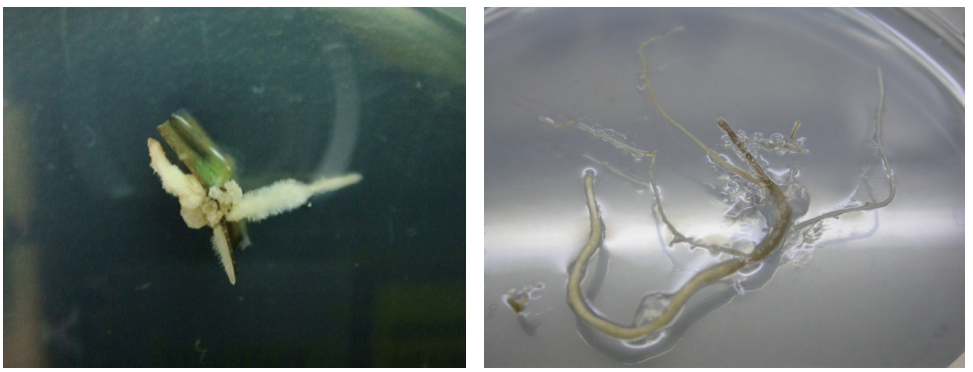

Fig. 1. (a) Hairy-root induction in leaf explants of *Cyclobalanopsis glauca* infected with *Agrobacterium rhizogenes* (BCRC 15785) after 45 d and (b) growth of an excised single root tip after 60 d.

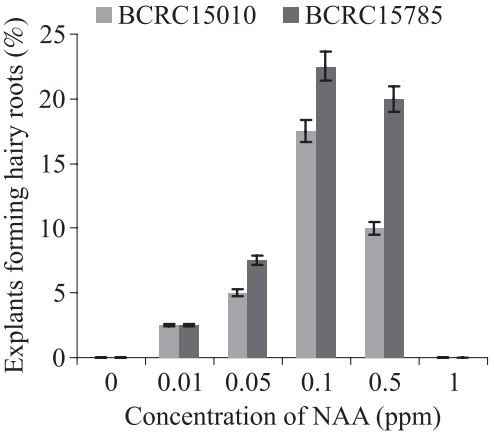

Fig. 2. Effect of different α-naphthaleneacetic acid (NAA) concentrations on leaf explants of *Cyclobalanopsis glauca* infected for 12 h with *Agrobacterium rhizogenes* (BCRC15010 and BCRC 15785).
*** Different letters represent classification results by Duncan's multiple-range test at a significance level of α = 0.05. Error bars represent standard deviations.**

Negative controls consisted of explants the were not co-cultivated with *A. rhizogenes*. They were maintained in MS medium with 0.1 mg L^{-1} NAA, and roots grew slowly (Fig. 3) with a low induction rate, and had a morphology of coarse (no tiny) root hairs.

Infection time and hairy root induction

Infection with strain BCRC15010 at cultivation times of 1, 6, 12, 24, and 30 h in MS medium with 0.1 mg L^{-1} NAA respectively resulted in hairy root growth in 0, 7.5, 17.5, 22.5, and 0%, of infected explants. Following infection with strain BCRC15785 under the same conditions, hairy root induction respectively occurred in 0, 5.0, 22.5, 32.5, and 0% of explants (Fig. 4). Results showed that infection for only 1 h failed to produce hairy roots, and infection extended to 24 h and beyond resulted in brown death.

Fig. 3. Induction of adventitious roots in *C*yclobalanopsis *glauca* explants cultured on MS medium with 0.1 ppm α-naphthaleneacetic acid.

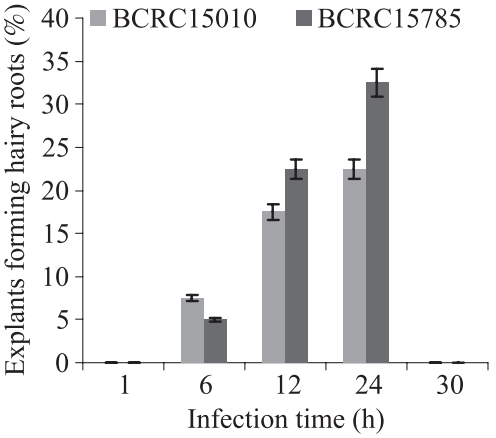

Fig. 4. Induction of hairy-root growth in leaf explants of *Cyclobalanopsis glauca* infected with *Agrobacterium rhizogenes* (BCRC 15010 and BCRC 15785) for 1~30 h in MS liquid media.
*** Different letters represent classification results by Duncan's multiple-range test at a significance level of α = 0.05. Error bars represent standard deviations.**

