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Production of lithospermic acid B and rosmarinic acid in hairy root cultures of *Salvia miltiorrhiza*

H Chen[1], F Chen[1], Y-L Zhang[2] and J-Y Song[2]

[1]*Department of Botany, The University of Hong Kong, Pokfulam Road, Hong Kong;* [2]*Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, Beijing 100094, PR China*

Hairy root cultures of *Salvia miltiorrhiza* were established by infecting sterile plantlets with *Agrobacterium rhizogenes* ATCC 15834, and the transformation was proved by direct detection of the inserted T-DNA by the polymerase chain reaction. As determined by HPLC, these hairy root cultures had the ability to produce lithospermic acid B (LAB), rosmarinic acid (RA) and other related phenolic compounds, the water-soluble active components of the plant. The effect of five different basal media, MS, MS-NH_4 (MS without ammonium nitrate), B5, WPM and 6,7-V on the root growth and phenolic compound production was studied. It was found that MS-NH_4 and 6,7-V media were superior to MS, B5 and WPM media in terms of both root growth and phenolic compound production. The time course of biomass accumulation and phenolic compound formation was also examined in the culture using MS-NH_4 medium. During cultivation, the content of RA in the roots was stable being approximately 0.48% of dry weight while the content of LAB fluctuated between 0.73% and 1.61% of dry weight, and decreased gradually at the stationary phase of growth. The highest production of LAB and RA was about 64 mg L^{-1} and 23 mg L^{-1}, respectively.

Keywords: *Salvia miltiorrhiza*; Lamiaceae; *Agrobacterium rhizogenes*; hairy roots; lithospermic acid B; rosmarinic acid

Introduction

Dan-shen, the dried roots of *Salvia miltiorrhiza* Bunge (Lamiaceae), is officially listed in the Chinese Pharmacopoeia and has been used for the treatment of disorders caused by poor blood supply such as coronary artery disease and angina pectoris [16,31]. The effective components of the roots, which could be classified as lipid-soluble and water-soluble ones, have been studied extensively and are well documented [16,31]. The lipid-soluble components are mainly tanshinones (diterpenoid quinones) which impart the reddish-brown colour to the roots while the water-soluble ones are caffeic acid and related phenolic compounds (Figure 1). Recently, much attention has been paid to the pharmacological activities of the water-soluble phenolic compounds [18,35,36]. Among these phenolic compounds, lithospermic acid B, a tetrametric derivative of caffeic acid (Figure 1) exhibits the endothelium-dependent vasodilator and hypotensive effects [14,15] while magnesium lithospermic acid B shows uremia and hepatitis preventive effects in animal models [10,30]. More recently, rosmarinic acid, an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, or its salts were reported to have anti-HIV activities by several research groups [2,17,20].

Plant cell culture has long been suggested as an alternative means for producing useful secondary metabolites usually unique to plants [7]. Compared to the numerous publications on tanshinone production in *S. miltiorrhiza* cell and organ cultures [4,12,21], however, there have been only a few reports on the production of phenolic compounds by *S. miltiorrhiza* cell and organ cultures. Morimoto *et al* firstly reported the formation of lithospermic acid B and rosmarinic acid in callus tissues and regenerated plantlets of *S. miltiorrhiza* [22]. However, in callus tissues, the content of lithospermic acid B disappeared completely by the fourth subculture, and the content of rosmarinic acid decreased gradually during subcultures [22]. In fact, poor secondary metabolite synthesis and culture instability of the undifferentiated tissues have prevented commercialization of many plant culture systems [6,27]. Hairy root culture has been demonstrated in many cases as a possible solution to these problems due to its genetic stability [1]. Hairy roots, also called transformed roots, are produced after infection of susceptible plant cells with a soil bacterium, *Agrobacterium rhizogenes*, and are associated with the integration of T-DNA from the bacterial Ri plasmid into chromosomal DNA in plant cells. Once produced, hairy roots may be cultured axenically in liquid medium in the absence of exogenous plant hormones. Characterized by a high degree of lateral branching and many meristemic root tips, hairy roots can usually grow as fast as unorganized plant cells and maintain the ability to produce root-derived metabolites [27]. Hairy root cultures of *Ocimum basilicum* and *Hyssopus officinalis* have been reported to produce rosmarinic acid and lithospermic acid B [23,29].

