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Exogenous ABA and polyamines enhanced salvianolic acids contents in hairy root cultures of *Salvia miltiorrhiza* Bge. f.alba

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Abstract

Salvia miltiorrhiza Bge. f.alba is a variety of *S. miltiorrhiza* Bunge which is a well-known and very important traditional chinese medicinal herb. In this study, we successfully induced hairy roots from leaves of *S. miltiorrhiza* Bge.f.alba by *Agrobacterium rhizogenes* ACCC10060. As determined by high performance liquid chromatography (HPLC), these hairy root cultures had the ability to produce salvianolic acid B and salvianic acid A, two main salvianolic acids in *S. miltiorrhiza* Bge.f.alba. The effects of ABA and polyamines (PAs, including putrescine (Put), spermidine (Spd) and spermine (Spe)) on salvianolic acids production were studied. The results showed that phenylalanine ammonia-lyase (PAL) activity, salvianolic acid B and salvianic acid A contents increased 1.8, 2.0 and 3.3 times after 80 μmol L^{-1} ABA treatment for 10 d, 12 d and 10 d, respectively. Similarly, Put was the most effective polyamine among the three PAs, and the effects of Put and Spd for salvianolic acids production were better than that of Spe. Moreover, hairy root cultures treated with 50 mg L^{-1} Put produced the highest levels of hairy root growth (13.23 g L^{-1} culture), salvianolic acid B (12.13 mg g^{-1} DW) and salvianic acid A (3.95 mg g^{-1} DW) among the three PAs. After treated with the mixture of Put and Spd (50 mg L^{-1}each) for 10 d, 12 d and 10 d respectively, PAL activity, salvianolic acid B and salvianic acid A production were about 1.82, 2.05 and 3.45 times as those in the control, respectively. These findings indicate that exogenous ABA and PAs can enhance two salvianolic acids production in hairy root cultures of *S. miltiorrhiza* Bge.f.alba.

Keywords: *Salvia miltiorrhiza* Bge.f.alba; hairy roots; ABA; polyamines; salvianolic acid B; salvianic acid A.
Abbreviation: ABA-abscissic acid; PAs-polyamines; Put-putrescine; Spd-spermidine; Spe-spermine; DW-dry weight; PAL-phenylalanine ammonia-lyase; HPLC-high-performance liquid chromatography; Cef-cefotaxime; MS-Murashige and Skoog; B5-Gamborg's B5; WPM-Woody Plant Medium.

Introduction

Production of secondary metabolites of distinct and complex structures in plant cell cultures has been extensively explored. Hairy root cultures are in vitro plant tissue cultures established by transformation of plant cells with *Agrobacterium rhizogenes*. Hairy root cultures have many advantages, including biochemical and genetic stability, independence from seasonal and geographical conditions, rapid growth, and the ability to produce secondary metabolites at the levels comparable to those of the plants grown naturally. As a result, these cultures have been used to produce pharmaceuticals, cosmetics and food additives from many plant species **(**Giri and Narasu, 2000). Various elicitors have been studied for the higher production of plant secondary metabolites. The most important PAs in higher plants are Put, Spd and Spm, all of which play important roles in growth process, cell division and plant response to abiotic stress (Cacho et al., 2010). The best known relationship between PAs and secondary metabolism is the production of hundreds of alkaloids (Cacho et al., 2010) and other secondary products, such as coumarins, which are phenolic compounds derived from the shikimate pathway, capsaicin, which are intermediates in the biosynthetic pathway of flavonoids (Sudha and Ravishankar, 2003), betalaine production in hairy root cultures of *Beta vulgaris* (Suresh et al., 2004), silymarin producdin the milk thistle (Cacho et al., 2010) and rosmarinic acid production in hairy root cultures of *Nepeta cataria* L. Abscisic acid (ABA) is an essential signal for plant resistance to pathogens and biosynthesis of secondary metabolites. Nagira et al. (2006) reported that changes in the amount of endogenous ABA may play an important role in the induction of anthocyanin synthesis and chlorophyll degradation in regenerated torenia shoots. Luo et al. (2001) showed that ABA stimulated the synthesis in paclitaxel in plant cell cultures which depended on the dosage of ABA as well as the growth stage of the cells. Our previous study suggested that a causal relationship between ABA release and both PAL activity and flavonoids accumulation under fungal endophytes treatment, and that ABA is involved in fungal endophytes-induced flavonoids accumulation in suspension cells of *Ginkgo biloba* (Hao et al., 2010). *Salvia miltiorrhiza* Bunge, named Dan Shen in China, is a well-known and very important traditional chinese medicinal herb that is used for the treatment of various cardiovascular diseases, such as menstrual disorders, blood circulation disturbance and inflammation (Chen et al., 2005; Wu et al., 2007) .White flowered *S. miltiorrhiza* Bge.f.alba grown only in Shandong province of China is a varietas of *S. miltiorrhiza* Bunge. The morphological difference between white flowered *S. miltiorrhiza* Bge.f.alba and *S. miltiorrhiza* Bunge is the color of flowers. The flower color of *S.miltiorrhiza* Bunge is purple, while that of *S. miltiorrhiza* Bge.f.alba is white. Except for morphological difference, *S. miltiorrhiza* Bge.f.alba also has special pharmacological effect for treatment of thromboangiitis obliterans (Hao et al., 2009). Studies showed

