Tanshinone Production in Adventitious Roots and Regenerates of Salvia miltiorrhiza

Koichiro Shimomura, Takashi Kitazawa, Nobuyuki Okamura, and Akira Yagi

J. Nat. Prod., **1991**, 54 (6), 1583-1587• DOI:
10.1021/np50078a014 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 54, No. 6, pp. 1583-1587, Nov-Dec 1991

TANSHINONE PRODUCTION IN ADVENTITIOUS ROOTS AND REGENERATES OF *SALVIA MILTIORRHIZA*

KOICHIRO SHIMOMURA,* TAKASHI KITAZAWA,

Tsukuba Medicinal Plant Research Station, National Institute of Hygienic Sciences, 1 Hachimandai, Tsukuba, Ibaraki 305, Japan

NOBUYUKI OKAMURA, and AKIRA YAGI

Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University, Fukuyama, Hiroshima 729-02, Japan

ABSTRACT.—The adventitious root culture of *Salvia miltiorrhiza* has been established on Gamborg B5 solid medium containing various combinations of phytohormones. The production of the four major tanshinones in the root cultures under different culture conditions was simultaneously determined by hplc. The roots grew rapidly when indole-3-acetic acid or 1-naphthaleneacetic acid in combination with or without benzyladenine was added to Gamborg B5 liquid or solid media. On the other hand, the highest production of tanshinones was obtained with the addition of indole-3-butyric acid to the medium. In Gamborg B5 liquid medium, over 80 mg/g dry wt of tanshinones (sixfold the amount produced in the roots of parent plant) were obtained from the root cultures. In addition, a method for rapid propagation of *S. miltiorrhiza* has been established. Plantlets micropropagated on Murashige and Skoog solid medium could be transferred to the field. The roots of the 6-month-old regenerates produced more tanshinones than the commercially available roots, which are usually from 3–4-year-old plants.

The root of *Salvia miltiorrhiza* ("tan-shen" in Chinese) is traditionally used in Chinese medicine to treat disorders caused by poor blood supply (1). Main constituents of the roots are red abietane-type diterpenoids with a naphthoquinone moiety (2). Of the various constituents, tanshinone I and cryptotanshinone have been reported to be effective in protecting the myocardium against ischemic disturbances, and in addition, cryptotanshinone is purported to be a remedy for angina pectoris (3,4).

Cell suspension cultures and immobilized cells of *S. miltiorrhiza* have been shown to have the capability to produce cryptotanshinone in vitro. The main product in those cultures, however, was ferruginol, presumably the precursor of tanshinones (5–7).

In this report we describe the establishment of adventitious root cultures of *S. miltiorrhiza* and the culture conditions for high-yield production of tanshinones in the adventitious roots. Furthermore, a method has been developed for rapid propagation of this plant using in vitro cultures, which allows multiplication of the high-producing clones of *S. miltiorrhiza* for cultivation in the field.

RESULTS AND DISCUSSION

Shoots of *S. miltiorrhiza* subcultured on various hormone-free solid media showed normal development on Murashige-Skoog (MS) (8) and Gamborg B5 (9) media, while in the low-ion strength media [half strength MS (½MS) and Woody Plant (WP) (10)] poor growth and vitrification were observed (Table 1). In preliminary experiments, the root tips of plantlets cultured on ½MS and WP solid media turned dark brown, which caused a low establishment rate for transplantation to soil (data not shown). Plantlets subcultured on B5 medium showed unstable growth of shoot (different height) which also resulted in a low transplantation rate. However, in the root system many branches were observed. Therefore, B5 medium was used for experiments to establish adventitious root cultures, while the plants obtained from MS medium were transferred to soil.

