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Enhanced tanshinone production in hairy roots of '*Salvia miltiorrhiza* Bunge' under the influence of plant growth regulators in liquid culture

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ABSTRACT. Tanshinone constituents are the most potent diterpene diketones used to treat several diseases. We studied the influence of different plant growth regulators (PGR) on the growth and production of three tanshinone constituents using hairy root culture of *Salvia miltiorrhiza* Bunge. Leaves of the medicinally-important plant *S. miltiorrhiza* were infected with *Agrobacterium rhizogenes* strain BCRC15010, which induced hairy roots in 78% of the explants. The best line of hairy roots was established on B5 liquid medium under dark conditions. The transformed nature of the root lines was confirmed by polymerase chain reaction using '*rol*' B and C gene-specific primers. Adding different PGRs i.e., auxins, cytokinins and abscisic acid (ABA), enhanced the production of cryptotanshinone, tanshinone I and IIA. Tanshinone I and cryptotanshinone accumulation was 5- and 7.5-fold higher with the addition of 1.0 mg/l ABA and TDZ, respectively, compared to root of greenhouse-grown plant. Cryptotanshinone content was 6.3-, 5.0- and 3.75-fold higher in hairy roots grown under the influence of TDZ, ABA and BA, respectively, compared to a commercial herb. The devised protocol can be used to enhance tanshinone production.

Keywords: Hairy root culture; *Salvia miltiorrhiza* Bunge; Tanshinone.

INTRODUCTION

Plant cell cultures are being considered as an alternative, more sustainable production method for valuable phytochemicals. Tissue culture has also become an alternative way to obtain products when conventional methods or economic viability are challenged. Furthermore, plant metabolites are affected by climatic and seasonal variations, thus their growth in controlled environments overcomes several of their production limitations. Organized cultures, especially root cultures, can make a significant contribution to secondary metabolite production. *A. rhizogenes* causes hairy root disease in plants. The neoplastic (cancerous) roots produced by *A. rhizogenes* infection are characterized by high growth rate, genetic stability and growth in hormone-free media (Srivastava and Srivastava, 2007). These genetically transformed root cultures can produce higher levels of secondary metabolites comparable to that of intact plants. It is a valuable tool for plant biotechnology and a promising tissue culture method for mass production of useful plant secondary metabolites (Giri and Narasu, 2000). The greatest advantage of hairy roots is that they often exhibit similar or greater biosynthetic capacity for secondary metabolite production as compared to their mother plants (Kim et al., 2002a, b). Many valuable secondary metabolites are synthesized in roots and often the synthesis is linked to root differentiation (Flores et al., 1999). Even in cases where a particular secondary metabolite accumulates only in the aerial part of an intact plant, hairy root cultures have accumulated this same metabolite (Wallaart et al., 1999). Hairy root cultures are also known to produce a spectrum of secondary metabolites that are not present in the parent plant (Veerasham, 2004). Medicinal plants have been widely explored for hairy root culture and their bioactive principles (Guillon et al., 2006; Zhou and Wu, 2006). *S. miltiorrhiza* Bunge (Lamiaceae) is a well-known Chinese herb and the rhizome is known as 'tanshen' or 'danshen', an ancient drug in traditional Chinese medicine (Duke and Avensu, 1985). It has been used widely to treat coronary heart disease, menstrual disorders, miscarriage, hypertension, and viral hepatitis (Chang and But, 1985). Quinones are biologically important compounds because of their cytotoxic activity and pharmacological action. More than 50 ortho-quinone diterpenes, called tanshinone, have been isolated from danshen. *S. miltiorrhiza* roots contain two major classes of chemicals,

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a class of lipid-soluble diterpene quinone pigments, generally known as tanshinone, and a class of water-soluble phenolic acids (Tang and Eisenbrand, 1992). Among these tanshinones, cryptotanshinone is a typical compound having an ortho-quinone skeleton. Cryptotanshinone acts as an effective topoisomerase I inhibitor (Gantchev and Hunting, 1997; Lee and Hong, 1998) and exhibits significant cytotoxicity against a number of cultured human tumor cell lines (Ryu et al., 1997). The antitumor activities of the quinones, including naphthoquinone, were revealed more than three decades ago when the National Cancer Institute published a report in which 1500 synthetic and natural quinones were screened for their anticancer activities (Driscoll et al., 1974). Many 1, 2-naphthoquinones were reported to be cytotoxic against a number of tumor cell lines. Because of their effectiveness in drug-resistant cells, these agents appear to hold promise as effective chemotherapeutic agents (Dolan et al., 1998). The hairy roots of *S. miltiorrhiza* have been established as an alternative method to obtain secondary metabolites without exploiting plants from their natural habitats (Hu and Alfermann, 1993; Chen et al., 1999). This work aims to enhance the accumulation of three tanshinones *i.e*. cryptotanshinone, tanshinone I and IIA in hairy roots of *S. miltiorrhiza* and to study the effects of different PGR on hairy root growth and tanshinone accumulation.