that caffeic acid-derived phenolic acids contents in white flower *S. miltiorrhiza* Bge.f.alba were about two times higher than those in *S. miltiorrhiza* Bunge (Hao et al.,2009). Qi et al. (2004) also reported that the trace elements in the contents of Fe, Mg, Mn in white flower *S. miltiorrhiza* Bge.f.alba were more than those in *S. miltiorrhiza* Bunge. Jiao et al. (2007) found that *S. miltiorrhiza* Bge.f.alba root preparation could inhibit the proliferation and induce the apoptosis of human gastric cancer cells. *S. miltiorrhiza* Bge.f.alba significantly increases cerebral blood flow, reduces neuronal apoptosis, and promotes neuronal regeneration in rats with cerebral ischemia/reperfusion impairment (Zhang et al., 2010). These results indicated that white flower *S. miltiorrhiza* Bge.f.alba has higher pharmaceutical values. Recently, our group successfully produced seedlings of *S. miltiorrhiza* Bge.f.alba from stems and leaves culture (Sun et al., 2008), and constructed full-length cDNA library of white flower *S. miltiorrhiza* Bge. f.alba root (Hao et al., 2009). There is no report about induction of hairy roots and salvianolic acids production in hairy root cultures of *S. miltiorrhiza* Bge. f.alba. In this study, hairy roots were successfully induced from leaves of *S. miltiorrhiza* Bge. f.alba by *A. rhizogenes* ACCC10060, and exogenous ABA and polyamines were used as elicitors to investigate the influence and/or induction effect on the synthesis of salvianolic acids in the culture of the induced hairy roots.

Results

Establishment of hairy root cultures of S. miltiorrhiza Bge.f.alba

Hairy root formation and culture system of *S. miltiorrhiza* Bge.f.alba for salvianolic acids production was established using *A. rhizogenes* ACCC10060. Two different explants, leaf and stem, were investigated for hairy roots induction. Leaf and stem explants of *S. miltiorrhiza* Bge.f.alba were inoculated with *A.rhizogenes* ACCC10060. After 2 days of co-cultivation with *A.rhizogenes*, two explant tissues were transferred to agar-solidified MS medium containing 400 mg L^{-1} cefotaxime (Cef) for removing *A. hizogenes.* Hairy roots emerged within 15 days of bacterial inoculation at the wounded edges of stem and leaf explants. Between the different explants used, leaf explants showed slightly better response in terms of transformation and initiation of hairy roots with the infection frequency 73%. The leaf was chosen (Fig.1), however, as the optimal explant tissue for co-cultivation with *A. rhizogenes*, as the frequency of bacterial infection was higher than that seen in stem explants, and the resulting hairy roots grew more rapidly than those derived from stem. Induced hairy roots (about 2-3cm long) were excised from the explant tissues and subculture on fresh agar-solidified MS medium containing 400 mg L^{-1} Cef. The growing hair roots were transferred to new MS solidified medium with gradual decrease of cefotaxime interval of 7 days. The hairy roots began to grow more rapidly after repeated transfer to fresh medium with gradual decrease of cefotaxime for 6-7 times. Mature hairy roots generally became thicker following subculture. Chen et al.（1999） reported that 6,7-V medium was superior to MS, B5 and WPM medium in terms of both root growth and phenolic compound production of *S.miltiorrhiza*. After repeated transfer to fresh medium for one-two months, hairy root clones with no *A. rhizogenes* were transferred to liquid 6,7-V culture medium. Hairy roots were growing rapidly in liquid 6,7-V medium (Fig.1).

