Tanshinones in Culture of *Salvia przewalskii* Maxim In Vitro

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The content of four tanshinones was determined in different in vitro cultures of *Salvia przewalskii*. Accumulation of tanshinones depended on the type and age of tissue and could be altered by growth conditions. Differentiated tissues (in vitro cultured shoots, shoots and roots of plantlets regenerated in vitro) contained more diterpenoids than undifferentiated tissues (i.e., the four callus lines). Root was the most important organ for tanshinone accumulation. The highest levels were achieved in roots of 4-week-old plantlets (5.1–5.6 mg g^{-1} DW); the shoots used for root induction were maintained on multiplication medium for 2.5–4 years with regular subcultures every 4 weeks.

Key words: In vitro cultures, tanshinones, *Salvia przewalskii*.

INTRODUCTION

Salvia przewalskii Maxim. (Lamiaceae) grows in the wild in limited regions of northwestern China (http://www.eFloras.org). The dried roots of the plant (locally known as *Hong Qin Jiao*), and dried *Salvia miltiorrhiza* roots, have been widely used in traditional Chinese and Japanese medicine for treatment of coronary artery disease (Li et al., 2008). The roots of both plant species contain abietane-type diterpenoids – tanshinones such as tanshinone I, tanshinone IIA and IIB, cryptotanshinone, dihydrotanshinone and methyltanshinone (e.g., Okamura et al., 1991; Wang et al., 2007; He et al., 2010). The compounds have been shown to have cardiovascular protective (Ling et al., 2005), neuroprotective (Lam et al., 2003), antioxidant (Weng and Gordon, 1992), cytotoxic (Ryu et al., 1997) and antibacterial (Lee et al., 1999) properties. They are also capable of reducing aggregation of blood platelets (Lee et al., 1987). Tanshinone IIA, one of main abietanoid diterpenoids of *S. miltiorrhiza* and *S. przewalskii* roots, as sodium sulfonate, has shown enough promise to be used in clinical trials for treatment of patients with cardiovascular disease and cerebral thromboembolism symptoms (Wang et al., 2007).

In recent years, plant cell, tissue and organ cultures have been seen as a valuable source of bioactive secondary metabolites, available regardless of seasonal and climatic conditions. Numerous studies have shown that tanshinones can be produced in culture in vitro of *S. miltiorrhiza* (e.g., Shimomura et al., 1991; Hu and Alfermann, 1993; Wu et al., 2003a; Lee et al., 2008). The only published report concerning tanshinone production in tissue culture of *S. przewalskii* is an earlier report on the content of tanshinone I and tanshinone IIA in plants regenerated in vitro after 10 weeks and 1 or 2 years of cultivation in soil (Skała and Wysokińska, 2005). Since *S. przewalskii* might be used in medicine instead of *S. miltiorrhiza* roots, we saw the need to establish various in vitro cultures of this plant and make comparative qualitative and quantitative studies of tanshinones (tanshinone I, tanshinone IIA, dihydrotanshinone, cryptotanshinone) in callus, cell suspension and shoot cultures as well as in shoots and roots of 4-week-old plantlets grown in vitro. We also wanted to determine the effect of long-term culture on the biosynthetic capacity of callus and shoot cultures of *S. przewalskii*. Tanshinones were also extracted from shoots and roots of 3-year-old *S. przewalskii* plants regenerated in vitro, grown in the field.

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MATERIAL AND METHODS

PLANT MATERIAL

Salvia przewalskii seeds provided by the Adam Mickiewicz University Botanical Garden in Poznań (Poland) were sterilized with 2% sodium hypochlorite solution for 10 min and then rinsed three times (15 min each) in sterile distilled water. For germination, seeds were placed on MS (Murashige and Skoog, 1962) agar (0.7%) medium supplemented with 3% sucrose, gibberellic acid (0.1 mg l^{-1}) and kinetin (0.02 mg l^{-1}).

