Production of Lithospermic Acid B and Rosmarinic Acid in Callus Tissue and Regenerated Plantlets of Salvia miltiorrhiza

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PRODUCTION OF LITHOSPERMIC ACID B AND ROSMARINIC ACID IN CALLUS TISSUE AND REGENERATED PLANTLETS OF *SALVIA MILTIORRHIZA*

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ABSTRACT.—Callus tissue from petioles of *Salvia miltiorrhiza* was obtained on Murashige and Skoog medium supplemented with indole-3-butyric acid and 6-benzylaminopurine. When the calli were subcultured, adventitious shoots formed. These shoots developed into normal plantlets with roots when transferred to hormone-free Murashige and Skoog medium. Clonal micropropagation was established by shoot tip culture and was maintained on Murashige and Skoog medium supplemented with kinetin and gibberellic acid A_3. Callus that was induced and maintained on supplements of 2,4-dichlorophenoxyacetic acid and 6-benzylaminopurine in the absence of light produced rosmarinic acid (1.24% dry wt) and lithospermic acid B (0.10% dry wt). Higher concentration levels of rosmarinic acid (6.96% dry wt) and lithospermic acid B (6.05% dry wt) were formed in the leaves of 15-week-old plants that were generated by micropropagation.

Salvia miltiorrhiza Bunge (Labiatae) is an important Chinese medicinal plant that grows wild in China. The dried root is prescribed in combination with other herbal drugs for chest congestion and coronary diseases (1). The root contains quinoid diterpenes (2–5) and depsides (6–9), the former having been reported to protect the myocardium against ischemia-induced derangements (5) and to promote a post-hypoxic recovery of the cardiac contractile force (6). Magnesium and ammonium-potassium lithospermate B were determined as being uremia-preventive active principles in our laboratory (10).

The formation of quinoid diterpenes has been a main goal of tissue culture studies (11–16). The production of rosmarinic acid (RA) has been investigated in tissue cultures of several species (17–21), although as of yet, there has been no reported attempt to produce lithospermic acid B (LA) by tissue culture. In our continuing study of the applications of tissue culture of medicinal plants to the production of secondary metabolites, we have adopted two approaches. Our first approach is that of breeding, such as of virus-free plants by meristem tip culture as with *Rehmannia* spp. (22), and other examples concerning *Aconitum* spp. and *Atractylodes* spp. (23,24). Our second approach is exemplified by the in vitro production of saponins in *Panax japonicus* (25) and glycosides in *Rehmannia glutinosa* (26). Herein, we report on the accumulation of LA together with its potential precursor, RA, in both undifferentiated and differentiated tissues of *S. miltiorrhiza*.

The petiole segments regenerated from callus were cultured for 7 weeks on Murashige and Skoog (MS) medium (27) supplemented with various auxins, either alone or in combination with 6-benzylaminopurine (BAP) as reported previously (16). On all media containing either 2,4-dichlorophenoxyacetic acid (2,4-D), alone or together with BAP, callus formation occurred within 2 weeks. All calli grew rapidly into a friable pale yellowish mass of cells without differentiation during an additional 5-week-culture. The medium supplemented with 2,4-D and BAP was most favorable for callus growth, and the growth rate of callus was constant by the fourth subculture on this same medium.

When petiole segments were cultured on MS media supplemented with indole-3-butyric acid (IBA), indole-3-acetic acid (IAA) or 1-naphthaleneacetic acid (NAA) with BAP, callus formation occurred.

Shoot bud formation over the whole callus was induced from MS medium supplemented with IBA, IAA, or NAA with BAP over a period of 5 weeks as described above. Differentiation from callus occurred on all media. However, the effect of adding NAA or IAA with BAP resulted in slightly less differentiation than that with IBA (data not shown). Supplements of IBA and BAP formed an average of 8.7 shoots per petiole. Individual subcultures of regenerated petioles were carried out under the same conditions and their primary culture had a shoot formation rate in the range of 7.8–8.5 shoots per individual callus.