MATERIALS AND METHODS

Plant materials and tissue culture

Salvia miltiorrhiza plants were collected from the mountains of China's Henan Province, Zhengzhou City, with the help of the Department of Traditional Chinese Medicine, China Medical University, Taiwan. *S. miltiorrhiza* leaves were washed with running tap water and excess moisture was removed with filter paper. Leaves were surface-sterilized with 70% v/v ethanol for 30 s; followed by three washes of sterile distilled water. Leaves were further sterilized with 0.5% (w/v) sodium hypochlorite (NaOCl) containing 0.02% Tween 20 for 10 min in an ultrasonic oscillator (15 Amp), followed by three washes of sterile distilled water (to remove traces of hypochlorite). The sterilized leaves were transferred aseptically on sterilized filter paper, to remove moisture from the leaf surfaces, and inoculated on MS media (Murashige and Skoog, 1962) supplemented with 3% of sucrose, 1.0 μM BA (6-Benzyladenine) and 0.1 μM NAA (naphthalene acetic acid).

Culture Conditions and Data Analysis

Uniform culture conditions were applied for all the experiments. The pH of the media was adjusted to 5.7 before autoclaving. The media was autoclaved for 15 min at a pressure of 1.05 kg cm^{-2} at 121°C. Cultures were incubated at 25±2°C in the dark. All experiments were repeated three times, each with 10 replicates. Data were analyzed statistically using Fisher's protected least significant difference (LSD) test at the 5% probability level.

Agrobacterium rhizogenes-mediated transformation and time course studies

Leaves were cut into 0.25 cm^2 pieces and a small cut was made perpendicular to the mid rib into the center of the squared leaf. Explants were precultured on MS basal medium for one day prior to infection. *A. rhizogenes* strains BCRC15010 (Food Industry Development Institute, Taiwan) were grown on BEP (beef extract and peptone) medium at 28°C in the dark overnight. The cultures were inoculated into fresh BEP media and grown for 8-10 h. Cells were collected by centrifugation and resuspended in liquid MS medium at an OD_{580} of 0.4. Precultured explants were submerged into the bacterial suspension for 30 min by shaking. After blotting off the excess bacteria suspension, discs were transferred to MS basal medium and co-cultivated for two days. They were then cultured on MS basal medium supplemented with 3% sucrose, cefotaxime (200 mg l^{-1}) and gelled with 0.9% agar (w/v). Hairy roots, which developed from the infected areas at the end of two weeks, were individually excised after three weeks and transferred to B5 medium (Gamborg et al., 1968) containing 200 mg l^{-1} of cefotaxime. The induced roots were successfully cleared of bacteria by several passes through medium containing decreasing concentrations of cefotaxime. Hairy roots obtained from a single clone were transferred to 70 ml B5 liquid medium in 250 ml flasks and kept at 100 rpm. The clones were selected on the basis of rapid growth. The cultures were subcultured every three weeks and used for further analysis.

Hairy roots obtained from suspension culture were cut (1.5 cm, ~5.4 mg dw) and transferred to 250 ml flask containing 70 ml of B5 liquid media. Roots were kept in growth chamber at 25±2°C at 100 rpm rotation in the dark as described by Ge and Wu (2004). The hairy roots were harvested every second week for 12 weeks and their dry weight (dw) was recorded. A growth curve was plotted between weeks of proliferation and total mass gain by the growing hairy roots (Figure 2).