The regenerates cultivated in the field showed satisfactory growth and developed a wide branched root system which showed a partially red color within one month, indi-

TABLE 1. Root and Shoot Development of *Salvia miltiorrhiza* Shoots Subcultured on Different Hormone-free Basal Media.[a]

Medium	No. of roots	No. of shoots	Growth
Half-strength Murashige-Skoog	3.00 ± 0.65	2.30 ± 0.24	dwarf[b]
Murashige-Skoog	2.45 ± 0.68	2.30 ± 0.14	normal[c]
Woody Plant	3.85 ± 0.46	5.75 ± 0.96	dwarf[b]
Gamborg B5	3.75 ± 0.36	3.45 ± 0.43	unstable[d]

[a]The numbers are average (±SE) of 20 experiments.
[b]Small in size (<5 cm height).
[c]Stable in size (8–10 cm height).
[d]Differing heights (4–8 cm).

cating the accumulation of tanshinones. After 6 months of cultivation, tanshinone II was the main constituent of those roots (ca. 5 mg/g dry wt), and was present at a level corresponding to that of the parent plant which had been maintained in the field (ca. 6.2 mg/g dry wt) (Figure 1). On the other hand, only one third as much cryptotanshinone (ca. 1.6 mg/g dry wt) was produced in the roots of the regenerates compared to those of the parent plant. The production of tanshinones in the roots of the regenerates was stable from 6 to 20 months of field cultivation. Tanshinone II and cryptotanshinone were produced in approximately 4 times and twice the amount, respectively, as compared to the yield in the commercial roots (ca. 3 mg/g dry wt total tanshinones), which were usually from 3–4-year-old plants (Figure 1).

Adventitious root cultures were established from petiole segments (ca. 1 cm) of

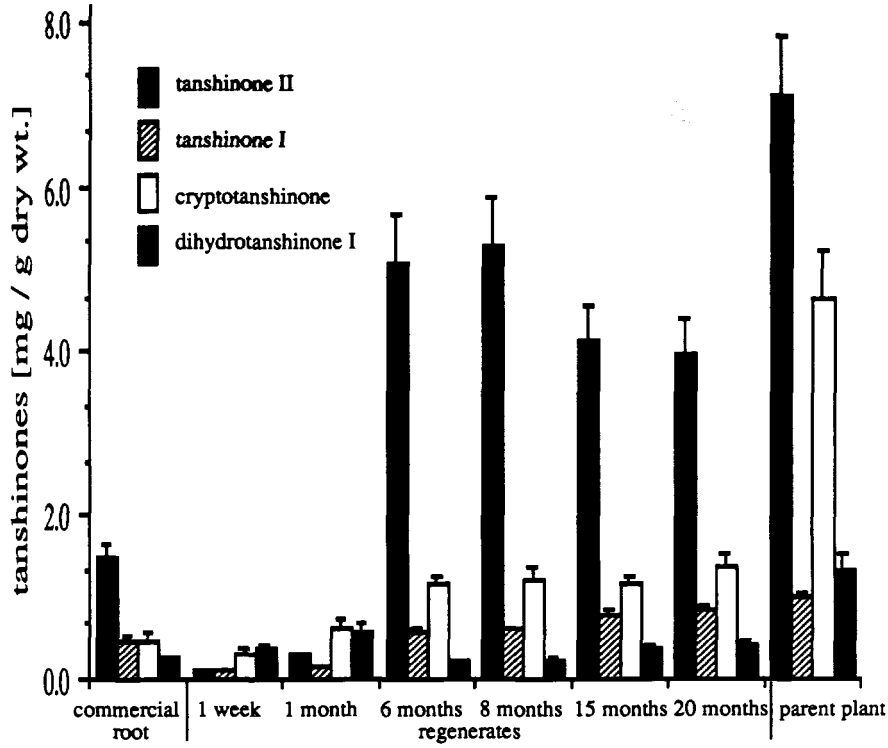

FIGURE 1. Tanshinone content in commercial "tan-shen," regenerates from in vitro cultures, and parent plant of *Salvia miltiorrhiza*. Bars indicate the standard error.

