Salvia milthiorrhiza Bunge *in vitro* cultivation in callus cultures

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S u m m a r y

Salvia milthiorrhiza root (Danshen) is one of the oldest and most traditional drug of Chinese origin, mainly used in the treatment of cardiovascular and cerebrovascular diseases. The tanshiniones (diterpenoids) and phenolic acids are the main biological active substances of *S. miltiorrhiza*. The aim of this study was to determine the optimal conditions for callus cultures and biosynthesis of the biological active compounds. The callus cultures (on solid medium, CCA in shake flask and CCA in bioreactor) were obtained and phytochemical studies on them were carried out. Total amount of phenolic acids determined in callus (solid medium) averaged from 2.58% to 5.72% of dry weight (DW). The callus cultured on solid medium and CCA (in flasks) did not produce tanshiniones. Culture conditions in the bioreactor enabled the biosynthesis of tanshiniones (0.27% of dihydrotanshinione, 0.12% of cryptotanshinone, 0.01% of tanshinione 2A and tanshinione 1). The obtained contents of rosmarinic acid in callus on solid medium and CCA (cultured in shake flasks) are relatively high and comparable to raw material. The callus cultured in bioreactor is eligible for tanshinione production, moreover the accumulation of them is comparable with the intact plants.

Key words: Salvia miltiorrhiza, compact callus aggregates (CCA), bioreactor, elicitation, tanshinones

INTRODUCTION

Salvia milthiorrhiza Bunge (family: Lamiaceae) is a perennial herb growing in China, Korea and Vietnam. The *S. miltiorrhiza* root (Danshen) is one of the oldest and most valuable drug in traditional Chinese medicine, mainly used in cardiovascular diseases treatment. Many pharmacological studies implicate cardiovascularprotective [1, 2], neuroprotectective [3-5], hepatoprotective [6, 7], anticancer [8, 9], antioxidant [10, 11] and antimicrobial activities [12, 13] of this herb and suggest it's implementation in cardiovascular and cerebrovascular diseases. Some activities of *S. miltiorrhiza* extracts have been proved in clinical studies [14, 15]. Although, most of them still need more randomized clinical trials and further research.

The *S. miltiorrhiza* roots contain two main groups of active compounds: phenolic compounds (caffeic acid, rosmarinic acid, isoferulic acid, lithospermic acid, salvianolic acid etc.) [16-20] and tanshinones (the phenanthrofurane quinone derivates: tanshinone 1, tanshinone 2A, tanshinone 2B, dihydrotanshinone 1, cryptotanshinone, etc.) [21-23]. The tanshinones and phenolic acids take part in defense against pathogens and their biosynthesis is effected by stress factors [36].

There are many *in vitro* studies on *S. miltiorrhiza* cultures, most of them concerning hairy roots [24-33]. The numbers of studies on *S. miltiorrhiza* callus cultures (including transformed cell cultures) are very limited [34-40]. The *in vitro* multiplication of *S. miltiorrhiza* was also elaborated by Shimoura et al. in 1991; Morimoto et al. in 1994 and Buchwald in 1999 [41-43].

The aim of this study was to determine optimal conditions for callus cultures and biosynthesis of biological active compounds.

MATERIAL AND METHODS

The callus culture was initiated from leaf of *S. milthiorrhiza* and established on solid MS medium with addition of NAA, BA and adenine chloride (fig. 1). The same line of callus was cultured on liquid medium in flasks on shakers (CCA) and in the bioreactor (air lift /5L) (fig. 2 and 3). The parameters of culture were: 23°C, fotoperiod – night: 8 h, day: 16 h (callus on solid medium and CCA in shake flasks), dark (CCA in shake flasks and in the bioreactor), 100 rpm (shakers and bioreactor). The callus on solid medium was subcultured every four weeks, CCA every two weeks. The obtained callus cultures were elicited by yeast extract (YE) in concentrations as follows: 2-7 mg/culture (on solid medium), 50mg/L and 100 mg/L (CCA in flasks and in the bioreactor).

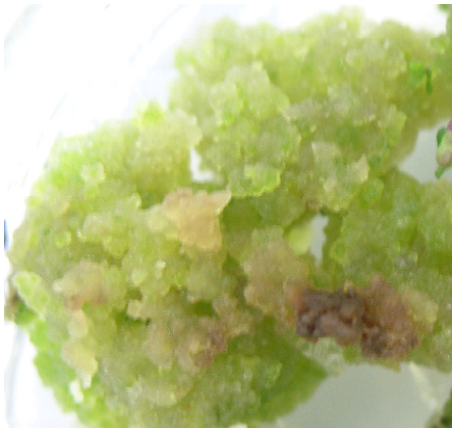

Figure 1. The *S. miltiorrhiza* callus culture on solid medium

Figure 2. Compact callus aggregates (CCA) in shake flask

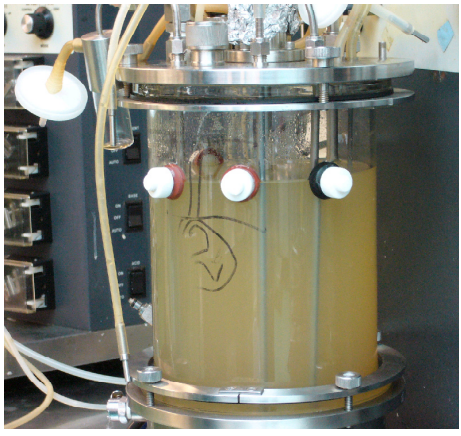

Figure 3. *Salvia miltiorrhiza* suspension culture in bioreactor

The proportion *inoculum* (in the bioreactor) approximated about 177g fresh weight per 1000 ml of medium. The pH medium in the bioreactor fluctuated from 5.32 to 6.02.