Due to the pharmacological importance of the phenolic compounds found in Dan-shen, it is worthwhile to examine the ability of the hairy root cultures of *S. miltiorrhiza* to produce these phenolic compounds. In this paper, we report the formation of lithospermic acid B and rosmarinic acid in hairy root cultures of *S. miltiorrhiza*.

Materials and methods

Plant material

Seeds of *S. miltiorrhiza* were collected from plants cultivated at the botanical garden of the Institute of Medicinal

Correspondence: Dr F Chen, Department of Botany, The University of Hong Kong, Pokfulam Road, Hong Kong
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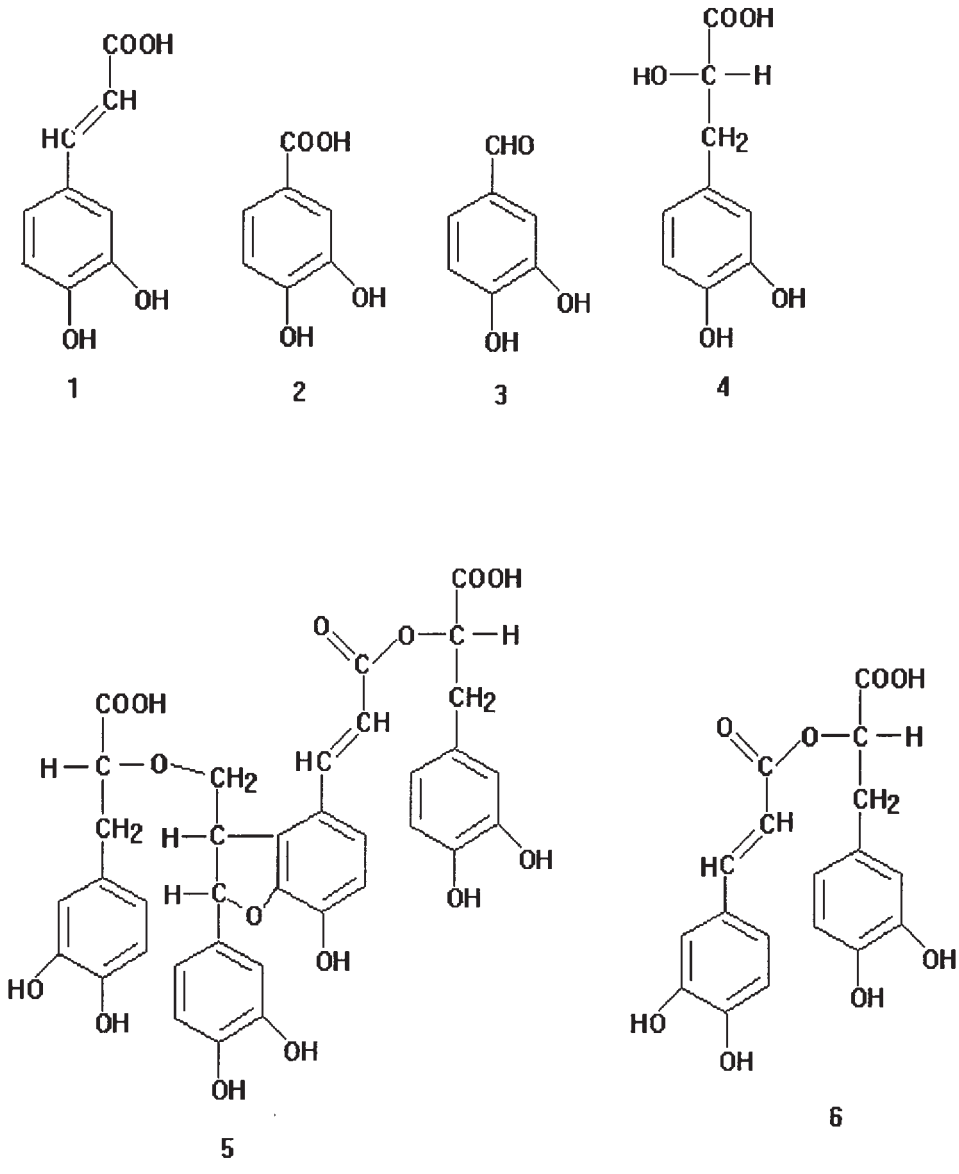