CALLUS AND CELL SUSPENSION CULTURES

Four-week-old seedling explants (hypocotyls, cotyledons, roots) and leaves (divided into petiole and lamina) of 4-week-old shoots grown on MS agar (0.7%) medium supplemented with 0.5 mg l^{-1} indole-3-cetic acid (IAA) and 1.0 mg l^{-1} 6-benzyladenine (BA) were used as explants. These explants were placed on MS agar (0.7%) medium supplemented with various types and concentrations of auxin (0.5 mg l^{-1} IAA; 0.5 mg l^{-1} indole-3-butyric acid (IBA); 0.5 mg l^{-1} and 0.2 mg l^{-1} α-naphthaleneacetic acid (NAA); 0.2 mg l^{-1} 2,4-dichlorophenoxy acetic acid, 2,4-D) and cytokinin (0.2 mg l^{-1} and 1.0 mg l^{-1} BA; 1.0 mg l^{-1} thidiazuron, TDZ). Additionally, for induction of callus tissue from leaf explants, Woody Plant (WP) (Lloyd and McCown 1980) agar (0.7%) medium supplemented with 2,4-D (0.5 mg l^{-1}) and BA (0.2 mg l^{-1}) was also used. For the callus cultures, tubes (25 x 180 mm) containing 25 ml medium were used. The four callus lines were obtained and labeled as follows: S1 and S2 – petiole-derived calli cultured on WP medium containing 0.5 mg l^{-1} 2,4-D and 0.2 mg l^{-1} BA; S3 – petiole-derived callus maintained on MS medium supplemented with 0.5 mg l^{-1} 2,4-D and 0.2 mg l^{-1} BA; and S4 – hypocotyl-derived callus cultured on MS medium supplemented with 0.2 mg l^{-1} NAA and 1.0 mg l^{-1} TDZ. Callus lines S1, S3 and S4 were maintained under a 16 h photoperiod (40 µmol m^{-2} s^{-1}) and S2 under darkness, at 26°C ±2°C in a culture room. The calli were regularly subcultured at 4-week intervals by transferring 0.2–0.3 g fresh weight to fresh medium of the same composition, and were maintained in vitro for 4 years. Fresh weight (FW) was measured after the 10th subculture over three subsequent passages, and 3 calli of each line were used for each subculture. The tanshinone concentrations in calli were determined by HPLC analysis. The age of the analyzed callus cultures was ~1, ~2.5 and ~4 years after the 15th, 35th and 55th passages respectively.

Calli of the S1 and S2 lines were used to initiate cell suspension cultures: ~4 g callus was transferred to 300 ml Erlenmeyer flasks containing 50 ml WP liquid medium supplemented with 0.5 mg l^{-1} 2,4-D and 0.2 mg l^{-1} BA. The cultures were incubated on a rotary shaker (100 rpm) at 26°C ±2°C under two light regimes: a 16 h photoperiod at 40 µmol m^{-2} s^{-1} (line SC1), or darkness (line SC2). Subcultures were made every 2 weeks by transferring 6 ml suspension (0.66–0.78 g/flask FW; 0.05 g/flask DW) to 50 ml fresh medium. After 1 year of subculturing (30 passages) the growth curves of the SC1 and SC2 lines were established by measuring DW every 3 days for 30 days, each point being the mean of three flasks. Dry weight was determined after drying the cells at 80 C to constant weight. Time course experiments were performed three times for each line. For tanshinone determination the cells (after 30 subcultures) were harvested on the 14th day.

SHOOT CULTURE AND PLANTLET REGENERATION

The procedure for establishment of *S. przewalskii* shoot culture, regeneration of plantlets and acclimatization in soil was as described in our previous work (Skała and Wysokińska, 2005; Skała et al., 2007). Shoot culture initiated from shoot tips of 4-week-old aseptically germinated seedlings of *S. przewalskii* was maintained on MS agar medium supplemented with 0.1 mg l^{-1} IAA and 0.5 mg l^{-1} BA (multiplication medium). The culture was incubated at 26°C ±2°C under a 16 h photoperiod (cool-white fluorescent light, 40 µmol m^{-2} s^{-1}). The culture was maintained on the same medium for 4 years with regular subcultures every 4 weeks. Shoots were harvested at the 10th, 30th and 50th passages and then either used directly for quantitative determination of tanshinones or else transferred to MS medium supplemented with 0.1 mg l^{-1} IBA for root formation. After 4 weeks of culture under those conditions the content of tanshinones in shoots and roots of plantlets was determined.