Isolation of genomic DNA

Genomic DNA from the hairy roots and normal roots (control) of *S. miltiorrhiza* were isolated following the procedure by Cocciolone and Cone (1993), with minor modifications. Fresh hairy root tissues (0.2 g) were harvested, frozen in liquid nitrogen, and ground into fine powder. The frozen powder was transferred to a 2 ml microcentrifuge tube containing 0.7 ml lysis buffer (0.30 M NaCl, 50 mM Tris-HCI pH 8.0, 20 mM EDTA, 7 M urea and 1% sarkosyl). The samples were mixed gently. One ml pheno1: chloroform: isoamyl alcohol (25:24:l) solution was added and mixed until the layers disappeared. The sample was kept at room temperature for 15 min followed by centrifugation at 5000 ×*g* for 10 min. The aqueous solution was transferred to a new tube and DNA was precipitated by adding 1/10 vol of 3 M sodium acetate, pH 5.2, and 1 vol

of isopropanol. DNA was collected by centrifugation (5000 ×*g*) for 10 min at 4°C. The supernatant was discarded and the pellet was washed with 500 µl of 70% ethanol followed by centrifugation for 30 s. The supernatant was discarded and the DNA pellet was re-suspended in 20 µl TE and stored at 4°C.

Isolation of plasmid DNA

Plasmid DNA from *A. rhizogenes* strain BCRC15010 was isolated using the alkaline lysis method (Sambrook et al., 1989).

PCR analysis

PCR analysis was carried out in an Eppendorf Mastercycler Gradient in 25 µl reaction mixtures containing commercial buffer, 2.5 mM $MgCl_2$, 200 µM dNTP, 3 U Taq polymerase, DNA (100 *ng* total DNA, or 10 *pg* plasmids), and oligonucleotide primers in 1.0 µM final concentration. To amplify internal '*rol*' B and C gene fragment (780 and 540 bp respectively; Bulgakov, 2008), the following primer pairs were used:

'*rol*'B-1: 5′-ATGGATCCCAAATTGCTATTCCCCC ACGA-3′

'*rol*'B-2: 5′-TTAGGCTTCTTTCATTCGGTTTACTG CAGC-3′

'*rol*'C-1: 5′-ATGGCTGAAGACGACCTGTGTT-3′

'*rol*'C-2: 5′-TTAGCCGATTGCAAACTTGCAC-3′

PCR reaction profiles involved pre-denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 45 s, annealing at 57°C for 30 s, extension at 72°C for 45 s, a final extension for 10 min at 72°C, and storage at 4°C. The amplified products were analyzed on 1% agarose-ethidium bromide gels.

Growth regulators and tanshinone accumulation

Selected lines were used to study the effect of PGR on growth and accumulation of tanshinone. B5 media supplemented with 1.0 mg l^{-1} concentration of different auxins (2,4 di phenoxy acetic acid {2,4-D}, indole 3 acetic acid {IAA}, NAA), cytokinins (BA, kinetin, thiadiazuron {TDZ}, zeatin) and indole butyric acid (IBA) were used to grow the hairy root. The increase in total biomass and tanshinone content were analyzed 12 weeks after subculture.

HPLC Analysis

Reagents, materials and conditions. HPLC-grade methanol was purchased from Merck (Germany). An L-2130 pump, L-2200 auto injector and L-2459 diode array detector system were purchased from Hitachi. Symmetry Waters column C18 (5 µm, 4.6 × 250 mm) were used for HPLC analysis. Milli Q water (Millipore, Milford, MA, USA) was used for all the analysis. Acetonitrile: water (58:42) was used for the mobile phase at room temperature. Samples were detected at the wavelength of 245 nm with flow rate of 1 ml min^{-1}.

Preparation of HPLC standard and root sample. Cryptotanshinone, tanshinone I and IIA were purchased from Jiuding Biotechnology Companies (Taiwan) for the standard. Standard solutions were prepared by dissolving 2 mg of each standard in 2 ml of MeOH. Dissolved solutions (1.0 mg ml^{-1}) were filtered through a 0.22 µm filter (Millipore, USA) and further diluted to the concentration of 0.25, 0.125, 0.0625, 0.03125, 0.015625 and 0.001 mg ml^{-1}. Calibration curves were established based on six points covering a concentration range of 0.25-0.001 mg ml^{-1} for all three standards. 10 µl of standard solution was used for HPLC injections. Calibration graphs were plotted based on linear regression analyses of the peak areas in response to concentrations of standards injected. The repeatability of the migration time and peak areas of cryptotanshinone, tanshinone I and II A in the experiment were determined by repeated injection (n = 3) of each standard mixture under the optimum conditions. The roots were collected from the culture flask and their fresh weight was recorded. The roots were then freeze-dried for 48 h and their dry weight was determined. About 1.0 gm of dried roots was crushed into fine powder and dissolved in 30 ml of methanol. It was ultra-sonicated for 30 min and filtered through Whatman No 1 filter paper. This process was repeated three times for each sample. After filtration, the combined methanol extracts were evaporated to dryness with the help of rotary evaporator. The residue was dissolved in 10 ml methanol and filtered through a 0.22 µm (Millipore, USA) membrane before analysis.