Figure 1 Chemical structures of caffeic acid and related phenolic compounds. **1.** Caffeic acid (CA); **2.** protocatechuic acid (PA); **3.** protocatechualdehyde (PE); **4.** Danshensu (3,4-dihydroxyphenyllactic acid); **5.** lithospermic acid B (LAB); and **6.** rosmarinic acid (RA).

Plant Development, Chinese Academy of Medical Sciences, Beijing, PR China. After being flushed with tap water for 24 h, the seeds were surface sterilized with 0.1% mercuric chloride for 10 min. Then, the seeds were rinsed with sterile distilled water three times, and germinated on 1/2 MS medium [24] solidified with 8 g L^{-1} agar. The cultures were incubated at 25°C for 1 month under continuous cool white fluorescent light with an intensity of 3000 lux before the shoot tips of seedlings were cut off and transferred onto the same fresh medium for subsequent sterile plantlet subculture.

Induction and maintenance of hairy root cultures

Agrobacterium rhizogenes strain ATCC 15834 subcultured on YEB agar medium [34] was transferred to YEB liquid medium and incubated overnight at 25°C in the dark on a rotary shaker at 100 rpm. The bacterial suspension was inoculated using a syringe at the cut of the sterile plantlet after removing the shoot tip and most of the leaves. Infected plantlets were incubated at 25°C under illumination (photoperiod 16 h with an irradiance of 3000 lux). After about 2 weeks, several hairy roots appeared at the infected sites. The hairy roots were subsequently cut off and placed on 1/2 MS solid medium containing 0.5 g L^{-1} sodium cefotaxime to eliminate bacteria. After several passages of subculture in the medium containing 0.5 g L^{-1} sodium cefotaxime, axenic hairy roots were obtained, which were maintained on antibiotic-free MS medium at 25°C in the dark.

Amplification of transformed DNA by PCR

DNA was extracted from fresh transformed and nontransformed tissues using a protocol based on Doyle [8]. DNA

quantification was done by fluorometric analysis and the extracted DNA was dissolved in TE buffer (pH 7.4).

Four oligonucleotides were designed according to References [3,28] and synthesized by Life Technologies (Hong Kong) to identify the insertion of TL-DNA or TR-DNA of the Ri-plasmid into the plant genome by PCR amplification. The primers for TL-DNA were 5′-ATGGAATTAGCCGGACTAAACG-3′ and 5′-ATGGATCCCAAATTGCTATTCC-3′, which are complementary to 5′ coding sequence of *rol* A and 3′ coding sequence of *rol* B, respectively [28]. And those primers for TR-DNA were 5′-CGGAAATTGTGGCTCGTTGTGGAC-3′ and 5′-AATCGTTCAGAGAGCGTCCGAAGTT-3′, complementary to 5′ and 3′ flanking sequences of the agropine synthase gene (*ags*), respectively [3]. For the positive control of TL and TR-DNAs, the relevant cosmid pLJ1 and pLJ85, kind gifts from Dr L Jouanin (INRA, Versailles, France) [13] were used as templates, respectively.