PCR amplification of *rol* B and *rol* C genes

Genomic DNA extracts from hairy roots grown in the presence of 2 different strains of *A. rhizogenes* and negative control roots underwent PCR amplification using *rol* B

(780 bp) and *rol* C (540 bp) primers followed by electrophoresis. Results indicated that the *rol* B and *rol* C bands appeared separately in transformed roots (Fig. 5). However, *rol* B and *rol* C did not appear in non-transformed roots of the negative control. The *rol* C gene used for the PCR was compared on the NCBI website, with accession no. EU642409.1, and the similarity was 99% (Fig. 6). Therefore, we confirmed the identity of *rol* C, and *C. glauca* leaf explants were successfully transformed with *A. rhizogenes*.

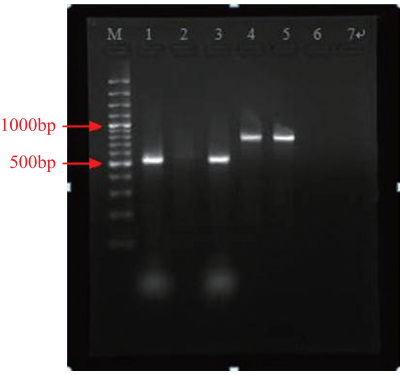

Fig. 5. Image of an electrophoretic gel of DNA extracted from *Cyclobalanopsis glauca* hairy roots infected by *Agrobacterium rhizogenes* (BCRC15010 and BCRC 15785). Lane M, molecular weight marker (100 bp DNA ladder); lane 2, DNA from non-transformed roots (negative control); lanes 1 and 3, *rol* C (540 bp) amplification products; lanes 4 and 5, *rol* B (780 bp) amplification products.

HPLC analysis

Quantitative analysis of callus samples showed an average level of catechin of 136.5 μg mL^{-1} in the methanol extract of non-transformed calli. Thus, every gram of callus had a catechin content of 3.413 mg. The average level of catechin in the methanol extract of

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Score =  957 bits (518),  Expect = 0.0
Identities = 535/542 (99%), Gaps = 5/542 (1%)
Strand=Plus/Plus

Query  1    ATGGCTCAAGACGACCTGTGTT-TCTCTCTTT-AAGCTTC-AAGTGGAGGATGTGACAAG  57
            ||||||||||||||||||||||| ||||| ||| |||| || ||||||||||||||||||
Sbjct  1    ATGGCTCAAGACGACCTGTGTTCTCTCTTTTTCAAGC-TCAAAGTGGAGGATGTGACAAG  59

Query  58   CAGCGATGAGCTAGCTAGACACATGAAGAACGCCTCAAATGAGCGTAAACCCTTGATCGA  117
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  60   CAGCGATGAGCTAGCTAGACACATGAAGAACGCCTCAAATGAGCGTAAACCCTTGATCGA  119

Query  118  GCCGGGTGAGAATCAATCGATGGATATTGACGAAGAAGGAGGGTCGGTGGGCCACGGGCT  177
            ||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  120  GCCGGGTGAGAATCAATCGATGGATATTGACGAGGAAGGAGGGTCGGTGGGCCACGGGCT  179

Query  178  GCTGTACCTCTACGTCGACTGCCCGACGATGATGCTCTGCTTCTATGGAGGGTCCTTGCC  237
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  180  GCTGTACCTCTACGTCGACTGCCCGACGATGATGCTCTGCTTCTATGGAGGGTCCTTGCC  239

Query  238  TTACAATTGGATGCAAGGCGCACTCCTCACCAACCTTCCCCCGTACCAGCATGATGTGAC  297
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  240  TTACAATTGGATGCAAGGCGCACTCCTCACCAACCTTCCCCCGTACCAGCATGATGTGAC  299

Query  298  TCTCGATGAGGTCAATAGAGGGCTCAGGCAAGCATCAGGTTTTTTCGGTTACGCGGATCC  357
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  300  TCTCGATGAGGTCAATAGAGGGCTCAGGCAAGCATCAGGTTTTTTCGGTTACGCGGATCC  359

