Tanshinone Production in Roots of Micropropagated *Salvia przewalskii* Maxim.

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Production of tanshinones (tanshinone I and IIA) was determined in roots of *Salvia przewalskii* micropropagated plants. It was found that the total tanshinone content (tashinone I and tashinone IIA) was dependent on the age of the analyzed plants. The roots of 2-year-old *in vitro* regenerated plants at flowering stage produced highest tanshinone levels (3.8 mg/g dry weight of tanshinone I and 7.6 mg/g dry weight of tanshinone IIA).

Key words: Salvia przewalskii, Tanshinone I and Tanshinone IIA

Introduction

Salvia przewalskii Maxim. is a herbaceous perennial plant endemic to north-western China (Li *et al*., 1991). The dried roots of this species are used as a drug under the name "Hong Qin Jiao" in the traditional Chinese and Japanese medicine for the treatment of disorders caused by poor blood supply, such as coronary artery disease (Wang *et al*., 1988). The roots of this plant have been used as a substitute for *Salvia miltiorrhiza* roots. It has been reported that the main chemical components of *S. przewalskii* roots are abietane-type diterpene pigments – tanshinones, namely cryptotanshinone, tanshinone I, IIA, IIB, przewalskin, przewaquinone A (Li *et al*., 1991; Xue *et al*., 2000). The tanshinones isolated also from *S. miltiorrhiza* roots have demonstrated antidermatophytic, anti-inflammatory, antioxidant, antimutagenic and antiplatelet aggregation activities (Chang *et al*., 1990; Ryu *et al*., 1997a, b). The antiproliferative activity of tanshinones against different human tumor cells has been shown. Tanshinones have also exhibited cardiovascular effects and are used in the treatment of some coronary heart diseases. They have demonstrated inhibitory activity against *Mycobacterium tuberculosis* (Baricevic and Bartol, 2000). Tanshinone I and cryptotanshinone prevent the complications of myocardial ischemia (Chen *et al*., 1997). Furthermore, cryptotanshinone and tanshinone IIB are reported to have bacteriostatic activity against *Staphylococcus aureus* (Dweck, 2000). Apart from tanshinones also other bioactive compounds have been found in roots of *S. przewalskii*. They include triterpenoids (przewanoic acid A and B, oleanolic acid and ursolic acid) (Wang *et al*., 1988) and phenolic derivatives (three lithospermic acid B esters) (Zhijun *et al*., 1999).

The aim of this study was to determine for the first time the production of tanshinone I and IIA (Fig. 1) in roots of micropropagated plants of *Salvia przewalskii*.

Experimental

Establishment of Salvia przewalskii plants

Plants of *Salvia przewalskii* were initiated from shoot tips obtained from 4-week-old seedlings and cultured on Murashige and Skoog (MS) (Murashige and Skoog, 1962) basal medium solidified with 0.7% agar containing 0.5 mg/l indole-3-acetic acid (IAA) and 1 mg/l 6-benzylaminopurine (BA). The cultures were kept under light (continuous cool-white fluorescent lamps at a PPFD of 40 μmol m^{-2} s^{-1}) at 26 ± 2 °C.

The shoots raised *in vitro* with the length over 1 cm were excised from shoot cultures and transferred into full- or half-strength MS agar medium without growth regulators or supplemented with 0.1 mg/l indole-3-butyric acid (IBA). The percentage of rooted shoots, the length of shoots and roots and the number of roots per shoot were recorded after 4 weeks (Table I). The rooted shoots were removed from the culture flasks, washed in tap-water to remove traces of agar attached to the roots and transplanted into pots (10 cm diameter) containing a sterilized mixture of soil, sand and peat (4:3:3 v/v/v). The number of plantlets trans-

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ferred into pots was 45. To maintain high humidity, the plantlets were covered with glass caps gradually opened during a 2 weeks period. They were watered with sterile water during the first seven days. The potted plants were grown in the growing chamber at 26 °C under continuous cool-white fluorescent tubes of 40 μmol m^{-2} s^{-1}. Survival of the rooted plantlets was recorded at 10 weeks. Then, the hardened plants were transferred to field conditions. They were grown to maturity in the Medicinal Plant Garden of the Medical University of Łódź. The species identity was confirmed in our Department based on the data of the Flora of China (Li and Hedge, 1994).