The amplification reaction mixture containing 10–20 ng template DNA, 10 mM Tris-HCl (pH 8.3 at 25°C), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTPs, 10 pmol oligonucleotide primers and 1.0 U Taq polymerase (Gibco BRL, Life Technologies Inc, Gaithersburg, MD, USA) in 25 μl. PCR reactions were performed in an MJ Research PTC-200 Peltier Thermal Cycler. The initial denaturation was carried out at 94°C for 2 min, followed by 35 cycles of amplification (denaturation at 94°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 2 min) and the final extension at 72°C for 10 min. The PCR reaction mixture was electrophoresed on 1% agarose gel and visualized under UV light after ethidium bromide staining.

Chemicals and standards

Caffeic acid, protocatechualdehyde and protocatechuic acid were purchased from Sigma (St Louis, MO, USA). Rosmarinic acid was purchased from ICN (Costa Mesa, CA, USA). Danshensu (3,4-dihydroxyphenyllactic acid) was kindly provided by Shanghai Medical University. Lithospermic acid B was a gift from Dr K Kamata (Hoshi University, Tokyo, Japan) and Dr Y M Xu (Shanghai Institute of Materia Medica, Academia Sinica, Shanghai, China). All other chemicals were purchased from Sigma.

Culture experiments involving hairy roots

The effects of different basal media on the growth and phenolic compound production in hairy root cultures were investigated by employing MS [24], MS-NH_4 (MS without ammonium nitrate), B5 [9], WPM [19] and 6,7-V [33] media. Hairy roots were cultivated in 250-ml shake flasks containing 50 ml of the hormone-free liquid basal medium. The pH of the medium was adjusted to 5.7 prior to autoclaving it at 121°C for 15 min. Cultivation was performed on an orbital shaker at 140 rpm in darkness at 25°C for 30 days. The inoculum size was controlled at about 0.5 g root tips (fresh weight) for each flask. At the end of cultivation, the growth rate and phenolic compound content of hairy roots in each flask were determined. The experiment was performed in triplicate.

In a time course study, 38 250-ml flasks were used, each containing initially 0.5 g root tips (fresh weight) and 50 ml MS-NH_4 medium supplemented with 30 g L^{-1} sucrose. Every 3 days, three flasks were harvested for measurement of biomass and phenolic compound contents.

Analyses

Fresh weight was measured after the hairy roots had been separated from the medium on Whatman No. 1 filter paper under vacuum and washed three times with distilled water. Dry weight was determined by freeze-drying the biomass to constant weight.

For HPLC analysis of methanol extractable phenolic compounds, dried samples (*ca* 50 mg) of hairy roots were mashed and extracted with MeOH (10 ml) for 16 h at room temperature. Each extract, after filtration through a 0.45-μm pore size filter, was subjected to HPLC analysis on a Beckman System Gold liquid chromatograph as previously described [37]. Briefly, the extract solution was separated and analyzed using a 250 × 4.6 mm Beckman Ultrasphere C_{18} (5 μm) column at 30°C. The mobile phase consisted of solvent A (methanol / water / formic acid, 14.0 : 85.2 : 0.8, v/v/v) and solvent B (methanol / water, 65 : 35, v/v). The following gradient procedure was used: 0% of B for 2 min; a linear gradient from 30% to 45% of B for 8 min. The flow rate was set at 1.0 ml min^{-1}. The detector wavelength was 280 nm.

Results

Development of hairy root cultures and formation of phenolic compounds

Direct infection of a sterile plantlet with *A. rhizogenes*, followed by antibiotic treatment, yielded several clones of axenic adventitious roots. One clone was selected for further experiments due to its rapid growth. The transformation was confirmed by PCR. PCR amplification with template DNA extracted from hairy root cultures and synthetic oligonucleotide primers gave positive results (Figure 2). The PCR products from the hairy roots for TL (*rol* A and B) and TR (*ags*) regions gave the expected 1.540-kb and 1.647-kb bands, respectively.