Salvianolic acids production of hairy root cultures of S. miltiorrhiza Bge.f.alba

Hairy roots were cultured in 6,7-V liquid medium for 18 days. Salvianolic acids production was investigated by harvesting hair roots at intervals of 2 days (Fig. 3). From our previous results, salvianolic acid B and salvianic acid A are the two main salvianolic acids in leaves and roots of *S. miltiorrhiza* Bge.f.alba (date not published)**.** So we assayed the production of salvianolic acid B and salvianic acid A in hairy roots of *S. miltiorrhiza* Bge.f.alba. Results showed that salvianolic acid B and salvianic acid A were detected in hairy roots (Fig. 2). In analysis of HPLC, the retention time of salvianic acid A was 11.96 min, and salvianolic acid B was 20.86 min (Fig. 2). So salvianolic acid B and salvianic acid A could be respectively separated and then monitored. During the 18 days culture period, the contents of salvianolic acid B and salvianic acid A dramatically rose. The maximum production of salvianolic acid B and salvianic acid A (8.98 mg g^{-1} DW and 1.21 mg g^{-1} DW respectively) were attained after 14 days of culture (Fig. 3).

Effects of ABA on growth, PAL activity and salvianolic acids production in hairy root cultures

To study the effects of ABA on growth and salvianolic acids production in hairy root cultures, hairy roots were grown for 12 days in media supplemented with various concentrations of ABA. Our results showed that salvianolic acid B and salvianic acid A contents were enhanced by exogenous ABA, and that ABA effect was dose-dependent. As shown in Fig. 4, both the salvianolic acid B and salvianic acid A contents increased with the addition of ABA up to 80 μmol L^{-1}. Above 80 μmol L^{-1}, both salvianolic acid B and salvianic acid A contents decreased. Among the concentration tested, the optimal concentration was 80 μmol L^{-1}, and salvianolic acid B and salvianic acid A production increased from 8.65 mg g^{-1} DW and 1.32 mg g^{-1} DW in the control to 16.14 mg g^{-1} DW and 3.99 mg g^{-1} DW in the treated hairy roots respectively. Our results also showed that ABA was not effective for hair roots growth, and the growth rate of hair roots decreased with the addition of ABA up to 80 μmol L^{-1}. The highest dry weight was obtained (10.72 g L^{-1}) in non-treated hairy roots. Based on these results, a concentration of 80 μmol L^{-1} was chosen for further experiments. Fig. 5 showed the PAL activity and salvianolic acids production in 80 μmol L^{-1} ABA treated hairy roots after different times. All of PAL activity, salvianolic acid B and salvianic acid A contents increased upon stimulation by ABA after 4 days ABA treatment. PAL activity, salvianolic acid B and salvianic acid A contents reached the peak levels of 176.56 nmol mg^{-1} h^{-1}, 17.31 mg g^{-1} and 4.35mg g^{-1} DW in about 10 d, 12 d and 10 d following treatment, respectively (Fig. 5). No obvious change of salvianolic acid B and salvianic acid A contents was observed between 12 d and 16 d. There was a gradual decline in PAL activity from 10 d to 16 d. These data indicated that exogenous ABA assuredly enhanced PAL activity and salvianolic acids contents of hairy root cultures of *S. miltiorrhiza* Bge. f.alba.

Effects of PAs on growth, PAL activity and salvianolic acids production in hairy roots

To examine the effects of PAs on hairy root growth and salvianolic acids production in hairy root cultures of *S. miltiorrhiza* Bge. f.alba, hairy roots were grown for 12 days in media supplemented with various concentrations of

different PAs. All three PAs (Put, Spd and Spe) increased growth, PAL activity and salvianolic acids production in hairy roots (Fig. 6). But Put was the most effective polyamine, and the effects of Put and Spe for salvianolic acids production were better than that of Spd. Hairy root cultures treated with 50 mg L^{-1} Put produced the highest levels of hairy root growth (13.23 g L^{-1} culture), salvianolic acid B (12.13 mg g^{-1} DW) and salvianic acid A (3.95 mg g^{-1} DW) among the three PAs. Like Put, 50 mg L^{-1} Spd also produced the higher levels of hairy roots growth (12.27 g L^{-1} culture), salvianolic acid B (11.34 mg L^{-1} DW) and salvianic acid A (4.01 mg L^{-1} DW) than those of Spe. For both Put and Spe, concentrations up to 50 mg L^{-1} increased the dry weight and salvianolic acids contents; however, higher concentrations decreased these effects. Based on these results, we used the mixture of Put and Spd (50 mg L^{-1} each) for further experiments. Time course for the PAL activity and salvianolic acids synthesis induced by the mixture of Put and Spd (50 mg L^{-1}each) is presented in Fig.7. Both PAL activity and salvianolic acids synthesis were significantly induced after the mixture of Put and Spd treatment for 6 d. As an overall trend, it is quite obvious that all of PAL activity, salvianolic acid B and salvianic acid A contents dramatically rose. PAL activity hit a peak with 179.56 nmol mg^{-1} h^{-1} after 10 d treatment, which was 1.82 times of the control (98.21 nmol mg^{-1} h^{-1}). Salvianolic acid B and salvianic acid A production reached the peak levels of 17.11 $mg.g^{-1}$ and 4.21mg g^{-1} DW in about 12 d and 10 d following treatment, which was about 2.05 and 3.45 times as that of the control, respectively (Fig. 7).