Also, over a period of 4 years we recorded the shoot multiplication (number of shoots per explant after 4 weeks of culture on multiplication medium), rooting of shoots 4 weeks after transfer to rooting medium (rooting frequency %), the mean number of roots per shoot, and mean length of roots. For comparison we also measured tanshinone content in shoots and roots of 3-year-old in vitro regenerated *S. przewalskii* plants collected from the field at flowering stage.

EXTRACTION AND DETERMINATION OF TANSHINONES

Dried and powdered material (250 mg) was extracted with methanol. Tanshinone extraction from the plant material and quantitative analysis of it fol-

TABLE 1. Tanshinone content in callus and cell suspension cultures of *S. przewalskii*

Culture type	Basal medium/ PGR (mg l⁻¹)	Number of subcultures (age of culture in years)	Tanshinone content (mg g⁻¹ DW)			
			TI	TIIA	DHT	CT
Callus line*						
S1	WP/	15 (1)	0.15 ± 0.001	0.04 ± 0.0003	n.d.	n.d.
	0.5 2,4-D	35 (2.5)	0.06 ± 0.002	n.d.	n.d.	n.d.
	0.2 BAP	55 (4)	t	n.d.	n.d.	n.d.
S2	WP/	15 (1)	0.12 ± 0.005	n.d.	n.d.	n.d.
	0.5 2,4-D	35 (2.5)	0.09 ± 0.004	n.d.	n.d.	n.d.
	0.2 BAP	55 (4)	t	n.d.	n.d.	n.d.
S3	MS/	15 (1)	0.13 ± 0.003	0.04 ± 0.001	0.06 ± 0.002	n.d.
	0.5 2,4-D	35 (2.5)	0.12 ± 0.003	t	t	n.d.
	0.2 BAP	55 (4)	0.04 ± 0.001	n.d.	t	n.d.
S4	MS/	15 (1)	0.15 ± 0.020	0.06 ± 0.008	0.12 ± 0.090	0.05 ± 0.001
	0.2 NAA	35 (2.5)	0.11 ± 0.003	0.04 ± 0.003	0.09 ± 0.002	t
	1.0 TDZ	55 (4)	0.06 ± 0.002	t	0.04 ± 0.001	t
Cell suspension lines **						
SC1	WP/ 0.5 2,4-D 0.2 BAP	30 (1)	0.22 ± 0.008	0.15 ± 0.010	n.d.	n.d.
SC2	WP/ 0.5 2,4-D 0.2 BAP	30 (1)	0.07 ± 0.002	n.d.	n.d.	n.d.

*Cultures were grown under a 16 h photoperiod (lines S1, S3, S4) or in darkness (line S2) for 4 weeks; **Cell suspension cultures were cultured under a 16 h photoperiod (line SC1) or in darkness (line SC2) for 2 weeks. Value are means ±SE (n=9); t – trace (<0.01 mg g⁻¹ DW); n.d. – not detected.

lowed methods described previously (Skała and Wysokińska, 2005). Tanshinones were identified by comparing their chromatographic peak retention times and UV spectra with those of commercially available standards of tanshinone I (TI), tanshinone IIA (TIIA), cryptotanshinone (CT), and dihydrotanshinone (DHT) (ChromaDex, USA). The retention times (min) were 8.34 ±0.03 for TI, 9.98 ±0.02 for TIIA, 7.94 ±0.03 for CT, and 6.55 ±0.03 for DHT. The calibration curves, constructed separately for TI, TIIA, CT and DHT, were linear above the range of 0.008–0.166 mg/ml for TI and TIIA and 0.009–0.458 mg/ml for CT and DHT. Tanshinone content is expressed as mg g⁻¹ DW. Total tanshinone content is the sum of four tanshinones (T I, TIIA, DHT, CT). The results are means ±SD of three replicates from three independent experiments.

STATISTICAL ANALYSIS

The results presented in Table 2 were tested by ANOVA and differences in means were assessed using the Mann Whitney U-test at P=0.05. All statistical analyses employed Statistica 5.0.