RESULTS

Initiation of hairy root culture and their molecular analysis

Hairy root initiations were observed in 75-80% infected leaves after three weeks of infection with *A. rhizogenes* strains BCRC15010. Hairy roots were isolated individually and grown on B5 solid medium supplemented with 200 mg l^{-1} cefotaxime. After 4-5 subcultures, the roots were able to grow without *A. rhizogenes* in the absence of cefotaxime and were negatively geotropic. Transformation was confirmed by polymerase chain reaction using gene-specific primers ('*rol*' *B* and *C*). PCR amplification with specific primers was used to show the integration of '*rol*' B and C genes in to the genome of *S. miltiorrhiza*. When amplified with specific primers, these isolated DNA showed '*rol*' *B* and *C* gene amplification of 540 and 780 bp respectively, while no amplification was observed in non-transformed roots (Figure 1).

Tanshinone accumulated during hairy root growth

There was a significant increase in dry weight of the hairy roots over consecutive weeks. After 12 weeks, the dry weight had increased 34 times (5.4 to 185 mg; Figure 2). HPLC analysis for the three compounds *i.e*. cryptotanshinone, tanshinone I and IIA, revealed their increased

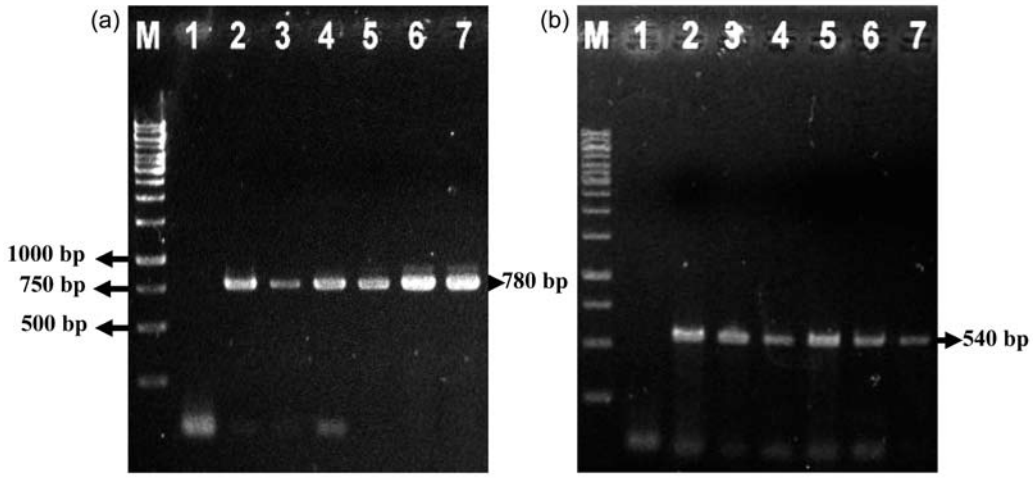

Figure 1. PCR confirmation of transgenic hairy roots of *Salvia miltiorrhiza* Bunge showing the amplification of '*rol*' *B* (780 bp) (a) and '*rol*' *C* (540 bp) (b) genes. Lane M: marker; Lane 1: non-transgenic line; Lanes 2-7: putatively transformed hairy root lines.

accumulation over consecutive weeks (Figure 3). Cryptotanshinone accumulation was observed four weeks after subculture and reached maximum accumulation at 10 weeks (1.301 mg g^{-1} dw). Accumulation of tanshinone I and IIA started six weeks after subculture and reached to maximum after 10 weeks (0.351 and 0.156 mg g-1 dw respectively).