Discussion

The soil-borne plant pathogen *A. rhizogenes* responsible for adventitious (hairy) root formation at the site of infection also causes certain biochemical changes in the plant metabolism. Hairy roots induced *by A. Rhizogenes* have been reported in a number of plant species （Lee et al., 2010; Georgiev et al., 2007. Hairy root cultures have several properties that have promoted their use for plant biotechnological applications. Their fast growth, genetic and biosynthetic stability offer an additional advantage for their use as an alternative to plant cell suspension cultures, for production of secondary metabolites of interest. Phenolic acid compounds production by hairy root culture have been reported in several plant species, such as tartary buckwheat (*Fagopyrum tataricum* Gaertn) (Kim et al., 2009), *Salvia officinalis* (Grzegorczyk et al., 2006), *Ocimum basilicum* (Tada et al., 1996), *Salvia miltiorrhiza* (Chen et al., 2001), *Lactuca virosa* (Stojakowska et al., 2012), *Fagopyrum tataricum (*Uddin et al., 2011), and *Agastache rugosa* (Lee et al., 2008). In the present study, we described, for the first time, an efficient *A.rhizogenes*-mediated transformation protocol for the establishment of *Salvia miltiorrhiza* Bge.f.alba hairy root cultures. Our results showed that leaf is better than stem for hairy root induction (Fig.1), like the hairy root induction of *Nepeta cataria* L (Lee et al., 2010). Specifically, we achieved salvianolic acid B and salvianic acid A production from the induced hairy roots of *S. miltiorrhiza* Bge.f.alba using *A. rhizogenes* ACCC10060 (Fig.2, 3). Our results indicate that hairy root culture is a biotechnological and valuable alternative approach for the production of salvianolic acid from *S. miltiorrhiza* Bge.f.alba. Various elicitors have been studied for the higher production of plant secondary metabolites. Elicitation is the induced or enhanced biosynthesis of metabolites due to addition of trace amounts of elicitors. Elicitor may be defined as a substance which, when introduced in small concentrations to a living

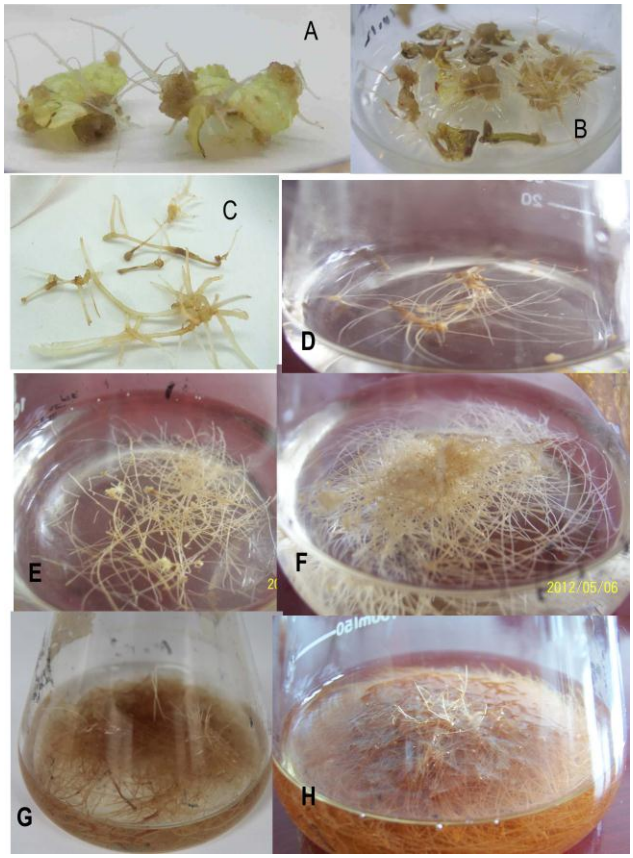

Fig 1. Establishment of hairy root cultures of *S. miltiorrhiza* Bge.f.alba. A: hairy roots emerged from wounded sites 15 days after infection with *A. rhizogenes* ACCC10060; B: hairy roots growing well 20 days after infection with ACCC10060; C: Rapidly growing hairy roots in solid MS medium; D-H: hairy roots cultured in liquid 6,7-V medium without plant growth regulator (D: 5 days culturing E: 10 days culturing; F: 15 days culturing G: 25 days culturing F: 40 days culturing).