RESULTS AND DISCUSSION

UNDIFFERENTIATED CULTURES

Among the explants tested, only hypocotyls (from 4-week-old seedlings) and leaf petioles (from 4-week-old multiple shoots) formed calli at high frequency (~90%), able to grow continuously when maintained on the media described above. For other explant types the callus induction frequency was much lower: 0–54% for leaf laminae, 0–65% for cotyledons and 0–40% for roots, depending on basal media (WP or MS) and the type and concentration of growth regulators. Irrespective of the medium composition, the calli derived from the explants were small, turned brown and showed no growth after two subcultures, most probably due to accumulation and oxidation of polyphenols. Our observations suggest

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TABLE 2. Tanshinone production in multiple shoots, in vitro regenerated plantlets, and field-grown plants (shoots and roots)

Material	Shoot culture after	Tanshinone content (mg g $^{-1}$ DW)				
		TI	TIIA	DHT	CT	Total
Multiple shoots *	10	0.65 ± 0.030[d]	0.25 ± 0.020[a]	0.73 ± 0.030[c]	0.34 ± 0.010[c]	1.97 ± 0.020[c]
	30	0.33 ± 0.020[b]	0.21 ± 0.001[a]	0.47 ± 0.020[b]	0.21 ± 0.009[b]	1.22 ± 0.010[b]
	50 subcultures	0.39 ± 0.007[bc]	0.17 ± 0.020[a]	0.33 ± 0.001[a]	0.15 ± 0.004[ab]	1.04 ± 0.008[a]
Plantlets in vitro** Shoots	10	0.37 ± 0.01[bc]	0.26 ± 0.02[a]	0.43 ± 0.02[b]	0.27 ± 0.004[bc]	1.33 ± 0.04[b]
	30	0.22 ± 0.008[a]	0.19 ± 0.01[a]	0.31 ± 0.01[a]	0.18 ± 0.004[b]	0.9 ± 0.03[a]
	50 subcultures	0.23 ± 0.01[a]	0.17 ± 0.009[a]	0.27 ± 0.02[a]	0.11 ± 0.01[a]	0.78 ± 0.03[a]
Roots	10	0.44 ± 0.01[c]	1.37 ± 0.03[b]	0.54 ± 0.04[b]	0.21 ± 0.01[b]	2.56 ± 0.25[d]
	30	0.97 ± 0.02[e]	2.17 ± 0.07[c]	1.04 ± 0.09[d]	0.89 ± 0.03[d]	5.07 ± 0.3[e]
	50 subcultures	1.07 ± 0.07[e]	2.13 ± 0.06[c]	1.23 ± 0.07[d]	1.19 ± 0.04[e]	5.62 ± 0.24[e]
Plants grown in field***						
Shoots		0.28 ± 0.050[ab]	0.17 ± 0.010[a]	0.20 ± 0.008[a]	0.12 ± 0.003[a]	0.77 ± 0.020[a]
Roots		4.40 ± 0.240[f]	8.30 ± 0.270[d]	4.33 ± 0.100[e]	1.85 ± 0.170[f]	18.88 ± 0.200[f]

*Shoots maintained during 10, 30 and 50 subcultures on MS agar medium supplemented with 0.1 mg l^{-1} IAA and 0.5 mg l^{-1} BA.
** Shoots for plantlet regeneration were taken from the 10th, 30th and 50th subcultures on MS agar medium supplemented with 0.1 mg l^{-1} IAA and 0.5 mg l^{-1} BA and transferred to MS agar medium containing 0.1 mg l^{-1} IBA for rooting.
*** 3-year-old plants micropropagated in vitro and grown in the field. Plants were field-cultivated from May 2003 and harvested in July 2006
Values are means ±SE (n=9). Means followed by the same letter within column do not significantly differ at P=0.05.

that callus formation and further development in *S. przewalskii* is strongly influenced by explant type. Such variable responses among explant types have been reported in other plant species such as *Rhodiola sachalinensis* (Wu et al., 2003b) and *Lilium* sp. (Mori et al., 2005).

On the basis of callus growth capability, the four culture lines of *S. przewalskii* (S1-S4) initiated from two types of explants (leaf petiole, lines S1-S3; hypocotyl, line S4), and cultured on two basal media (WP, lines S1 and S2; MS, lines S3 and S4) were chosen for the other experiments described above. All examined callus lines were undifferentiated. After the 10th subculture (~1 year after initiation), fresh biomass was highest (5.8 g; 23-fold increase over initial inoculum after 4 weeks) for callus line S2 grown on WP medium supplemented with 2,4-D (0.5 mg l^{-1}) and BA (0.2 mg l^{-1}) in the dark. It was 30–40% higher than the fresh weight of callus line S1 maintained on the same medium composition but under a photoperiod (4.5 g; 15-fold increase over initial inoculum after 4 weeks) and callus line S4, cultured on MS medium in combination with NAA (0.2 mg l^{-1}) and TDZ (1.0 mg l^{-1}) (4.9 g; 20-fold increase over initial inoculum after 4 weeks). Biomass production was lowest for callus line S3 (2.8 g; 18-fold increase over inoculum biomass after 4 weeks) grown on MS agar medium with the same composition of growth regulators as calli of lines S1 and S2.