Using HPLC [37], the main phenolic compounds found in Dan-shen such as caffeic acid (CA), protocatechuic acid (PA), protocatechualdehyde (PE), danshensu (3,4-dihydroxyphenyllactic acid), rosmarinic acid (RA) and lithospermic acid B (LAB) were successfully determined in a single run. The HPLC profile of the methanol extract from hairy root cultures of *S. miltiorrhiza* is shown in Figure 3. LAB (lithospermic acid B) was the main constituent of the extract and RA (rosmarinic acid) was the second highest. CA and PE existed only in trace amounts while danshensu and PA were not detected (Figure 3 and Table 1). The accumulation of these phenolic compounds was intracellular as no detectable phenolic compounds were found in the culture medium.

Effects of basal media

Of the five basal media tested, MS-NH_4 and 6,7-V were superior to MS, B5 and WPM media in terms of both root growth and phenolic compound production (Figure 4 and Table 1). The profiles of phenolic compounds formed in these cultures were similar to each other with LAB and RA

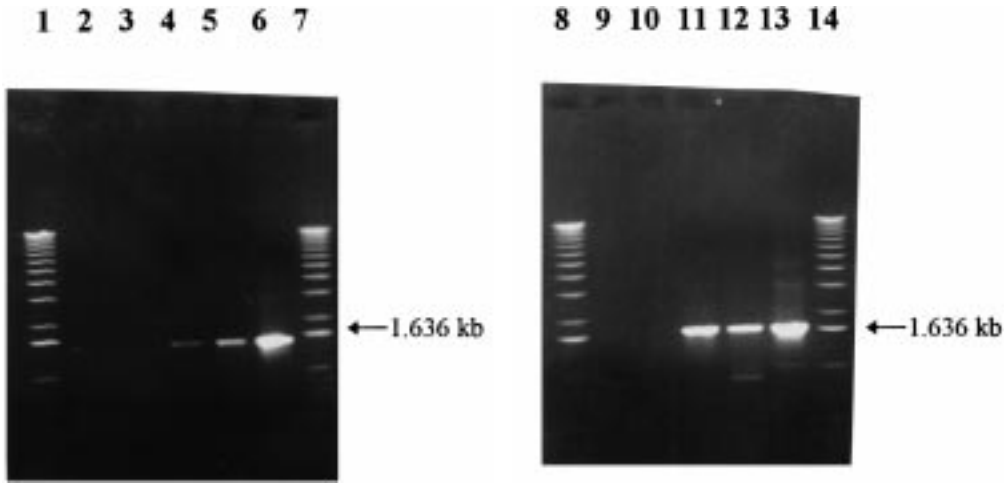

Figure 2 Detection of T-DNA by PCR amplification of DNA from transformed and non-transformed tissues of *S. miltiorrhiza*. Two sets of oligonucleotide primers were used for TL and TR regions, respectively. **1.** 1-kb DNA ladder; **2.** non-transformed root; **3.** Ti C58 transformed cells [4]; **4.** hairy root clone 1; **5.** hairy root clone 2; **6.** pLJ1; **7.** 1-kb DNA ladder; **8.** 1-kb DNA ladder; **9.** non-transformed root; **10.** Ti C58 transformed cells [4]; **11.** hairy root clone 1; **12.** hairy root clone 2; **13.** pLJ85; and **14.** 1-kb DNA ladder. Arrow heads indicate the position of 1.636 kb.

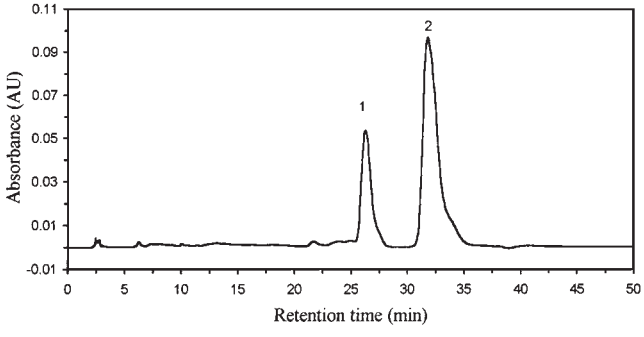

Figure 3 HPLC profile of the methanol extract from hairy roots of *S. miltiorrhiza*. Peaks: 1. rosmarinic acid and 2. lithospermic acid B.