Statistical analysis

The data were recorded as means ± standard error. Significant differences between means were assessed by Duncan multiple range test at a 5% probability level.

Determination of tanshinones

For quantitative determination of tanshinones, the roots of *S. przewalskii* micropropagated plants were used. The air-dried roots (250 mg) were extracted five times at room temperature for 3 h with 30 ml of methanol. The extraction was performed in the dark. The combined methanolic extracts were evaporated to dryness under reduced pressure. The residue of methanolic extract (5 mg of dry weight) was dissolved in acetonitrile/methanol (2:1 v/v) (10 ml). The solution was filtered through 0.45 μm filters and analyzed by HPLC using a Varian ProStar apparatus coupled to a UV-Vis detector. A Nucleosil C18 (5 μm) column (250 × 4.6 mm) was used with two solvent systems: A, H_2O containing 5 mM K_2PO_4 (pH 2.6), and B, acetonitrile/methanol (2:1 v/v). The elution programme was as follows: 0.0 to 12.5 min 70–95% B (linear change); 12.5 to 15.0 min 95% B; 15.0 to 16.0 min 95–70% B; 16.0 to 20.0 min 70% B. The flow rate was 1.5 ml min^{-1}. The absorbance was measured at 270 nm. The identification of tanshinone I and tanshinone IIA was carried out by comparison of the chromatographic peak retention times and UV spectra of the test solution with those of authentic compounds. Using the above-mentioned conditions, two tanshinones were identified as tanshinone I and tanshinone IIA (Table II). The quantification of these tanshinones was achieved using calibration curves prepared with pure compounds. The detector response was linear from 0.008 to 0.166 mg of tanshinone I ($y = 1117.88x - 2.4$; $r = 0.999$) and tanshinone IIA ($y = 4440.44x - 8.8$; $r = 0.999$). Tanshinone I (TI) and tanshinone IIA (TIIA) standards were purchased from ChromaDex (USA). The content of TI and TIIA was expressed in mg/g dry weight. In this paper the sum of two tanshinones (TI and TIIA) was referred to as the total tanshinone content (TT).

Results and Discussion

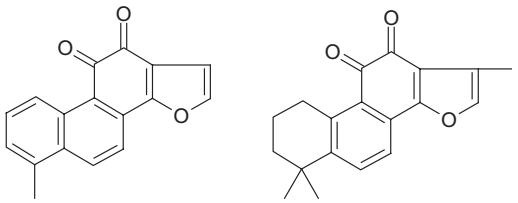
The shoots proliferated on MS medium containing 0.5 mg/l IAA and 1 mg/l BA were rooted within 4 weeks on full- or half-strength MS media alone or in the presence of 0.1 mg/l IBA (Table I). The maximum rooting percentage (97%) and the length of roots (4.1 cm) were achieved on full-strength MS medium supplemented with 0.1 mg/l IBA (Table I). The rooting plantlets could be successfully acclimatized in pots containing a sterilized mixture of soil, sand and peat. About 80% of the plants survived the transfer to the field and flowered in the next season.

Levels of tanshinone I (TI) and tanshinone IIA (TIIA) (Fig. 1) were measured in roots of micropropagated plants of *S. przewalskii* by HPLC (Ta-

Table I. Rooting of *Salvia przewalskii* shoots on MS and ½ MS media without growth regulators or supplemented with auxin (IBA, indole-3-butyric acid) after 4 weeks of culture.