shoot cultures on B5 solid medium containing auxins in combination with or without benzyladenine (BA). The best growth (ca. 60-fold in 8 weeks) was observed when the roots were cultured on B5 solid medium supplemented with 1-naphthaleneacetic acid (NAA) without or with low addition of BA (0.5 mg/liter NAA, 0 or 0.1 mg/liter BA). On the other hand, only 0.3–1.2 mg/g dry wt of tanshinones were produced in those roots. The maximal content (1.2 mg/g dry wt) was obtained with the combination of 0.1 mg/liter BA (Figure 2). Tanshinone II was the main constituent of those roots. The same results were obtained when indole-3-acetic acid (IAA) (3.0 mg/liter) was used as auxin. In this case, in combination with 0.1 mg/liter BA, approximately 1.8 mg tanshinones/g dry wt were produced and tanshinone II was the major compound. In both experiments, the addition of 0.5 mg/liter BA to the cultures reduced the growth rate and the tanshinone production.

The addition of indole-3-butylic acid (IBA) (0.1–1.0 mg/liter) to the culture medium suppressed the growth (Figure 2). In this case best growth (ca. 12-fold in 8 weeks) was observed with 0.2 mg/liter IBA in the combination with 0.1 mg/liter BA. The production of tanshinones, however, was highly stimulated by the supply of IBA. Over 12 mg/g dry wt of tanshinones were obtained with the addition of 0.1 mg/liter IBA. In combination with BA (0.1 mg/liter) less tanshinones were produced, except with the addition of high concentrations of IBA (1 mg/liter). Unlikely in the roots of the parent plant and roots cultured with IAA and NAA, the 1,2 dihydrofuran derivatives cryptotanshinone and dihydrotanshinone I were the main constituents in the adventitious roots cultured on B5 solid medium containing low concentrations of IBA (up to 0.5 mg/liter without the addition of BA). Maximal dihydrotanshinone I (4 mg/g dry wt) and cryptotanshinone (5.2 mg/g dry wt) were produced in the adventitious roots cultured with 0.1 mg/liter IBA (Figure 2).

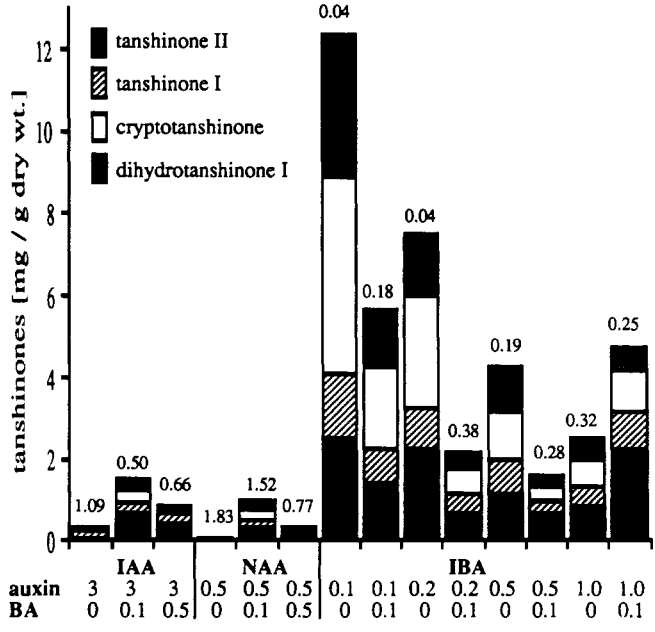

FIGURE 2. Tanshinone production in adventitious roots of *Salvia miltiorrhiza* cultured on Gamborg B5 solid medium with different phytohormones for 8 weeks. Each culture was inoculated with ca. 30 mg fresh roots. The numbers on the columns indicate the fresh wt (g).