The growth of two lines of cell suspension cultures of *S. przewalskii* (SC1 and SC2) was studied during 30 days of cultivation by DW determination. As shown in Figure 1, the growth curves of the two cultures were similar. The growth of line SC1, cultured under 16 h light, was nearly exponential between the third and twelfth days of cultivation, with growth rate μ=0.17 day^{-1} and doubling time (*td*) 4 days; the corresponding values for cell line SC2, cultured in darkness, were μ=0.15 day^{-1} and *td*=4.7 days during the exponential growth phase between the 6th and 15th days of the growth cycle. Accumulated dry biomass reached maximum on day 18 for SC1 (10.99 g l^{-1}) and day 15 for SC2 (9.93 g l^{-1}) (Fig. 1). There was a 10–11-fold increase over the initial inocula.

SHOOT CULTURE AND PLANTLET REGENERATION

Shoot culture was obtained through proliferation of shoot tips of *S. przewalskii* on MS agar medium containing IAA (0.1 mg l^{-1}) and BA (0.5 mg l^{-1}). This combination of growth regulators was based on earlier experiments (Skała at al., 2007). After the 10th subculture on multiplication medium, an average 3.3 shoots per explant developed from preexisting meristems after 4 weeks. Plantlets were regenerated after transferring shoots to MS agar medium supplemented with 0.1 mg l^{-1} IBA. Under these condi-

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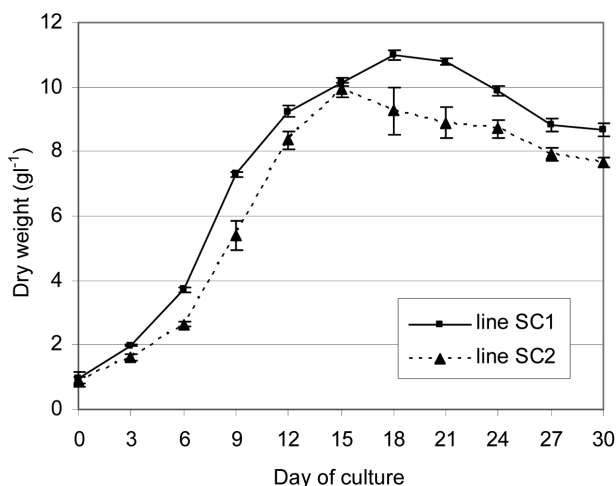*Skała et al.*
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Fig. 1. Growth curves of two cell suspension cultures of *S. przewalskii* (SC1 and SC2). The cultures were grown in WP liquid media supplemented with 0.5 mg l^{-1} 2,4-D and 0.2 mg l^{-1} BA. Line SC1 was cultured under a 16 h photoperiod and line SC2 in darkness. Dry weight was measured at 3-day intervals for 30 days. Each time point represents three replicates (n=9).

tions, 96% of the shoots developed roots, averaging 3 roots per shoot after 4 weeks. These roots reached 4.1 cm mean length. *S. przewalskii* shoot culture was successfully maintained on multiplication medium for more than 4 years (50 subcultures), although shoot proliferation efficiency and the frequency of shoot rooting after transfer to MS medium supplemented with 0.1 mg l^{-1} IBA was lower in long-term culture. The number of multiple shoots was 2.2 per explant and rooting frequency decreased to 74%. Among the parameters tested, only mean length of roots was not significantly affected when shoots from the 4-year-old culture were used for rooting.