Query  358  TATGCGGAGCGCCTACTTCGCTGCATTTTCTTTCCCTGGGCGTGTCATCAAGCTGAATGA  417
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  360  TATGCGGAGCGCCTACTTCGCTGCATTTTCTTTCCCTGGGCGTGTCATCAAGCTGAATGA  419

Query  418  GCAGATGGAGCTAACTTCGACAAAGGGAAAGTGTCTGACATTCGACCTCTATGCCAGCAC  477
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  420  GCAGATGGAGCTAACTTCGACAAAGGGAAAGTGTCTGACATTCGACCTCTATGCCAGCAC  479

Query  478  CCAGCTTAGGTTCGAACCTGGTGAGTTGGTGAGGCATGGCGAGTGCAAGTTTGCAATCCG  537
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |
Sbjct  480  CCAGCTTAGGTTCGAACCTGGTGAGTTGGTGAGGCATGGCGAGTGCAAGTTTGCAATC-G  538

Query  538  GC  539
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Sbjct  539  GC  540
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Fig. 6. Sequence analysis of the *rol* C gene amplified by a PCR and comparison to the NCBI website with accession no. EU642409.1.

transformed hairy roots was 168.1 μg mL^{-1}; thus, every gram of hairy root provided a catechin content of 4.203 mg. The average level of catechin in the methanol extract of non-transformed seedling roots was 186.7 μg mL^{-1}; thus, every gram of seedling root had a catechin content of 4.668 mg (Fig. 7) (Table 1).

DISCUSSION

Inducing the growth of hairy roots does not usually require a plant growth regulator; however, researchers reported that increased levels of auxin in conjunction with infection by *A. rhizogenes* enhanced the efficiency of

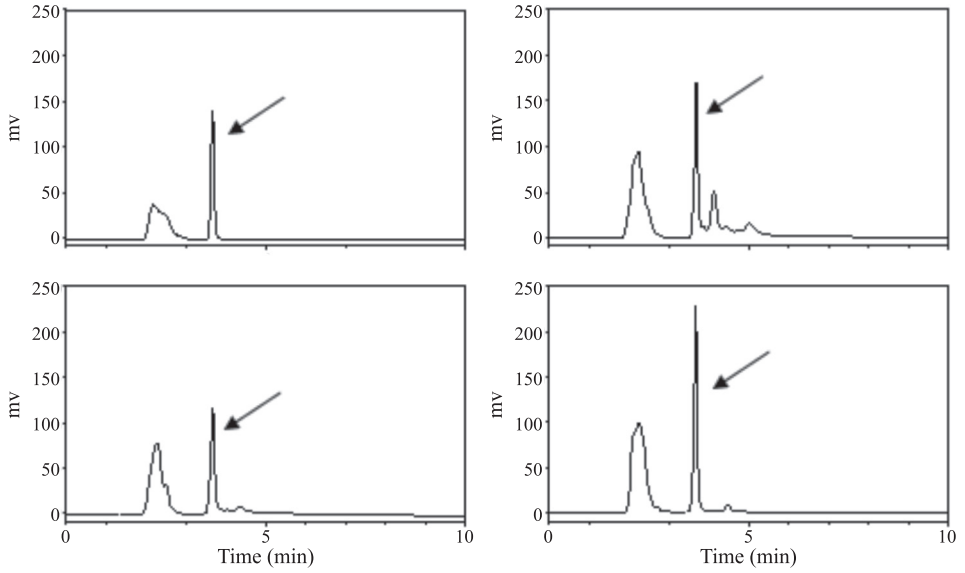

Fig. 7. HPLC chromatograms showing separation of catechin from various extracts. Separation was carried out on a C18 reverse-phase column using acetonitrile with 50 mM KH_2PO_4 in H_2O (30: 70) as the mobile phase, and a flow rate of 1.0 mL min^{-1}. Catechin was detected at 280 nm. (a) Catechin standard; (b) hairy-root methanol extract; (c) callus methanol extract; and (d) seedling-root methanol extract. Catechin contents were determined by comparing peak areas with that of standard catechin. Computed catechin content values were 4.203 mg g^{-1} of dry root powder; 3.413 mg g^{-1} of dry callus powder; and 4.668 mg g^{-1} of dry seedling-root powder.