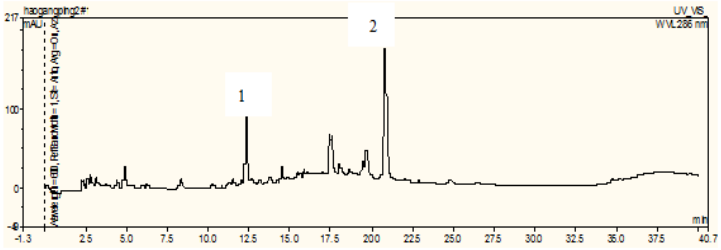

Fig 2. HPLC analysis of salvianic acid A and salvianolic acid B produced by hairy roots of *S. miltiorrhiza* Bge.f.alba . 1 is salvianic acid A, and 2 is salvianolic acid B.

cell system, initiates or improves the biosynthesis of specific compounds. Elicitors can be classified on the basis of their 'nature' like abiotic elicitors or biotic elicitors, or on the basis their 'origin' like exogenous elicitors and endogenous elicitors (Namdeo, 2007). Accumulating evidence suggested that ABA is an important signal molecule involved in the plant response to pathogens, and ABA also involved in plant secondary metabolite synthesis. The involvement of ABA on secondary metabolism accumulation is an intriguing field of research, but the literature on this subject is still controversial,

although some discrepancies might be easily explained, taking into account the different plant species and tissues analysed. With respect to anthocyanin synthesis, ABA application promoted anthocyanin synthesis in leaf discs of *Vitis vinifera* (Pirie and Mullins, 1976) and excised axes of *Phaseolus vulgaris* (Walton and Sondheimer, 1968). The highest paclitaxel production was obtained after exogenous ABA application to *Taxus chinensis* suspension culture cells (Luo et al., 2001). Our previous study also showed that ABA is an essential signaling molecule for triggering the endophyte fungal-induced PAL activation and flavonoid synthesis in *G.biloba* cells (Hao et al., 2010). Reversely, Sun reported that exogenous supplements of ABA to M9 medium significantly decreased the formation of shikonin in the cultured cells during the entire course of culturing (Sun et al., 2007). Results of the present study indicated that 80 μmol L^{-1} ABA could enhance salvianolic acid B and salvianic acid A production. Our results also showed that ABA was not effective for hair roots growth, and growth rate of hair roots decreased with the addition of ABA up to 80 μmol L^{-1}. It has been well established that ABA generally modifies plant growth by inhibiting shoot and root growth as an antagonist of auxin (Himmelbach et al., 1998). The role of ABA in modulating the growth rate of roots has been intensely studied and was found to be concentration-dependent. Low concentrations of ABA can even stimulate root growth, while high levels of ABA inhibit overall plant growth (Himmelbach et al., 1998). This explanation is consistent with our result.

The present study revealed that exogenous supplements of ABA could also induce the activation of PAL and synthesis of salvianolic acid, indicating that ABA was sufficient for triggering PAL activation and salvianolic acid synthesis in hairy roots of *S. miltiorrhiza* Bge.f.alba (Fig 4, 5). That is the same as our previous study which showed that direct treatment with ABA induced the activation of PAL and synthesis of flavonoids in *G. biloba* cells (Hao et al., 2010). The activation of phenylpropanoid pathway as a response to a wide diversity of stress-factors has led to its use as a genetic marker for the induction of plant defense responses (Pellinen et al., 2002). Phenylalanine ammonialyase (PAL) catalyzes the first step in phenylpropanoid biosynthetic pathway (Jones, 1984). Mounting evidence suggests that ABA plays an ambivalent role in defense responses to pathogens. Adie et al. (2007) have showed that ABA is an essential signal for plant persistence to pathogens affecting JA biosynthesis and the activation of defenses in Arabidopsis. Treatment with exogenous ABA could mimic the effect of β-amino butyric acid (BABA) and resulted in priming for callose and resistance to *P. cucumerina* (Ton and Mauch-Mani, 2004). Take together, this study indicate that ABA is involved in salvianolic acids accumulation in hairy roots of *S.*

S. miltiorrhiza Bge.f.alba..

PAs are known to play significant roles in plant development, control of flowering, metabolite synthesis in response to plant viral infections and other defense mechanisms (Suresh et al., 2004). PAs regulate developmental processes, such as root development, in plants (Couée et al., 2004). Exogenous PAs also stimulate growth and production of secondary metabolites in hairy root cultures. High levels of free PAs have been reported to influence growth by cell division and low levels with cell expansion (Egea-Cortines and Mizrahi, 1991). Moreover, the endogenous concentration of PAs can