TANSHINONE PRODUCTION

Table 1 summarizes the results from quantitative and qualitative analyses of tanshinones in the four established *S. przewalskii* callus lines, which were subcultured at 4-week intervals for ~1, ~2.5 and ~4 years. The metabolite profiles varied widely among the individual lines. Only callus S4 synthesized all four tanshinones after ~1 year of cultivation. Three tanshinones (TI, TIIA, DHT) were detected in callus S3. No dihydrotanshinone or cryptotanshinone were found in calli S1 and S2. In S2 culture only TI was accumulated (0.12 mg g^{-1} DW). This diterpenoid was also predominant in the other tested callus lines (S1, S3, S4), ranging from 0.13 to 0.15 mg g^{-1} DW. DHT was produced in callus S4 at a similar level (0.12 mg g^{-1} DW). The other two tanshinones (TIIA, CT) were accumulated in very low

amounts (0.04–0.06 mg g^{-1} DW). These differences in synthesis of secondary metabolites in vitro can be attributed to the state of morphological differentiation of explants used to initiate tissue cultures, as suggested by Sharada et al. (2007) for withanolide production. In the case of *S. przewalskii* callus culture it might also be explained by the differences in medium composition, for example the presence (lines S1-S3) or absence (line S4) of 2,4-D. Wu et al. (2003a) and Lee et al. (2008) reported that 2,4-D inhibited tanshinone production in *Salvia miltiorrhiza* callus tissue.

The results in Table 1 show that tanshinone biosynthesis in all examined callus lines was not stable when these calli were subcultured. Only callus S4 retained the ability to synthesize all four diterpenoids during long-term culture (4 years); it contained measurable amounts of TI and DHT and only traces of TIIA and CT. Reduction, loss or changes in secondary metabolite production over time have been reported in cultures of different plant species, for example rosmarinic and lithospermic acid production in callus culture of *Salvia miltiorrhiza* grown in darkness (Morimoto et al., 1994); rosmarinic acid content gradually decreased during subculturing, and lithospermic acid disappeared completely after the fourth subculture.

The qualitative composition of tanshinones in liquid cell cultures (SC1, SC2) and the corresponding callus tissues (S1, S2) was similar; the same tanshinones (TI, TIIA) were identified in both types of culture (Tab. 1). However, diterpenoid content differed between the liquid and solid cultures. For example, after one year of regular subcultures at 2-week intervals the content of TIIA was ~4 times higher in SC1 cell suspension culture (0.15 mg g^{-1} DW) than in samples from 1-year-old callus S1 (0.04 mg g^{-1} DW) (Tab. 1). Diterpenoids are secondary metabolites that can be biosynthesized in plastids (nonmevalonate pathway). For this reason we studied the tanshinone formation in *S. przewalskii* callus and cell cultures under different light conditions. Our observations suggest that light promoted biosynthesis of tanshinones. Cell culture SC1 grown under periodic light produced ~3 times more TI (0.22 mg g^{-1} DW) than cell suspension line SC2 cultured in the dark (0.07 mg g^{-1} DW). TIIA was found in light-grown culture (0.15 mg g^{-1} DW) but cells grown in darkness did not produce a detectable amount of it. Light also boosted tanshinone production in *S. przewalskii* callus cultures although the quantitative differences between cultures grown in the light (line S1) and in darkness (line S2) were less evident (Tab. 1). Our results stand in contrast to those reported in transformed cell suspension culture of *Salvia miltiorrhiza*, where light inhibited tanshinone biosynthesis (Chen et al., 1997).

The very low levels of tanshinones found in undifferentiated calli and cell suspension cultures suggest that biosynthesis of these compounds requires differentiated organs. To verify this hypothesis we examined tanshinone production in shoot culture and in vitro regenerated plantlets of *S. przewalskii*. We found that multiple shoots cultured on MS agar medium supplemented with IAA (0.1 mg l⁻¹) and BA (0.5 mg l⁻¹) produced TI, DHT, TIIA and CT, the first two compounds occurring in greater amounts (Tab. 2). The ability to biosynthesize tanshinones was maintained for at least 4 years of continuous subculturing of shoots on medium of the same composition but the quantities declined with time of cultivation: total tanshinones averaged 1.97 mg g⁻¹ DW in the first year of culture and 1.04 mg g⁻¹ DW in the fourth year, a significant decline (Tab. 2), but the latter amount was still ~2.5-fold higher than the total from the most productive callus line (S4) after the first year of cultivation (0.38 mg g⁻¹ DW) (Tabs. 1, 2).