Effects of different growth regulators on tanshinone accumulation

Stable transgenic lines with high multiplication rates were selected for analysis. An 18-fold (98.67 mg from 5.4 mg) increase in dry weight (dw) was observed after 12 weeks culture in PGR-free MS medium. We investigated the effects of exogenous PGR on the growth and tanshinone accumulation in transformed roots of *S. miltiorrhiza*. Hairy roots were cultured in medium containing various concentrations of PGR and the results are summarized in Table 1. There was no significant difference in hairy root multiplication with ABA, IAA, NAA and kinetin (101.67, 103.00, 116.67 and 118.33 mg) compared to the control (98.67 mg). However a significant difference in root multiplication was observed with IBA, BA and TDZ (137.00, 139.33 and 185.00 mg respectively). The multiplication of hairy roots was in direct proportion to the increased rate of root branching and elongation. The total dry weight gains were 25-, 25- and 34-fold after 12 weeks with 1.0 mg l^{-1} IBA, BA and TDZ, respectively. Although ABA had minimal influence on hairy root multiplication, we observed 2-fold increase in tanshinone I accumulation and 3-fold higher tanshinone IIA and cryptotanshinone. The total tanshinone content was thus 2.5 times higher in roots grown under the influence of ABA compared to the control. On other hand, TDZ not only influenced hairy root multiplication but also tanshinone accumulation. We observed 2.0-, 1.5- and 3.5-fold higher tanshinone I, tanshinone IIA and cryptotanshinone, respectively with TDZ. Hence, the total

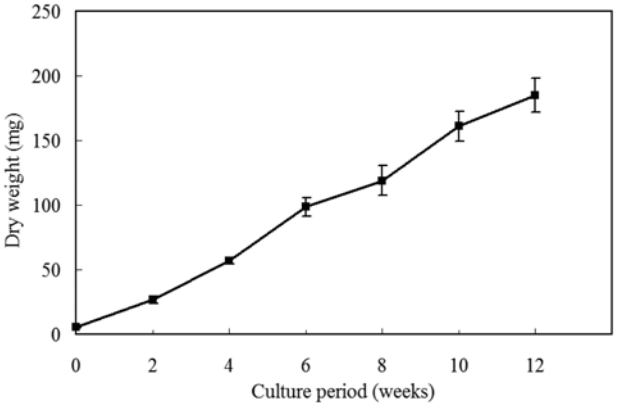

Figure 2. The growth course of hairy root of '*Salvia miltiorrhiza* Bunge' in liquid medium* containing 1.0 mg l^{-1} TDZ. *B5 basal salts supplemented with 3% sucrose. Observations were recorded after 12 weeks of culture. Error bars for standard errors (SE), n=3.

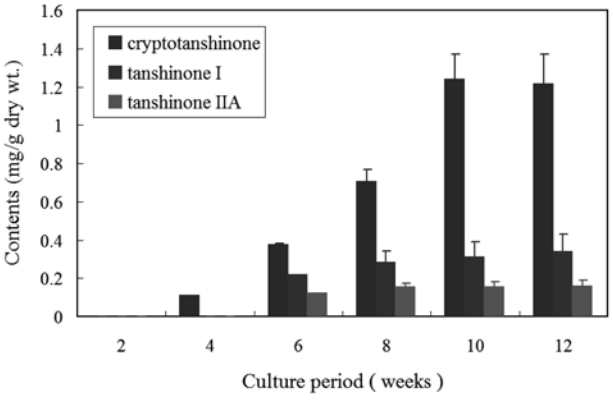

Figure 3. Effect of different culture periods on the production of tanshinone in hairy roots of *Salvia miltiorrhiza* Bunge. Error bars for standard errors (SE), n=3.

Table 1. Effect of different PGRs on hairy root production and tanshinone accumulation in *Salvia miltiorrhiza* in liquid medium*.

PGR constituents	Average dry weight after 12 weeks (mg)	Weight gain (folds)	TI[a] (mg g^{-1} of dw)	TII[b] (mg g^{-1} of dw)	CT[c] (mg g^{-1} of dw***)
0	98.67±7.22[b]	18	0.216±0.06	0.120±0.02	0.374±0.06
NAA (5.37 µM)	116.67±8.95[ab]	21	ND	0.069±0.01	0.158±0.04
IAA (5.71 µM)	103.00±10.07[b]	19	0.134±0.05	0.097±0.02	0.249±0.05
IBA (4.90 µM)	137.00±10.15[a]	25	ND	0.069±0.02	0.195±0.02
BA (4.44 µM)	139.33±8.76[a]	25	0.430±0.06	0.300±0.04	0.772±0.09
Kinetin (4.65 µM)	118.33±11.02[ab]	22	0.316±0.06	0.210±0.02	0.534±0.07
TDZ (4.54 µM)	185.00±8.14[a]	34	0.393±0.03	0.195±0.03	1.310±0.17
ABA (3.76 µM)	101.67±6.69[b]	19	0.451±0.05	0.341±0.02	1.038±0.11