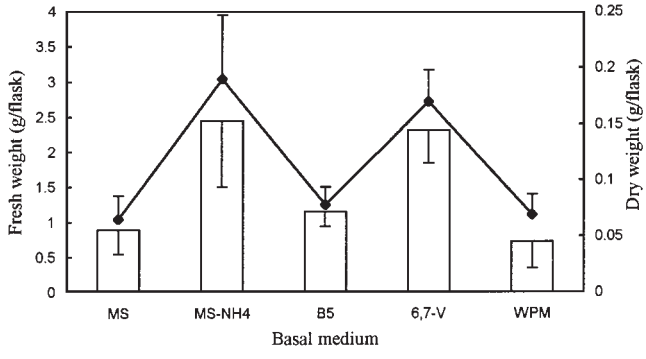

Figure 4 Effects of different basal media on the growth of hairy root cultures of *S. miltiorrhiza* in shake flasks. □, Fresh weight; -◆- dry weight. Values are means of triplicate results and error bars show standard deviations.

Table 1 Phenolic compounds in hairy roots of *S. miltiorrhiza* cultivated in different basal media[a]

Basal medium	Phenolic compound content (mg g^{-1} dry weight)					
	Danshensu	CA	PE	PA	RA	LAB
MS	0	0.039	0.035	0	3.41	3.72
MS-NH_4	0	0.011	0.010	0	7.05	14.01
B5	0	0.019	0.011	0	4.66	6.29
6,7-V	0	0.005	0.009	0	6.77	11.36
WPM	0	0.007	0.006	0	4.39	5.56

[a]Hairy roots were cultivated in 250-ml shake flasks containing 50 ml of the hormone-free liquid basal medium with an inoculation size of about 0.5 g fresh roots. After 30 days' cultivation, the growth and phenolic compound content of each culture were determined. Values are means of triplicate results. CA, caffeic acid; PE, protocatechualdehyde; PA, protocatechuic acid; RA, rosmarinic acid; LAB, lithospermic acid B. Danshensu is the common name of 3,4-dihydroxyphenyllactic acid.

as the dominant products. The LAB content in MS-NH_4 medium was higher than that in 6,7-V medium (1.4% *vs* 1.1% of dry weight) while the RA content was about the same (0.71% *vs* 0.68% of dry weight). Based on these findings, MS-NH_4 medium was employed for the time course study.

Time course of shake-flask culture in MS medium without ammonium nitrate

Biomass accumulation and phenolic compound production are presented in Figure 5. As shown in Figure 5a, after about 6 days of lag phase, the roots grew rapidly until entering the stationary phase on the 27th day of cultivation.