Tanshinone content was highest in in vitro regenerated *S. przewalskii* plantlets grown on MS agar medium supplemented with 0.1 mg l⁻¹ IBA after 4 weeks. Their roots and shoots accumulated all four tanshinones but the total amount of diterpenoids was significantly higher in roots (up to 5.62 mg g⁻¹ DW) than in shoots (max. 1.3 mg g⁻¹ DW) (Tab. 2). The content of individual diterpenoids differed between plantlet shoots and roots. The major tanshinone accumulated in roots was TIIA, occurring at around double the concentration of the other compounds. In shoots the predominant components were TI and DHT (Tab. 2).

Interestingly, the age of shoot culture played an important role in tanshinone production in regenerated plantlets. Total tanshinones were highest (5.62 mg g⁻¹ DW) in roots of plantlets developed from shoots maintained through 50 subcultures over a period of 4 years on multiplication medium (Tab. 2). It was lowest (2.56 mg g⁻¹ DW) in roots of plantlets derived from shoots in the first year of cultivation on MS multiplication medium. The complexity of the biosynthetic pathways and our insufficient knowledge of secondary metabolism regulation make it difficult to pinpoint the cause of the observed variation. A previous study of in vitro regenerated *S. przewalskii* plants grown in the field also indicates that the tanshinone level strongly depends on the plant's age. The roots of 2-year-old plants produced twice the amount of TI and TIIA (11.4 mg g⁻¹ DW) than the roots of 1-year-old plants (5.0 mg g⁻¹ DW) (Skała and Wysokińska, 2005). DHT and CT production followed a similar trend. The roots of older plants accumulated 3.05 mg g⁻¹ DW DHT and 1.23 mg g⁻¹ DW CT, 1.5 times more than the roots of younger, 1-year-old plants (data not shown). In the current study the content of all four tanshinones in the roots of micropropagated *S. przewalskii* plants grown in the field for 3 years

was even higher. In roots the total tanshinone content was 19 mg g⁻¹ DW, more than 24 times higher than found in shoots (0.77 mg g⁻¹ DW) (Tab. 2). Li et al. (2010) reported that tanshinone content in roots of *S. przewalskii* plants grown naturally in different regions of China, calculated as the sum of DHT, CT, TI and TIIA, varied from 8 mg g⁻¹ DW to 18 mg g⁻¹ DW, depending on environmental conditions. They found only traces of the compounds in shoots. However, data on the age of those plants was not given.

This is the first report of establishment of tissue cultures of *S. przewalskii*. Our results demonstrate that diterpenoid biosynthesis in differentiated tissue differs both qualitatively and quantitatively from that of undifferentiated tissue. Callus and shoot culture experiments showed that the profile and level of tanshinones was influenced by the age of the cultures, and that multiple shoots maintained in vitro for a long time can be used for regeneration of plantlets whose roots contain a high level of tanshinones (up to 6 mg g⁻¹ DW). These results and those from our earlier work indicate that total tanshinone content can be increased to even 16–19 mg g⁻¹ DW when in vitro culture-derived plants are transplanted to soil and field-cultivated for 2–3 years. Shimomura et al. (1991) reported that roots of in vitro-raised plants of *Salvia miltiorrhiza* produced ~7 mg g⁻¹ DW of tanshinones after 20 months of field cultivation. Commercially available powdered 3.5-year-old roots of the species, known as Danshen in Chinese medicine, contain only ~3 mg g⁻¹ DW tanshinones, calculated as the sum of TI, TIIA, DHT and CT (Okamura et al., 1991; Shimonura et al., 1991). For this reason, roots of in vitro-developed plants of *S. przewalskii* can be recommended as suitable plant material for production of these pharmacologically important compounds. The use of micropropagated plants as raw material will reduce the dependence of the pharmaceutical industry on natural populations of *S. przewalskii*. Another option to increase yields of tanshinones would be to use root cultures of *S. przewalskii*, though in spite of much effort our cultured roots did not grow well (data not shown).

AUTHORS' CONTRIBUTION

ES obtained in vitro cultures of *S. przewalskii*, performed the experimental work, prepared samples for HPLC analysis and wrote the manuscript; WM performed HPLC analysis; HW was responsible for verifcation of the paper. The authors declare that there are no conflicts of interest.

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