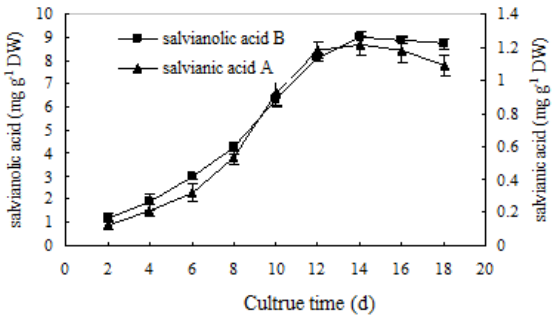

Fig 3. Time course studies of salvianolic acids production in hairy root cultures of *S. miltiorrhiza* Bge.f.alba grown in 6,7-V liquid medium for 18 days. Data are means ± SE, N = 3

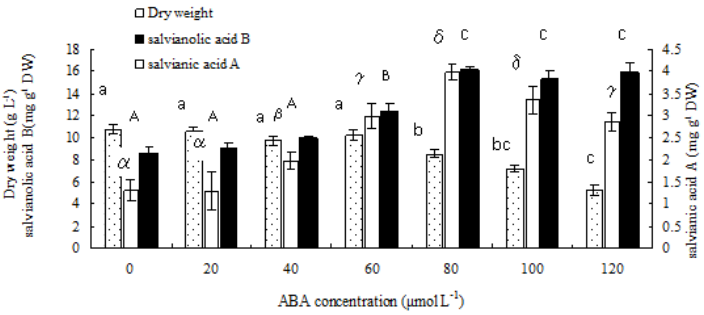

Fig 4. Effects of ABA concentrations on dry weight, salvianolic acid B and salvianic acid A production of hairy root of *S. miltiorrhiza* Bge. f.alba. Hair roots were collected after 12 days treatment with different concentrations of ABA. Data are means ± SE, N = 3. Within each set of experiments, bars with different letters are significantly different at $P<0.05$. A-C, α - δ and a-d indicate the comparisons between the salvianolic acid B, salvianic acid A and dry weight production under different ABA concentrations treatment, respectively.

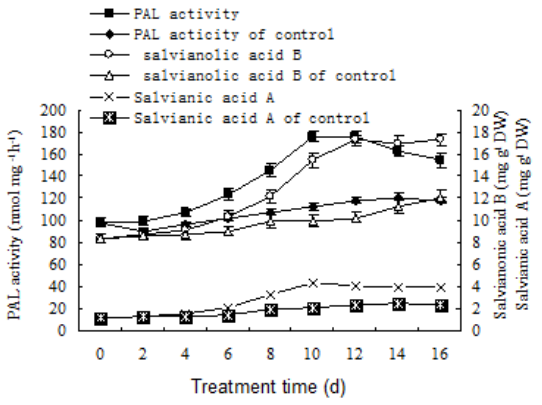

Fig 5. The time-course of PAL activity, salvianolic acid B and salvianic acid A production of hairy roots of *S. miltiorrhiza* Bge. f.alba under 80 μmol L^{-1} ABA treatment. Data are means ± SE, N = 3.

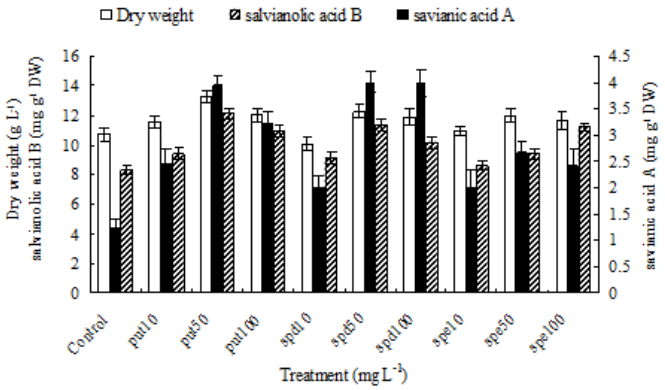

Fig 6. Effect of different concentration of polyamines on growth, production of salvianolic acid B and salvianic acid A in hairy roots of *S. miltiorrhiza* Bge. f.alba. Hair roots were collected after 12 days treatment with different concentrations of PAs. Data are means ± SE, N = 3. Control means no polyamines treatments. Asterisk shows significant differences of dry weight, salvianolic acid B and salvianic acid A at the 0.05 level comparisons between 0, 10, 50 and 100 mg L^{-1} Put treatment.