For clonal propagation, the regenerated shoot tips were subcultured on MS medium supplemented with IBA and BAP. However, the propagation rate was low. Therefore, the regenerated shoot tips were tested on MS medium supplemented with kinetin and GA as indicated in Table 1. From these results the combination of kinetin and gibberellic acid A_3 (GA) was the most effective for shoot propagation, resulting in 8.8 shoots per segment over 5 weeks. The shoots rooted when transferred to hormone-free MS medium and cultured for 5 weeks as reported previously (16).

The concentration levels of LA and RA in callus culture in total darkness or under 16-h light (4000 lux)/8-h dark cycles are presented in Figure 1. The content of RA gradually decreased in callus grown in complete darkness, while the content of LA was almost constant through the third subculture in the dark, but disappeared completely during the fourth subculture. Under light conditions, the RA concentration decreased during the third subculture and LA increased during the third, and then decreased during the fourth culture.

Because it is well known that secondary metabolite production can be enhanced by the induction of organogenesis, the in vitro shoots and the regenerated plants were analyzed. To determine the distribution of both compounds in each organ, the petioles, leaves and stems from the in vitro propagated plants were separately analyzed. The results showed that both compounds accumulated predominantly in the leaves (LA 1.75% dry wt, RA 3.76% dry wt) rather than in the petioles (LA 0.21% dry wt, RA 1.23% dry wt) or the stems (LA 0.43% dry wt, RA 1.62% dry wt). Figure 2 shows a comparison of LA and RA content in the in vitro shoots, in young regenerated plants cultivated either in vermiculite (Procedure 1), or first in vermiculite,

TABLE 1. Effect of Plant Hormone on Propagation of *Salvia miltiorrhiza* Using Regenerated Shoot Tips.[a]

Hormone (mg/liter)		Multiple shoot forming no.	Ave. shoot no.	Ave. fresh weight (g) per shoot complex
Kinetin	GA			
0.5		4	1.0	0.16
0.5	0.2	7	3.5	0.28
0.5	0.5	8	7.9	1.23
1.0		2	1.0	0.12
1.0	0.2	8	2.3	0.25
1.0	0.5	8	8.8	1.09
2.0		4	1.2	0.13
2.0	0.2	8	3.2	0.30
2.0	0.5	9	6.5	0.64
5.0		4	1.0	0.11
5.0	0.2	7	1.0	0.12
5.0	0.5	7	1.5	0.15

[a]Culture conditions: MS media, 16 h light (4000 lux), 25±1°, 5 weeks. Ten shoot tips were cultured for propagation in individual medium.

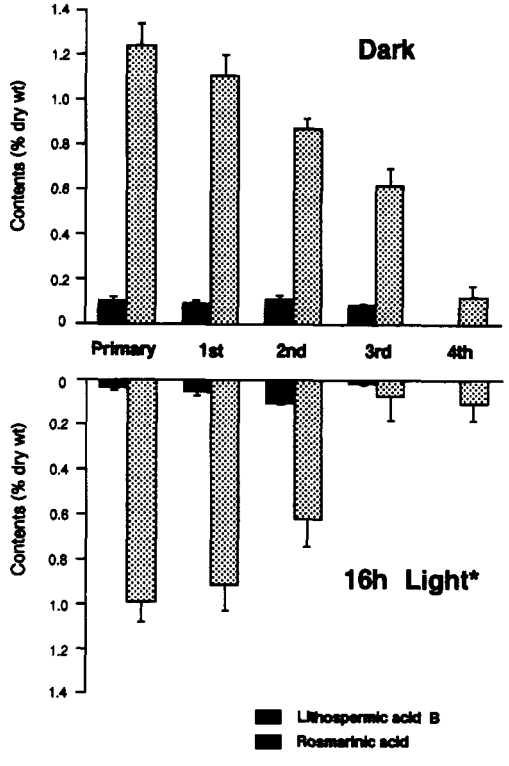

FIGURE 1. Formation of lithospermic acid B and rosmarinic acid in callus culture of *Salvia miltiorrhiza*. Culture conditions: MS media containing 2,4-D (0.5 mg/liter) and BAP (0.5 mg/liter), 25±1°, 5 weeks, *: 4000 lux. Bars show standard errors.