Table 1. Contents of (+)−catechin in hairy roots, calli, and seedling roots of *Cyclobalanopsis glauca*

Different line	content (mg g^{-1})				
	I	II	III	IV	Average
Hairy root (BCRC15010)	-	-	-	-	-
Hairy root (BCRC15785)	3.980±0.013	4.080±0.01	4.563±0.019	4.188±0.008	4.203
Callus	2.943±0.061	3.838±0.022	3.468±0.012	3.403±0.043	3.413
Seedling root	5.033±0.083	4.955±0.053	4.290±0.028	4.393±0.019	4.668

* Data of hairy roots (BCRC15010) were not analyzed by HPLC.

hairy root transformation (Cardarelli et al. 1987). Puddephat et al. (2001) used *A. rhizogenes* to infect *Brassica oleracea* L. and reported that 2,4-D enhanced the transformation of hairy roots. In experiments in which *Camellia sinensis* (L.) O. Ktze was co-cultivated with *A. rhizogenes*, John et al. (2009) showed that culture media supplemented with indole-3-acetic acid (IAA) and 2,4-D increased the growth rate of hairy roots, and that plant growth regulators played an important role in the induction of hairy roots in cultured tea explants. Rudrappa et al. (2005) co-cultivated red beets (*Beta vulgaris* L.) with various strains of *A. rhizogenes* to induce growth of hairy roots and various strains of hairy roots in MS liquid medium containing 0.5 or 1.0 mg L^{-1} NAA. They found that different lines of hairy roots had different responses depending on the concentration of NAA. Our experiment showed that *C. glauca*, infected with the BCRC15785 strain and cultivated for 24 h in MS medium containing 0.1 mg L^{-1} NAA and 300 mg L^{-1} cefotaxime, produced the highest hairy root induction rate, while MS culture medium containing different concentrations of 2,4-D only promoted callus formation. Other co-cultivated explants in MS media without plant growth regulators induced hairy roots and calli very slowly. Although 2,4-D did not induce the growth of hairy roots, NAA produced considerable hairy root growth, with little callus formation. Experimental results indicated that *C. glauca* requires a plant growth regulator to enhance the induction of hairy roots, and the selection of an appropriate plant growth regulator can result in the growth of hairy roots.

Co-cultivation is crucial to the processes of transformation. Bacterial attachment and T-DNA integration occur during co-cultivation (De et al. 2000), and excessive prolongation of this period resulted in brown death of co-cultivated explants. Cheng et al. (1997) studied wheat transformation mediated by *A. tumefaciens* and found that increasing the durations of infection and co-cultivation was beneficial to the transfer of DNA, but resulted in cell injury. Tao and Li (2006) studied the transformation of *Torenia fournieri* L. mediated by *A. rhizogenes* and found that the transformation frequency increased with an increase in infection times of 5~20 min. Their study indicated that an increased co-cultivation time enhanced hairy root induction, but co-cultivated bacteria underwent excessive proliferation. Our study of *C. glauca* explants cultivated with strain BCRC15785 showed that when the infection time was prolonged, explants died. Our study also showed that increasing the infection time enhanced the success of explant transformation. In addition, this experiment demonstrated that different bacterial strains variably influenced transformation success.

Methanol extracts from calli and hairy roots exhibited different levels of catechin. Co-cultivated hairy root extract produced catechin at a rate of 4.203 mg g^{-1}, while calli produced approximately 3.413 mg g^{-1}, and natural seedling root extract produced 4.668 mg g^{-1}. Due to the relationship between the accumulation of plant metabolites and plant cell differentiation (Dornenburg and Knorr 1995), elevated catechin contents in hairy roots may be the result of the roots having divided. Satdive et al. (2006) used *A. rhizogenes* to infect *Azadirachta indica* A. Juss and found that the azadirachtin content in hairy roots was comparable to that found in general roots. In a study of *Gmelina arborea* Roxb., Dhakulkar et al. (2005) reported that different parts of the plant produced different compositions and showed that the verbascoside content was highest (8.4 mg g^{-1}) in natural roots. The small young root content was 0.55 mg g^{-1},

and the hairy root content was the lowest (0.13 mg g^{-1}). Our results support previous observations indicating the possibility of different contents of secondary metabolites occurring in different tissue cultures or different parts of the same plant species.

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