*B5 basal salts supplemented with 3% sucrose. Observations were recorded after 12 weeks of culture.
**Value are means ± standard error, n = 3. Means followed by the same letter are not significantly different at 5% level by LSD (least significant difference) test.
***Dry weight.
[a]Tanshinone I; [b]Tanshinone IIA; [c]Cryptotanshinone; ND- not detected.

tanshinone content was 2.7-fold in hairy roots grown under the influence of TDZ.

Comparative analysis of tanshinones

Three different tanshinones (cryptotanshinone, tanshinone I and IIA) were measured from the normal roots grown in greenhouse and from the commercial herb available in the market. The contents were compared with the contents of hairy roots grown under the influence of different PGRs. Tanshinone I content was approximately 4-fold (0.384 mg g^{-1}) higher in the commercial herb, while, it was 5.0- (0.451 mg g^{-1}), 4.6- (0.430 mg g^{-1}), 4.2-fold (0.393 mg g^{-1}) higher in the hairy root grown under the influence of ABA, BA and TDZ respectively, compared to the root of greenhouse-grown plant (0.093 mg g^{-1}). Tanshinone IIA content in the commercial herb (0.452 mg g^{-1}) was similar to the root of the greenhouse-grown plant; and no significant influence of ABA, BA and TDZ on tanshinone IIA accumulation was observed. However, these regulators had profound influence on cryptotanshinone accumulation in hairy roots, cryptotanshinone content increased 7.6-fold (1.310 mg g^{-1}), 6-fold (1.038 mg g^{-1}) and 4.5-fold (0.772 mg g^{-1}) under the influence of TDZ, ABA and BA respectively, compared to roots from the greenhouse-grown plants. There were no significant difference in tanshinone I and IIA content in PGR-influenced hairy roots compared with commercial herbs, however a significant increase in cryptotanshinone content was observed in PGR-influenced hairy roots compared to commercial herbs. Cryptotanshinone content was 6.3- (1.310 mg g^{-1}), 5.0- (1.038 mg g^{-1}) and 3.75- (0.772 mg g^{-1}) fold higher in hairy roots grown with TDZ, ABA and BA respectively (Tables 1 and 2).

Table 2. Comparative analysis of tanshinone contents from greenhouse-grown plant and commercial herb of *Salvia miltiorrhiza*.

Source	Tanshinone content		
	TI[a] (mg g^{-1})	TII[b] (mg g^{-1})	CT[c] (mg g^{-1})
Root from greenhouse-grown plant	0.093±0.01	0.445±0.09	0.173±0.04
Commercial herb	0.384±0.02	0.452±0.04	0.205±0.04

**Value are means ± standard error, n = 3. Means followed by the same letter are not significantly different at 5 % level by LSD (least significant difference) test.
[a]Tanshinone I; [b]Tanshinone IIA; [c]Cryptotanshinone.

DISCUSSION

Salvia miltiorrhiza contains a large number of therapeutically-useful metabolites including diterpenes, quinones and phenolics. Since these are primarily located in the roots, the hairy root culturing method provides a promising means for large scale metabolite production. Several investigations have been carried out on *in vitro* regeneration of roots using leaves as the starting material (Zhi and Alfermann, 1993; Kintzios et al., 2004; Tao and Li, 2005; Yan et al., 2005; Lee et al., 2007b). The success of hairy root induction depends on the type of explants and *Agrobacterium* strains. Different strains of *A. rhizogenes* have differentially influenced hairy root induction in 'golden flax *Linum flavum*' leaves, (Lin et al., 2003). Sudha et al. (2003) have reported that leaf tissue produced more hairy roots than other plant tissue upon induction in *Rauwolfia micrantha*. We have found that amongst two different strains of *A. rhizogenes* (BCRC15010 and BCRC15722), the former responded better to hairy root induction (data not shown). Approximately 78% of leaves responded to hairy root induction. The response of hairy root proliferation on hormone-free B5 solid media was similar to that reported by Liao and Jhuang (2007) for Paulownia (*Paulownia*