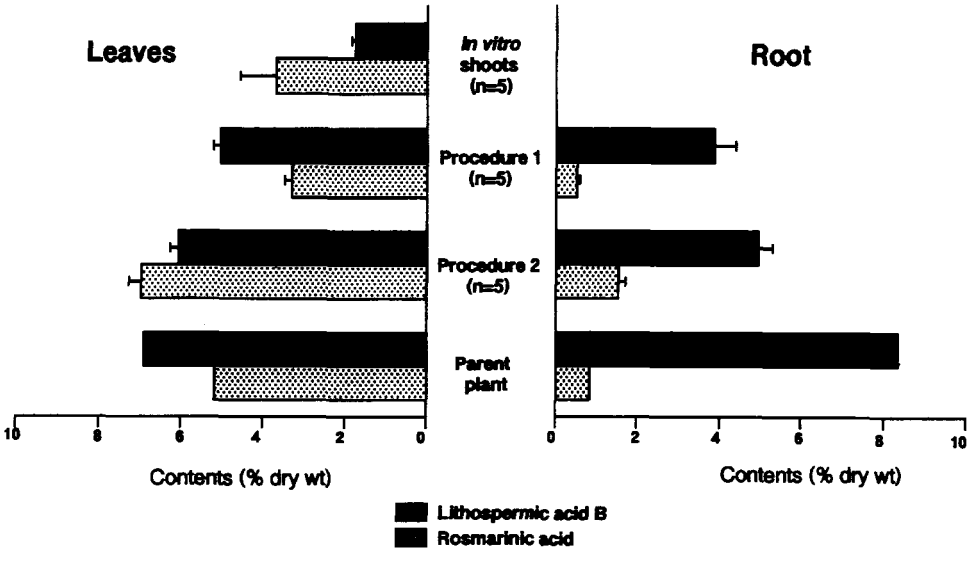

FIGURE 2. Formation of lithospermic acid B and rosmarinic acid in in vitro shoots, regenerated plants, and parent plant of *Salvia miltiorrhiza*. Procedure 1: cultivated in vermiculite for 10 weeks. Procedure 2: cultivated in vermiculite for 5 weeks, followed by 5-week-cultivation in soil. Bars show standard errors.

then in soil (Procedure 2) and in the parent plant. The concentration levels of LA and RA in in vitro shoots and regenerated plants were higher than in callus (Figure 1). The content of both LA and RA in the in vitro shoot regenerated from callus was stable throughout the fourth subculture as indicated in Figure 3.

Figure 2 shows that in the systems investigated, the content of RA in the leaves was higher than in the roots. In the plantlets cultivated by Procedure 1, increased accumulation of LA (5.05% dry wt) occurred, but decreased accumulation of RA (3.31% dry wt) was observed in the cultivated leaves when compared to the leaves of the in vitro-generated shoots. In the roots produced by cultivation according to Procedure 1, LA accumulated to a larger extent than did RA, as was also observed in the parent plant. When plantlets were transferred to soil and cultivated an additional 5 weeks (Procedure 2), quantities of LA (6.05% dry wt) and RA (6.96% dry wt) were found to be comparable to those in the parent plant. The LA and RA concentration levels in the leaf material produced by Procedure 2 were higher than those values found in the comparable root material (LA 4.93% dry wt; RA 1.54% dry wt). In addition, the accumulation of RA in the plantlets generated by Procedure 2 exceeded that in both the leaves and the roots of the parent plant, thus demonstrating a superior production system for RA. The accumulation of LA (8.36% dry wt) in the roots of the parent plant, however, was greater than that found in the regenerated roots produced by Procedure 2. A possible reason for this difference in the LA content of roots of the parent and the regenerated plants may simply be due to the age difference of the plant material. The parent plants were 3 years old, whereas the regenerated plants were only 10 weeks old.

When comparing the coefficient of variation for both compounds in the two propagation systems, callus culture (Figure 3) and shoot tip (Figure 4), the level of RA in the leaves of in vitro clonally propagated shoots was clearly smaller than that from the callus culture. However, the coefficient of variation of LA was almost the same in the two propagation systems tested.