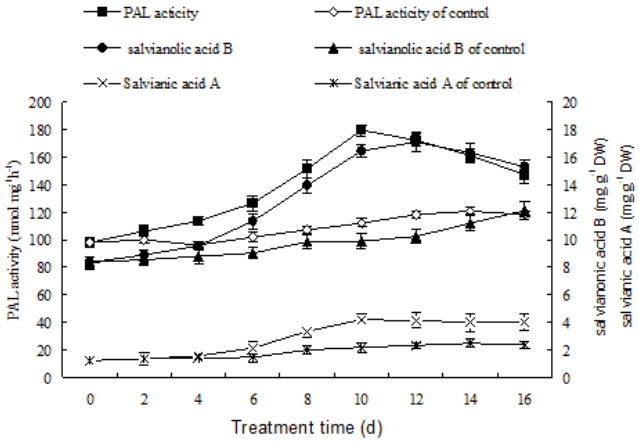

Fig 7. The time-course of PAL activity, salvianolic acid B and salvianic acid A production of hairy roots of *S. miltiorrhiza* Bge. f.alba under the mixture of put and spd (50 mg L^{-1} each) treatment. Data are means ± SE, N = 3

be growth limiting as reported by Smith (1982). Exogenous incorporation of PAs, and Put in particular, have been shown to stimulate growth of several higher plants. In these experiments, PAs are believed to act merely as a source of nitrogen when stimulating growth, although this is unlikely in the concentrations of less than 100 mM in standard media. In present work, Put was the most effective polyamine for growth and salvianolic acids accumulation in hairy roots of *S. miltiorrhiza* Bge.f.alba, and the effects of Put and Spd for salvianolic acids production were better than that of Spe (Fig. 6). This was consistent with previous study in other species. For example, Put increased growth and production of esculin and esculetin in hairy root cultures of witloof chicory (Bais et al., 1999). Moreover, Put was one of the most effective polyamines that stimulate hairy root growth and synthesis of coumarin, betalaine and rosmarinic acid in *Cichorium intybus*, *Beta vulgaris* and *Nepeta cataria* L**.** (Bais et al., 2004; Suresh et al., 2004). Using *B. vulgaris* hairy roots in shake flasks suggested that combined feeding of Spd and Put (0.75mM each) resulted in a higher yield of biomass as well as betalaine production (Bais et al., 2000; Suresh et al., 2004) . Our present study also showed that both PAL activity and salvianolic acids synthesis were significantly induced after the mixture of Put and Spd (50 mg L^{-1} each) treatment (Fig.6). Bais et al. (2000) stated that the increase in biomass was because of enhanced primary root length and extended branching. Furthermore, they observed that *B. vulgaris* hairy root cultures treated with PAs developed more tertiary branching and the maximum number of tertiary roots when Put and Spd (0.75mM each) were used, which were 2.07 fold higher than that of the control. In summer, PAs, especially Put and the mixture of Put and Spd, could stimulate hairy root growth as growth factor and induce salvianolic acids biosynthesis of *S. miltiorrhiza* Bge.f.alba.

Materials and methods

Plant materials

Seeds of *S. miltiorrhiza* Bge.f.alba were collected from the Experimental Farm of Taishan Medical University. Seeds were kept at 4°C for further use. The germination percentage was more than 80% and there was no seed dormancy in tests.

Seed sterilisation and germination

Seeds of *S. miltiorrhiza* Bge.f.alba were surface-sterilised with 70% (v/v) ethanol for 1 min and 4% (v/v) sodium hypochlorite solution for 10 min, and then rinsed three times in sterilised water. Twenty seeds were placed on 25 mL of MS medium solidified with 0.8% (w/v) agar in Petri dishes. The seeds were germinated in a growth chamber at 25°C under standard cool-white fluorescent tubes with a flux rate of 35 μmol s^{-1} m^{-2} and a 16-h photoperiod.

Growth of Agrobacterium rhizogenes

The culture of *A. rhizogenes* strain ACCC10060 was initiated from glycerol stock and grown overnight at 28°C with shaking (180 rpm) in liquid YEB medium to mid-log phase (OD_{600}=0.5). The bacterial cells were collected by centrifugation for 8 min at 3000 rpm, and resuspended at a cell density of OD_{600}=0.5 in liquid inoculation medium (MS salts and vitamins containing 30 g.L^{-1} sucrose).

General procedures to establish hairy root cultures

The establishment and maintenance of hairy roots cultures was performed as described in Chen et al. (1999) with some changes. Young leaves and stems of *S. miltiorrhiza* Bge.f.alba were taken from six-leave stage plants grown in *vitro*. Excised leaves and stems were pre-cultured in MS solidified medium for two days in a growth chamber at 25°C with 16-h photoperiod. These pre-cultured leaves and stems were dipped into *A. rhizogenes* ACCC10060 culture in liquid inoculation medium for 10 min as explant infection, blotted dry on sterile filter paper, and incubated in the dark at 25°C on agar-solidified MS medium. After 2 days of co-cultivation, the explant tissues were transferred to a hormone-free medium containing MS salts and vitamins, 30 g L^{-1} sucrose, 400 mg L^{-1} Cef, and 8 g L^{-1} agar for hairy induction and removing ACCC10060. Numerous hairy roots were observed emerging from the wound sites within 2 weeks. The hairy roots were separated from the explant tissues and subcultured