Chromatograhpical procedures

In this study HPLC with UV detector was used for chemical analyses. Samples of 100 mg for HPLC analysis were extracted with 5 ml methanol for 30 min in ultrasonic bath and filtered through a membrane filter (nominal pore size 0.45 μm).

HPLC analysis was performed on Agilent 1100 HPLC system, equipped with photodiode array detector. For all separation a Lichrospher 100 RP18 column (125 x 4 mm, 5 μm, Merck) was used. The mobile phase consisted 0.1% trifluoroacetic acid (TFA) in water (A) and acetonitrile (B), applied in gradient elution (table 1).

Table 1.

Scheme of gradient elution

time [min]	0.1% TFA_{aq} [%]	acetonitrile [%]
0.00	95.0	5.0
5.00	95.0	5.0
15.00	40.0	60.0
30.00	25.0	75.0
35.00	22.5	77.5

The follow rate was adjusted to 0.5 ml/min, the detection wavelength was set to DAD at λ=250.4 nm, and 20 μL of sample was injected. All separations were performed at the temperature of 25^0C. Peaks were assigned by spiking samples with standard compounds in comparison to the UV-spectra and retention times.

The content of biological active compounds are shown in percentage values of dry weight.

RESULTS

Total amount of phenolic acids determined in callus (solid medium) averaged from 2.58% (on light) to 5.72% dry weight (DW) (in dark). The rosmarinic acid was accumulated in amounts of 1.66% (light) – 1.54% of DW (in dark) on solid medium, 0.89% (light) – 1.14% (dark) of DW CCA in shake flasks and 0.03% (CCA in the bioreactor) of DW (fig. 4). The callus cultured on solid medium and CCA (in shake flasks) did not produce tanshiniones. The culture conditions in the bioreactor enabled the tanshinones biosynthesis (0.27% dihydrotanshinone, 0.12% cryptotanshinone, 0.01% tanshinone 2A and tanshinone 1, see fig. 5). The tanshinones and phenolic acids were not released into the medium.

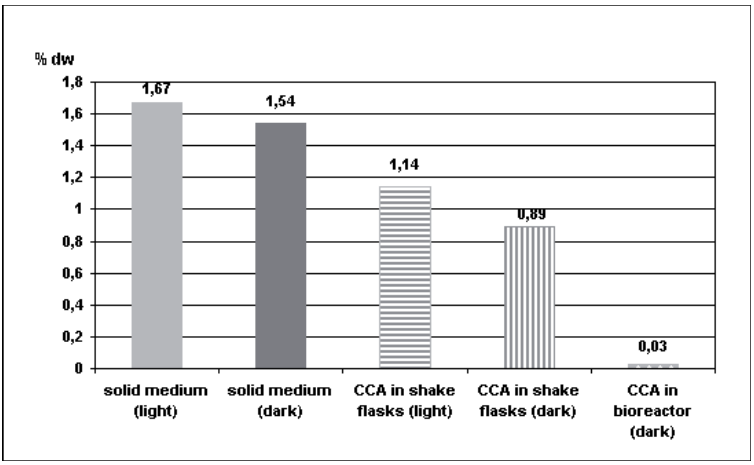

Figure 4. Content of rosmarinic acid in *S. milthiorrhiza* cultures (on solid medium, in shake flasks and in bioreactor)

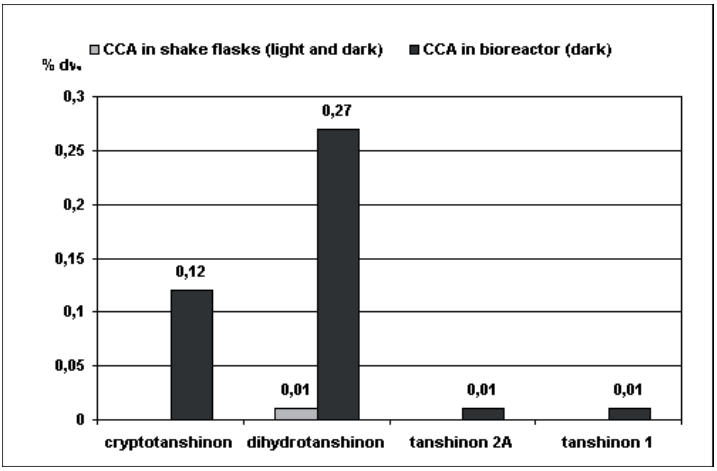

Figure 5. Content of tanshiniones in callus (CCA) cultured in liquid medium

The elicitation did not result in significant increase of the content of active compounds. The increase of the tanshinones content in trace amount was noticed: 0.01%–0.02% of DW dihydrotanshinone in CCA (YE dose: 50 mg/L), 0.01%–0.03% of DW in callus cultured on solid medium (YE dose: 5 mg/culture, see fig. 6). The decrease of rosmarinic acid content in elicited tissues was noticed on solid medium.