RA production in plant cell culture

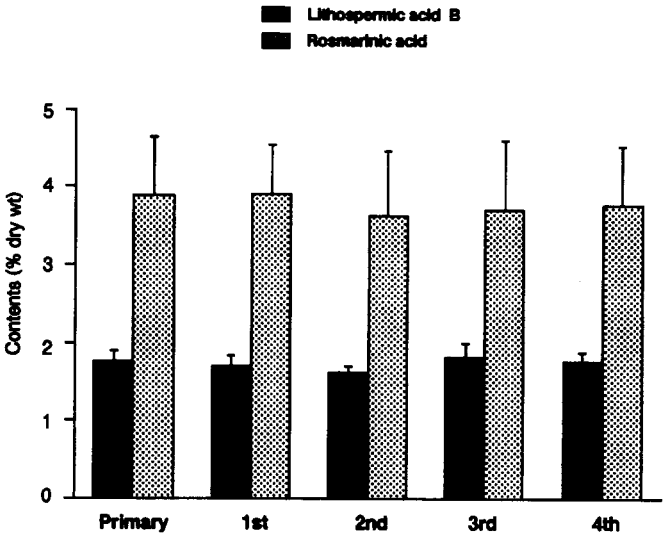

FIGURE 3. Formation of lithospermic acid B and rosmarinic acid in in vitro shoots regenerated from callus of *Salvia miltiorrhiza*. Culture conditions: MS media containing IBA (0.5 mg/liter) and BAP (0.5 mg/liter), 16 h light (4000 lux), 25±1°, 5 weeks. Bars show standard errors.

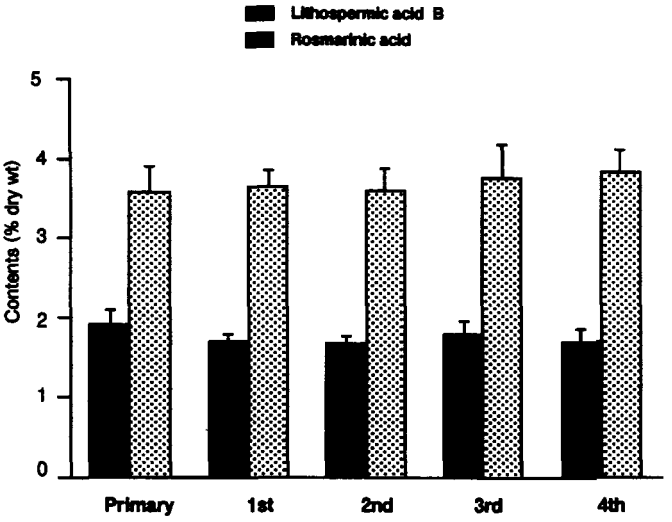

FIGURE 4. Formation of lithospermic acid B and rosmarinic acid in in vitro clonally propagated shoots of *Salvia miltiorrhiza*. Culture conditions: MS media containing kinetin (1.0 mg/liter) and GA (0.5 mg/liter), 16 h light (4000 lux), 25±1°, 7 weeks. Bars show standard errors.

has been investigated using various species (22–26). The formation of LA and RA in individual developmental stages such as callus tissue, regenerated shoots, and cultivated plants has been determined here. The production of LA by callus culture was almost insignificant when compared to the RA production reported here and as previously indicated in several species (22–26). In contrast, the in vitro plantlets contained higher amounts of both compounds than callus tissue.

This is the first report of a substantial accumulation of LA in leaves of young plants by short-term-cultivation.