fortunei). The proliferating roots were subcultured every three weeks to select the best line. During the subculture most of the hairy root growth was arrested, they turned brown and some died. A few roots grew rapidly with multiple branching and negative geotropism. The thick and fast-growing hairy roots without buds were maintained on B5 liquid medium and used for further experiments. Hairy root growth response varied with different transgenic lines and even in the branches of the same lines; a similar phenomenon has been reported by Kang et al. (2006) in Aralia (*Aralia elata*) hairy roots, where the growth and multiplication of hairy roots depended on the expression of the '*rol*' gene. The multiplication rate of *Datura* hairy roots varied between different lines and doubling time took 24 to 90 h (Payne et al., 1987). The doubling time of *S. miltiorrhiza* hairy root was 18 days. Many studies have pointed out that hairy root growth and metabolite production depend on '*rol*' gene expression (Palazon et al., 1998; Shkryl et al., 2008; Bulgakov, 2008). A growth curve was plotted to monitor the growth of hairy root and metabolite accumulation (Figure 2). Only cryptotanshinone accumulated four weeks after subculture (0.109 mg g^{-1} of dw), while, tanshinone I and IIA have started accumulating six weeks after subculture (0.218 and 0.122 mg g^{-1} of dw respectively). The maximum accumulations of cryptotanshinone, tanshinone I and IIA was observed 10 weeks after culture (Figure 3).

In vitro plant cell culture usually requires the presence of PGR, mainly auxins and cytokinins. As for hairy roots, one characteristic of their phenotype is the rapid hormone- independent growth. As a result, the media used to culture hairy roots generally lacks PGR. Even more, as demonstrated in transformed roots of '*Datura stramonium*', cultures treated with 2.0 and 0.2 mg l^{-1} NAA and kinetin respectively have induced root tissue de-differentiation (Ford et al., 1996). In several experiments, this de-differentiation have influenced a significant decrease or even the cessation of alkaloid production (Robbins et al., 1996). However, a more recent systematic test on the effects of different types of phyto-hormones upon root growth and secondary metabolite production showed that some of them have enhanced either growth or metabolite production. The growth of hairy roots of *Artemisia annua* (Weathers et al., 2005) has been evaluated under the influence of five different hormones: auxins, cytokinins, ethylene, gibberellins (GA) and abscissic acid (ABA). The highest biomass was obtained with 1-5 mg l^{-1} ABA, while 0.5-1.0 mg 1^{-1} 2-isopentenyladenine inhibited root growth but enhanced artemisinin accumulation. Different auxins have different effects on hairy root morphology and root formation. Washida et al. (2004) have reported that increased ginsenosides (ginsenoside) content was observed in ginseng (*Panax ginseng*) hairy root culture medium supplemented with individual or combined auxins. However, Lee et al. (2007a) have reported the inhibitory effect of different auxins on Ludin (rutin) levels in buckwheat (*Fagopyrum esculentum* M.). Similarly, auxins have an inhibitory effect on different tanshinone accumulations in *S. miltiorrhiza* hairy roots. The studies by Yoshimatsu et al. (2004) on Australian eggplant (*Duboisia myoporoides*) showed a negative correlation between auxin (IAA) concentration and secondary metabolite accumulation. The highest number of roots, and thus metabolites accumulation, was obtained when the leaf of *Plumbago zeylanicas* was cultured on MS medium with 1.0 mg l^{-1} IBA and 0.5 mg l^{-1} NAA (Sivanesan and Jeong, 2009). *S. miltiorrhiza* hairy roots showed similar responses. ABA can inhibit seed germination, leaf aging, stomatal closure, and trigger defensive gene activation (Zhao et al., 2005), but it has no significant effect on hairy root growth in henbane (*Hyoscyamus muticus*) or, as we found, in *S. miltiorrhiza*. ABA does trigger metabolite accumulation and all three tanshinones together increased 2.6-fold with 1.0 mg 1^{-1} ABA. There were no significant differences in tanshinone I and IIA accumulation in PGR-influenced hairy roots and the commercial herb, however differences were found in their cryptotanshinone content. Cryptotanshinone content in hairy roots grown under the influence of TDZ, ABA and BA, respectively, was 6.3- (1.310 mg g^{-1}), 5.0- (1.038 mg g^{-1}) and 3.75-fold (0.772 mg g^{-1}) that of the commercial herb. Zhang et al. (2004) have reported the total tanshinone content 0.54, and 2.50 mg g^{-1}, when hairy roots of S. miltiorrhiza were supplemented with sucrose alone or in combination with Ag^{+}. However, we reported total tanshinone content in hairy root of *S. miltiorrhiza*, which was 1.898 and 1.830 mg g^{-1} with TDZ and ABA, respectively. The total tanshinone content have increased 4.5- and 10-fold, when hairy root of *S. miltiorrhiza*, supplemented with either sorbitol or sorbitol and yeast elicitor together (Shi et al., 2007). Hence, the synergistic effects of biotic and abiotic elicitors are more effective for tanshinone accumulation. We have not yet tried or studied elicitors for their effects on tanshinone accumulation, but since they may increase total tanshinone content, they may be the subject of further research. Plant secondary metabolite analysis is an important way to measure their responses to biotic and abiotic stresses. Plants use multiple signal pathways to mediate their response to different stresses, and these signal pathways can converge at certain points, leading to common cellular processes (Zhao et al., 2005). The results also showed that different PGRs induced higher cryptotanshinone accumulation.