in the dark at 25°C on agar-solidified MS medium with gradual decrease of cefotaxime interval of 7 days. After repeated and transfer to fresh medium with gradual decrease of cefotaxime (from 400 mg L^{-1} to 350, 300, 200, 100 and 50 mg L^{-1}), rapidly growing hairy root cultures were obtained. These isolated root clones were transferred to 30 ml of 6,7-V liquid medium, containing 30 g L^{-1}sucrose, in 100 ml flasks. Root cultures were maintained at 25°C on a gyratory shaker (100 rpm) in a growth chamber. Hairy roots were harvested at intervals of 2 days until 18 days and salvianolic acids contents were determined.

ABA and PAs treatment conditions

ABA and PAs purchased from Sigma-Aldrich were used in treating hairy root cultures of *S. miltiorrhiza* bge f.alba. ABA were sterilised by filtration and added to the fresh autoclaved medium to give the 20-120 μM ABA. In addition, thress PAs (Put, Spd and Spe) were tested at 3 different concentrations (10, 50, and 100 mg L^{-1}). The controls received solvent only. Various chemical compounds were added to 8-day old hairy root cultures. After 12 days treatment with different concentrations of ABA and PAs, hair roots were collected for dry weight and salvianolic acids contents determination. Hair roots were collected at intervals of 2 days until 16 days for time course for the PAL activity and salvianolic acids synthesis analysis. Three flasks were used for each treatment condition.

PAL activity assay

Hairy roots (0.5 g FW) were homogenized at the extraction buffer (0.5 mL g^{-1}) containing 50 mM Tris-HCl (pH 8.0), 10 mM 2-mercaptoethanol, 4.0 mM EDTA and 1.0 μM leupeptin. The homogenate was centrifuged at 10,000 *g* for 20 min, and the supernatant was collected for enzyme assay. PAL activity was determined based on cinnamic acid production according to Ochoa-Alejo and Gomez-Peralta's method (1993). Briefly, 1 mL of the extraction buffer, 0.5 mL of 10 mM L-phenylalanine, 0.4 mL of double distilled water, and 0.1 mL of enzyme extract were incubated at 37 °C for 1 h. The reaction was terminated by the addition of 0.5 mL of 6 M HCl, and the product was extracted with 15 mL ethyl acetate, followed by evaporation to remove the extracting solvent. The solid residue was suspended in 3 mL of 0.05 M NaOH and the cinnamic acid concentration wherein was quantified with the absorbance measured at 290 nm. PAL activity was calculated in nmol cinnamic acid mg^{-1} protein h^{-1}.

Preparation of samples for HPLC analysis of salvianolic acids

Harvested hairy roots (1 g) were frozen in liquid N_2, ground to a fine powder using a mortar and pestle, and extracted twice with dH_2O (10 ml) for 24 h at room temperature. Extracts were reduced to dryness under vacuum dried, and dissolved in methanol. The extracts were analyzed by HPLC on a C18 reverse phase column (4.6 x 250 mm; Ultrasphere, Beckman-Coulter) at temperature 30°C. The solvent gradient used in this study was formed through with proportion of mix of solvent A (methanol) and solvent B (0.5 % acetic acid in water)：0-5min 80%B, 15-30min 57％B, 40min 20％B. The flow rate of the solvent was kept constant at 1.0 $ml.min^{-1}$. Samples (20 μ l) were detected at wave lengths of 286 nm.

We identified the salvianolic acids by matching the retention times and spectral characteristics to those from single HPLC run of a known salvianolic acid B and salvianic acid A standard.

Statistical analysis

Each result shown in the figures was the mean of three replicated treatments. Data are analyzed using SigmaPlot software (version 8.0, SYSTAT Software Inc., Richmond, California, USA). All mean comparisons were subjected to one-way analysis of variance (ANOVA) using SAS software (Version 6.2, SAS Institute Inc., North Carolina, USA). In all cases the confidence coefficient was set at $P<0.05$ level.

Conclusion

In previous study, we successfully developed the method for hairy roots induction of *S. miltiorrhiza* Bge.f.alba. Two main salvianolic acids in *S. miltiorrhiza* Bgune, salvianolic acid B and salvianic acid A, were detected in the induced hairy roots by HPLC. In addition, our results indicate that exogenous ABA and PAs can enhance two salvianolic acids production in hairy roots culture of *S. miltiorrhiza* Bge.f.alba. As a result, hairy root cultures of *S. miltiorrhiza* Bge.f.alba may be a valuable alternative approach for producing salvianolic acids.

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