Medium	Number of explants	Shoots of rooting (%)	Mean number of roots/shoot ± SE	Mean root length ± SE [cm]	Mean shoot length ± SE [cm]
MS	30	86.7	2.31 ± 0.17 [a]	3.21 ± 0.13 [a]	3.57 ± 0.10 [a]
½ MS	30	43	2.9 ± 0.04 [a]	3.57 ± 0.13 [ac]	1.34 ± 0.07 [b]
MS + IBA 0.1 mg/l	29	96.5	2.86 ± 0.14 [a]	4.13 ± 0.11 [bc]	2.56 ± 0.16 [c]
½ MS + IBA 0.1 mg/l	33	75.8	2.64 ± 0.31 [a]	3.45 ± 0.03 [a]	1.35 ± 0.04 [bd]

Means followed by the same letters are not significantly different ($p \leq 0.05$) using Duncan multiple range test.



Tanshinone I Tanshinone IIA

Fig. 1. Structures of tanshinone I and IIA.

Table II. Separation of tanshinones in methanolic extract obtained from the roots of *Salvia przewalskii* micropropagated plants by HPLC method. Conditions are described in the section Experimental.

Compound	Retention times [min]
Tanshinone I	8.013
Tanshinone IIA	9.620

ble II). It was found that the total tanshinone content (TI and TIIA) varied from 2.7 to 11.4 mg/g on a dry weight basis. The roots of 2-year-old *in vitro* regenerated plants grown in the field and harvested at the flowering stage, produced the highest tanshinone levels, namely 3.8 mg/g dry weight of TI and 7.6 mg/g dry weight of TIIA. The contents found were twice higher in comparison with the roots of one-year-old micropropagated plants which contained 1.8 mg/g dry weight of TI and 3.2 mg/g dry weight of TIIA (Table III). The lowest level of both TI and TIIA was found in roots of 10-week-old *in vitro* regenerated plants of *S. przewalskii* grown in pots. These differences in tanshinone production could be due to differences in the age and development stage between potted and field-grown plants. When the plants were grown in the field a 3-fold increase in the average leaf number (53 leaves/plant), a 2-fold increase in the size of leaves (29 cm) and root length (14 cm) as well as its diameter (about 5 mm) were noted. The surface of the roots showed a red color which suggests that tanshinones are localized mainly in the periderm and cortex layer.

TI and TIIA have been previously isolated from roots of *S. przewalskii*, but this is the first report showing the quantitative analysis of the tanshinones in the species. So far such studies have been restricted to *in vitro* cultures and roots of *Salvia miltiorrhiza* (Okamura *et al*., 1991; Hu and Alferman, 1993). For example studies carried out by Okamura *et al.* (1991) showed that the TI and TIIA content estimated in roots of one-year-old regenerated plants and roots of parent plants of *S. miltiorrhiza* was 1.27% and 1.67% on a dry weight basis, respectively. The values were comparable with these found by us in roots of regenerated plants of *S. przewalskii* although relative proportions between TI and TIIA in *S. przewalskii* and *S. miltiorrhiza* roots were different. The results are of particular interest since roots of *S. miltiorrhiza* are the most important source of tanshinones. The present study showed that also roots of *S. przewalskii* provide a suitable material for a high production of tanshinones, particularly that of TIIA. The compound such as sodium tanshinone IIA sulfonate (STS) has shown a sufficient promise to be used in clinical trials for treatment of patients with cardiovascular diseases and cerebral thromboembolism symptoms (Baricevic and Bartol, 2000).

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Table III. Content of tanshinones in roots of *in vitro* regenerated plants of *Salvia przewalskii*.

Material	Content[a] [mg/g dry weight] Tanshinone IIA	Tanshinone I	Total
Roots of 10-week-old plants[b]	1.999 ± 0.180	0.687 ± 0.162	2.686 ± 0.330
Roots of one-year-old plants[c]	3.231 ± 0.299	1.758 ± 0.197	4.989 ± 0.349
Roots of two-year-old plants[d]	7.588 ± 0.336	3.780 ± 0.241	11.368 ± 0.453

[a] Results are means ± standard error from three independent experiments.
[b] Cultured in the pots.
[c] Cultured in the field since May 2003 and harvested in September 2003.
[d] Cultured in the field since May 2003 and harvested in July 2004.

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