The dry weight of the leaves and that of the roots obtained by Procedure 2 was 319 mg and 568 mg per plant, respectively. From the content of LA in leaves (6.05% dry wt) and in roots (4.93% dry wt), the production potential of LA in a whole plant would be approximately 47.3 mg. For rooting and cultivation of clonally propagated shoots, 5 and 10 weeks were needed, respectively. Therefore, it is theoretically possible to obtain more than 1.04×10^7 clonal plants from a single shoot tip in the remaining period (37.1 weeks) of one year. Such results suggest that approximately 492 kg of LA could be theoretically biosynthesized by the cultivation of plants clonally propagated from a single shoot tip in one year. It is, therefore, suggested that this system may provide a useful resource for the important, pharmacologically active compounds, LA and RA. Until now, the crude drug preparation from *S. miltiorrhiza* was produced by a seed propagation system and required three years before harvest. Moreover, since the variation of RA content in the leaves of in vitro plantlets propagated by a multiple shoot formation system was small, this method provides a more stable and efficient means of producing RA and LA.

EXPERIMENTAL

PLANT MATERIALS.—*Salvia miltiorrhiza* was grown at the herbal garden, Faculty of Pharmaceutical Sciences, Kyushu University. Shoot buds for callus culture of *S. miltiorrhiza* were obtained from the plant stored at 4° for 1 month to break dormancy. Voucher specimens are kept in the herbal garden, Faculty of Pharmaceutical Sciences, Kyushu University. Commercial crude drugs of *S. miltiorrhiza* were purchased from Nakai-Koshindo (Kobe, Japan).

TISSUE CULTURE.—Portions of the shoot bud (10 mm long) were removed, sterilized with 2% NaOCl solution containing 0.1% Tween 80 for 15 min, then with 70% EtOH for 30 sec, and finally washed three times thoroughly with sterile distilled H_2O. The shoot tips were dissected from the shoot buds under a binocular microscope. The shoot tips were cultured on Murashige Skoog (MS) (22) medium supplemented with IBA-BAP (0.5 mg/liter each) for 5 weeks to form adventitious shoots through callus formation. The petioles obtained from the regenerated shoots were used for further experiments. The petiole segments regenerated were cultured on MS medium supplemented with 2,4-D and BAP (each 0.5 mg/liter) for 5 weeks to form callus. Regenerated shoots were subcultured on MS medium supplemented with kinetin (1.0 mg/liter) and GA (0.5 mg/liter) to form multiple shoot complexes. Regenerated shoots were transferred to a hormone-free MS medium to develop into plantlets. Culture conditions were the same as described in a previous paper (18).

CULTIVATION.—Plants cultured on hormone-free MS medium were transferred to vermiculite and cultivated under 16 h light condition (4000 lux) at 25° for 10 weeks (Procedure 1). The same plants were transferred to vermiculite and cultivated for 5 weeks under the same conditions. The established plants were transplanted to soil and cultivated for an additional 5 weeks under the same conditions (Procedure 2). The two types of cultivation (Procedures 1 and 2) were investigated in a growth chamber at the Faculty of Pharmaceutical Sciences, Kyushu University.

QUANTITATIVE ANALYSIS OF LITHOSPERMIC ACID B (LA) AND ROSMARINIC ACID (RA) BY HPLC.—LA and RA were isolated from the roots of *S. miltiorrhiza* as indicated in a previous paper (10).

Quantitative analysis was carried out by hplc as follows. The dried tissue (50–150 mg) of callus, regenerated plantlets, or cultivated plants was extracted 3 times with 60% Me_2CO (5–10 ml) for 30 min under sonication at 35°. The solution was centrifuged (2000 rpm, 10 min, 5°). To the supernatant, Celite-545 (300 mg) was added, and the solvent was then evaporated *in vacuo*. To the residue, H_2O (20 ml) was added and filtered. The filtrate was taken to dryness *in vacuo*, and the residue was redissolved in a known amount of mobile phase. Duplicate 10 μl samples were injected onto the hplc. Analyses were performed on a Model CCPM (Tosoh Co., Ltd) instrument equipped with a YMC-Pack Polymer C18 (4.6×250 mm, YMC Co., Ltd) and a variable-wave length detector (detection set at 280 nm). The mobile phase was 35% CH_3CN. LA (1.0 mg) and RA (1.0 mg) were dissolved individually in the mobile phase (1.0 ml), diluted stepwise, and 10 μl aliquots were injected into the hplc. Calibration plots were obtained from the peak heights of LA and RA.

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