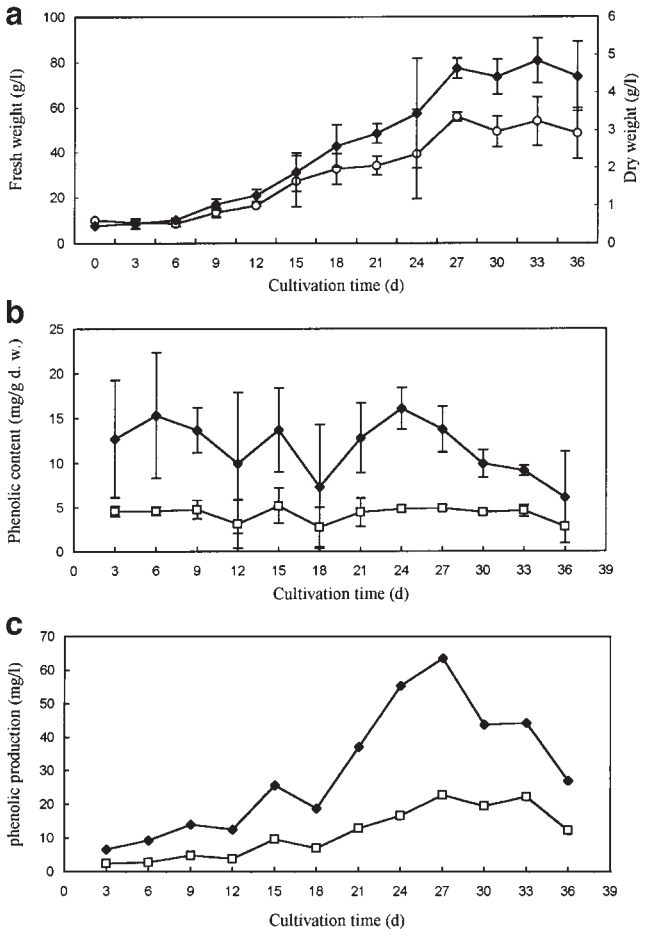

Figure 5 Time course for biomass accumulation (a), phenolic content (b) and phenolic compound production (c) in hairy root cultures of *S. miltiorrhiza*. (a) -○- Fresh weight; -◆- dry weight. (b and c) -◆- LAB; -□- RA. In the experiment, 250-ml flasks each containing initially 0.5 g fresh roots and 50 ml MS medium without ammonium nitrate were used. Every 3 days, triplicate flasks were harvested for the measurement of biomass and phenolic compound contents. Values are means of triplicate results and error bars show standard deviations.

The highest biomass recorded during the batch cultivation was about 5.4 g L^{-1} on a dry weight basis.

The content of RA in the root was stable (about 0.48% of dry weight) (Figure 5c) while the content of LAB fluctuated between 0.73% and 1.61% at first and decreased gradually at the stationary phase of growth (Figure 5b). The increase in RA production in the culture was mainly attributed to root growth while the production of LAB increased with root growth initially and decreased subsequently during the stationary phase (Figure 5c). The highest production of LAB and RA was about 64 mg L^{-1} and 23 mg L^{-1}, respectively.

Discussion

Hairy root cultures of *S. miltiorrhiza* are good producers of tanshinones [12]. MS-NH_4 medium was superior to MS medium in terms of both root growth and tanshinone production. Our observation confirmed that the hairy roots grew better in MS-NH_4 medium than in MS medium, and also showed that phenolic compound production was improved in MS-NH_4 medium.

Due to the pharmacological value of rosmarinic acid, extensive research has been carried out for its production in plant cell cultures [5,11,26,32]. However, little information is available about LAB production in plant cell cultures. This is probably due to the fact that LAB and its multiple pharmacological activities were discovered only recently [10,14,15,30,35]. In the hairy root cultures of *S. miltiorrhiza* established in the present study, the content of LAB and of RA (1.61% and 0.48% of dry weight, respectively) was one third of that found in the roots of the regenerated plants (4.93% and 1.54%) [22]. In contrast to the instability of the callus tissues of *S. miltiorrhiza* in producing RA and LAB [22], hairy root cultures of *S. miltiorrhiza* retained the ability to produce both RA and LAB after more than 2 years' subculture. With strain improvement and optimization of culture conditions, the production of secondary metabolites using hairy root culture could be greatly enhanced [7]. In addition, the development of a fermentation process for hairy roots is much easier than that for *in vitro* shoots or regenerated plantlets.

In hairy root cultures of *Ocimum basilicum* and *Hyssopus officinalis* (Lamiaceae), RA was the main phenolic compound [23,29]. In contrast, in the hairy roots of *S. miltiorrhiza* (Lamiaceae), the content of LAB was much higher than that of RA. Recently, Omoto *et al* [25] measured LAB and RA in the leaves and roots of plants from 26 species in Lamiaceae and found that high levels of LAB only existed in the roots of *S. miltiorrhiza* (5.1% and 0.15% of dry weight in the roots and leaves, respectively). Thus *S. miltiorrhiza* is unique in accumulating LAB. Consequently, it is understandable that LAB was the main component of phenolic compounds in hairy roots of *S. miltiorrhiza*. Our results indicated that hairy roots of *S. miltiorrhiza* provide a useful system for the production and biosynthetic study of phenolic compounds, especially LAB.

Acknowledgements

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