CONCLUSION

Tanshinones are the major compounds of danshen and are used to treat Alzheimer's disease, insomnia, vascular dementia, memory loss, attention disorders, heart disease, cancer, etc. The present study was designed to enhance the production of tanshinones in hairy roots of Salvia in liquid culture using PGRs. *A. rhizogenes* (BCRC 15010) was used for hairy root induction. The putative transformed roots were grown on PGR-free B5 media. Gene integration was confirmed by polymerase chain reaction. The hairy roots were grown under the influence of different auxins (IAA, IBA and NAA), cytokinins (BA, kinetin and

TDZ) and ABA. Three different tanshinones, i.e. cryptotanshinone and tanshinone I and IIA, were analyzed using HPLC. Different PGRs had inhibitory effect on tanshinone IIA accumulation in hairy roots, whereas it enhanced cryptotanshinone and tanshinone I accumulation. Tanshinone I content have increased 5-fold (0.451 mg g^{-1}) in the hairy roots grown under the influence of 1.0 mg l^{-1} ABA, whereas, cryptotanshinone content increased 7.5-fold under the influence of 1.0 mg l^{-1} TDZ. The present work aims to increase the tanshinone I and cryptotanshinone contents by tissue culture methodologies, which may be helpful to produce cost-effective drugs. The use of different biotic and abiotic elicitors may increase the total tanshinone content and may be the subject of further research.

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植物生長調節劑對丹參毛狀根液態培養累積丹參酮之影響

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丹參 (*Salvia miltiorrhiza* Bunge) 所含之二萜類丹參酮化合物於現代藥理研究證實其對許多疾病具有良好的療效。本研究以丹參葉片爲材料，建立毛狀根培養系統，並探討植物生長調節劑對丹參毛狀根生產 3 種丹參酮 (tanshinone-I, tanshinone-IIA, cryptotanshinone) 之影響。以農桿根群菌 (*Agrobacterium rhizogenes*）BCRC15010 菌系感染丹參葉片培植體可誘導毛狀根形成率達 78%。轉殖毛狀根之鑑定則利用農桿根群菌 *rolB* 及 *rolC* 基因序列爲引子，進行 PCR 反應並比對其基因片段序列後加以證實。誘導所得之丹參毛狀根於無光照條件下培養於 B5 液體培養基中具有生長迅速的特性。丹參毛狀根培養在含不同植物生長調節劑 (auxins, cytokinins 與 abscisic acid) 的培養基中均可增加丹參酮類產生；毛狀根培養在含 1 mg/L ABA (abscisic acid) 或 TDZ (thidiazuron) 培養基中時，其 tanshinone-I 與 cryptotanshinone 含量分別是溫室植株根部含量的 5 與 7.5 倍；添加 TDZ, ABA 或 BA (benzyladenine) 於培養基中可提升 cryptotanshinone 之含量，其值分別爲市售藥材之 6.3, 5.0 與 3.75 倍。本研究結果顯示添加植物生長調節劑可作爲提升丹參酮產量之有效策略。

關鍵詞：丹參；毛狀根培養；丹參酮。