The adventitious roots grown on solid media were transferred to liquid media containing the same phytohormones. The roots cultured in the liquid media showed faster growth as compared to roots cultured on solid media (Figure 3). The maximal growth (ca. 75-fold in 8 weeks) was obtained in medium with 3 mg/liter IAA. The addition of BA reduced the growth rate. The production of tanshinones by the root cultures was also enhanced in liquid media. Comparable to the results obtained on solid media, the adventitious roots cultured in media with the addition of IAA and NAA yielded only low levels of tanshinones (maximal 24 mg/g dry wt in B5 liquid medium containing 0.5 mg/liter NAA and 0.1 mg/liter BA). Nevertheless, the production of tanshinones in liquid medium was ca. 15-fold the production on solid media.

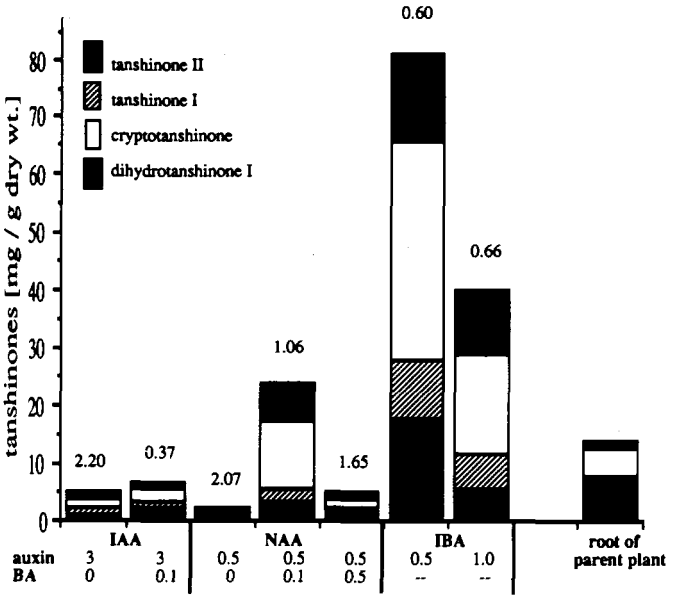

FIGURE 3. Tanshinone production in adventitious roots of *Salvia miltiorrhiza* cultured in Gamborg B5 liquid medium (30 ml/100 ml flask) with different phytohormones for 8 weeks. Each culture was inoculated with ca. 30 mg fresh roots. The numbers on the columns indicate the fresh wt (g).

On the other hand, the adventitious roots cultured in B5 liquid medium containing only IBA grew more slowly (ca. 20-fold in 8 weeks) but produced high amounts of tanshinones (Figure 3). With the addition of 0.5 mg/liter IBA, over 80 mg tanshinones/g dry wt were obtained, which corresponded to 4 times the content of the roots of the parent plant. Cryptotanshinone was the main constituent, yielding 38 mg/g dry wt, followed by tanshinone II (19 mg/g dry wt).

The production of cryptotanshinone in cell suspension culture at a yield of 36 mg/dry wt has been reported (6). The growth of the cells was completely suppressed under the culture conditions needed for this productivity. Consequently a two-stage system was needed for production of cryptotanshinone by cell suspension culture (6,7). The adventitious root cultures showed the same high productivity and fast growth in B5 liquid medium containing 0.1 mg/liter IBA. Therefore, the adventitious root cultures of *S. miltiorrhiza* seem to be an applicable system for production of cryptotanshinone in vitro.

EXPERIMENTAL

PLANT MATERIAL.—Nodal segments (3 cm) were disinfected with 75% EtOH for 30 sec and rinsed once with sterilized H_2O. The segments were sterilized with 3% NaOCl (1 drop Tween 20/40 ml) for 10 min and washed with sterilized H_2O 3 times. Axillary buds (1–2 mm) were cut off and cultured on MS solid medium (8) supplemented with BA (1 mg/liter) for 6 weeks in a 16 h light (70 $\mu E \cdot m^{-2} S^{-1}$): 8 h dark cycle at 25°. Shoots were subcultured on hormone-free MS (pH 5.7) solid medium under the same culture conditions to obtain sufficient material for the experiments. Adventitious roots were induced from petiole segments of shoot cultures on B5 (9) solid medium containing various auxins in combination with and without BA. The adventitious roots thus obtained were removed and subcultured every 8 weeks in B5 liquid medium containing the same phytohormone combination as used for root induction. Voucher specimens are kept at the Tsukuba Medicinal Plant Research Station. Commercial roots, grown in China, were purchased from Nakai-Koshindo (Kobe, Japan).