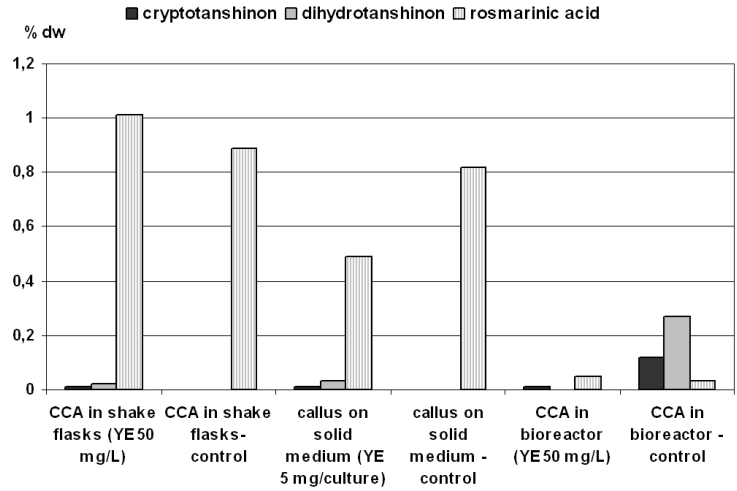

Figure 6. Content of tanshiniones in *S. miltiorrhiza* cultures elicited by yeast extract (YE)

DISCUSSION

According to available reports, the production of tanshinones in callus cells is not stable and depends on cell line, *Agrobacterium* transformation, nutrient factors, elicitor's stimulation and methods of culture [34-40, 44]. Tanshinones content in intact roots (cultivated in Polish climatic conditions) varies from 0.01 to 0.26% [45]. Rosmarinic acid concentration in roots approximates from 2.06 to 7.27% [45].

The detected tanshiniones content (mainly cryptotanshinone) in callus cells ranged from 0.06% to 0.08% of DW (suspension culture of transformed cells) [36], from 0.03% to 0.46% of DW in nontransformed cells [34], from 0.8% to 4% of DW [37], 87.4 – 110 mg/L in cell suspension [38], from 13.7% to 3.6% of DW [39] and from 0.26 to 0.1 mg/g in transformed cells suspension in the bioreactor [35]. The accumulation of rosmarinic acid cultures varies in different kinds of callus culture. The highest obtained rosmarinic acid concentration was 5% [36] and 4.59% [37] in cell suspension cultures (*Agrobacterium* transformed cells). The rosmarinic acid content in bioreactor cultures approximated from 107.3 to 749.4 mg/L [34, 35]. In the studies [35] also was found that yeast elicitor reduced the rosmarinic content and it corresponds with the results in this experiment.

The obtained contents of rosmarinic acid in callus on solid medium and CCA (cultured in shake flasks) are relatively high and comparable to raw material. The callus cultured in bioreactor is eligible for tanshinone production. Moreover, their accumulation is comparable to the intact plants.

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UPRAWA SZAŁWII CZERWONOKORZENIOWEJ *IN VITRO* NA POŻYWKACH KALUSOWYCH

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S t r e s z c z e n i e

Szałwia czerwonokorzeniowa (*Salvia miltiorrhiza*, Danshen) należy do najstarszych i najcenniejszych ziół używanych w chińskiej medycynie ludowej. Stosowana jest głównie w chorobach układu krążenia. Korzenie szałwi zawierają dwie grupy związków biologicznie aktywnych: tanszinony oraz kwasy fenolowe. Celem badań było określenie optymalnych parametrów hodowli oraz warunków biosyntezy związków aktywnych. Kultury kalusowe hodowano na pożywce stałej oraz płynnej: w kolbkach na wytrząsarkach i w bioreaktorze (agregaty CCA). Suma kwasów fenolowych w kalusie hodowanym na pożywce stałej wynosiła 2,58%–5,72% suchej masy (s.m.). Kalus hodowany na pożywce stałej oraz agregaty w kolbkach nie syntetyzowały tanszinonów. Agregaty hodowane w bioreaktorze produkowały tanszinony na poziomie: 0,27% s.m – dihydrotanszinon, 0,12% s.m. – kryptotanszinon, 0,01% s.m. – tanszinon 2A i tanszinon 1. Zawartość kwasu rozmarynowego w kalusie hodowanym na pożywce stałej oraz w hodowli suspensyjnej w kolbkach była stosunkowo wysoka i porównywalna z roślinami z gruntu (uprawianymi w polskich warunkach klimatycznych). Warunki hodowli w bioreaktorze umożliwiły syntezę tanszinonów porównywalną z roślinami z gruntu.

Słowa kluczowe: *Salvia miltiorrhiza, agregat CCA, bioreaktor, elicytacja, tanszinony*