CULTURE EXPERIMENTS.—Shoots (1 cm length) were subcultured onto various solid media (MS, ½MS, B5, WP, 30 ml/tube 40 mm × 150 mm and 20 tubes/medium). After 10 weeks, growth and number of shoots and roots obtained on the different media were determined.

Adventitious roots (5 roots, ca. 30 mg fresh wt) were subcultured in or on B5 liquid (30 ml/100 ml flask) or solid (0.2% Gelrite) media containing various phytohormone combinations. After 8 weeks of culture in the dark (25°, 100 rpm for liquid cultures), the fresh wts were determined and the roots were lyophilized. The tanshinone content was measured by hplc. The pH of all media was adjusted to 5.7 before autoclaving. All experiments were carried out in triplicate.

PROPAGATION AND CULTIVATION IN SOIL.—Shoots (1.5 cm length) subcultured on hormone-free MS solid medium were inoculated on different basal media [½MS, MS, B5, and WP (10) medium] for 8 weeks at 25° in a cycle of 16 h light (70 $\mu E \cdot m^{-2} S^{-1}$): 8 h dark. Plants grown on hormone-free MS solid medium were placed into pots [soil-leaf mold (1:4)] and cultivated in phytotron (25°, 16:8 h light-dark, 6000 lux) for 1 week. After 2 weeks of additional cultivation in the greenhouse, the plants were transferred to the field (May 1988) at Tsukuba Medicinal Plant Research Station. One to 20 months later, five plants were harvested and the roots were lyophilized for determination of tanshinone content. The experiment was repeated the next year.

HPLC ANALYSIS.—Lyophilized roots (10–50 mg dry wt) were homogenized with 10 volumes of $CHCl_3$-MeOH (2:1). Further sample preparation and hplc conditions were the same as previously described (11).

ACKNOWLEDGMENTS

The authors express their appreciation to Dr. Martina Sauerwein for preparing the manuscript.

LITERATURE CITED

1. M. Nakanao and T. Fukushima, *J. Pharm. Soc. Jpn.*, **54**, 844 (1934).
2. A.-R. Lee, W.-L. Wu, W.-L. Chang, H.-C. Lin, and M.-L. King, *J. Nat. Prod.*, **50**, 157 (1987), and references cited therein.
3. A. Yagi, K. Fujimoto, K. Tanonaka, K. Hirai, and S. Takeo, *Planta Med.*, **55**, 51 (1989).
4. Z.-X. Chen, *Kagaku no Ryoiki*, **35**, 494 (1981).
5. T. Nakanishi, H. Miyasaka, M. Nasu, H. Hashimoto, and K. Yoneda, *Phytochemistry*, **22**, 721 (1983).
6. H. Miyasaka, M. Nasu, T. Yamamoto, Y. Endo, and K. Yoneda, *Phytochemistry*, **25**, 637 (1986).
7. H. Miyasaka, M. Nasu, T. Yamamoto, Y. Endo, and K. Yoneda, *Phytochemistry*, **25**, 1621 (1986).
8. T. Murashige and F. Skoog, *Physiol. Plant.*, **15**, 473 (1962).
9. O.L. Gamborg, R.A. Miller, and K. Ojima, *Exp. Cell. Res.*, **50**, 151 (1968).
10. G.B. Lloyd and B.H. McCown, *International Plant Propagators' Society. Combined Proceedings*, **30**, 421 (1980).
11. N. Okamura, K. Kobayashi, A. Yagi, T. Kitazawa, and K. Shimomura, *J. Chromatogr.*, **542**, 317 (1991).

Received 9 April 1991