Production of the secondary metabolites in *Salvia miltiorrhiza in vitro* cultures

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S u m m a r y

Salvia miltiorrhiza (family: *Lamiaceae*) is well known as Danshen in traditional Chinese medicine. It is used mainly in the treatment of cardiovascular diseases. A number of pharmacological studies have proved its wide spectrum of pharmacological activities: cardiovascular protective effect, antioxidant, anti-inflammatory, neuroprotective, antimicrobial and anticancer. The roots of *S. miltiorrhiza* contain two main groups of active compounds: phenolic constituents and abietane-type diterpenoids (tanshinones). The studies on *S. miltiorrhiza in vitro* cultures have been focused on secondary metabolites production for over two decades. Both cultures, undifferentiated and transformed, are able to synthesize the active compounds but their content is low. The elicitation treatment significantly enhances the metabolites content at a level close or much higher than in the intact plants. The induction effect depends on many factors: the kind and dose of the elicitor, the type of the culture and its susceptibility, time and ways of administration, the growth state of tissues etc. The yeast extract and some heavy metal ions effectively induce tanshinones biosynthesis such as cryptotanshinone, whereas methyl jasmonate stimulate mainly phenolic compounds – lithospermic acid B and demonstrated limited effect on diterpenoids accumulation. Nowadays, the much attention has been paid to the biosynthetic pathways and genes including expression profiling and cloning. The recognition of the genes pathways and the transcription factors (including the signal transduction steps level) will be helpful

in better understanding of the regulatory mechanism and improvement of the production of the interesting secondary metabolites and eventually appliance in the industry.

Key words: *Salvia miltiorrhiza, elicitor, hairy root, crown gall culture, tanshinones, PAL (phenylalanine ammonia-lyase), MVA (mevalonic acid)*

INTRODUCTION

Salvia miltiorrhiza (family: *Lamiaceae*) is a perennial plant growing in China, Korea, Vietnam and Japan. Its roots have been used since ancient times in the traditional Chinese medicine to treat cardiovascular diseases. Numerous pharmacological studies have revealed a variety of activities including: cardiovascular protective effect [1], anti-inflammatory [2], hepatoprotective [3], neuroprotective [4], antioxidant [5], anticancer [6-7], antimicrobial [8] and antiviral [9] activity. *Salvia miltiorrhiza* extracts are applicable in the therapy of coronary artery disease, angina pectoris, hypertension, myocardial infraction and stroke [10]. There are also some reports suggesting the potential usage of this plant in Alzheimer's disease [11], diabetes [12], cancer [13], hyperlipidemia [14] and in alcoholism [15].

The roots of *S. miltiorrhiza* contain two main groups of active compounds: phenolic constituents (rosmarinic acid, salvianolic A and B acids, lithospermic acid, caffeic acid, protocatechuic acid and aldehyd etc.) [16-20] and tanshinones (abietane-type diterpenoids: T-I, T-IIA, T-IIB, dihydrotanshinon, cryptotanshinon - CT) [21-23]. The content of active constituents depends on the origin and age of the plants, quality of crops, climatic conditions etc. The content of tanshinones in raw material cultivated in Poland vary from 0.01% to 0.30% and rosmarinic acid from 1.06% to 7.27% [24]. The material of Chinese origin (from field cultivations) contains from 0.26% to 0.62% of tanshinones and from 3.34% to 6.13% of phenolic compounds [25]. According to Chinese Pharmacopoeia [26] the content of salvianolic acid B and tanshinone IIA in dried roots and rhizomes of *S. miltiorrhiza* should not be lower than 30 mg/g (0.3%) and 2 mg/g (0.02%), respectively.

The increasing demand for a high quality raw material and limited natural resources contributed to the searching of the alternative ways of valuable compounds production. Traditional field cultivations are time-consuming and labor cost process effected by environmental factors like: climate, pathogens and pets. *S. miltiorrhiza* plants are suitable for harvesting after three years of the cultivation. Up to day, the plant cell cultures became an alternative and efficient source of valuable bioactive compounds produced in the large scale, such as taksol®, berberine or shikonine [27-29].

For over two decades, the studies on *S. miltiorrhiza in vitro* cultures have been focused on secondary metabolites production. The finding that tanshinones and phenolic compounds take part in the defense response against pathogens and their biosynthesis is effected by stress factors had a great impact and promoted the studies concerning the elicitation procedures.

The studies on *S. miltiorrhiza in vitro* cultures have been focused on the efficient methods of cultivation, transformation with *Agrobacterium*, production of the secondary metabolites and the enhanced biosynthesis induced by biotic and abiotic elicitors. Nowadays, much attention has been paid to the studding of the biosynthetic pathways and involved genes including expression profiling and cloning [30-31]. The article presents the review of the main achievements and the current state of the *in vitro* biotechnological studies on *S. miltiorrhiza*.

THE UNDIFFERENTIATED CELL CULTURES

The early studies of *S. miltiorrhiza* cultures were established by Nakanishi and coworkers [32] in Japan. They induced six undifferentiated cell lines from the seedlings on MS medium and received one capable for production of cryptotanshinone and ferruginol. Cryptotanshinon was synthesized in the trace amount and decreased gradually in successive subcultures. In the later studies Miyasaka and his group [33-36] developed the methods of cultivation and the medium composition. They found that ferruginol biosynthesis was inversely related with cell divisions and noted the inhibitory effect of 2,4-D and light on it [33]. Different nutritional factors were examined on cryptotanshinon and ferruginol production and MS medium was modified, limiting its composition to 10 constituents. They also reported that sucrose, organic nitrogen and thiamine were essential for diterpene biosynthesis while the phosphates, manganese sulfur and kinetin had hardly a favorable effect on it [34-35]. The modification of the medium resulted in higher content of cryptotanshinone – 110 mg/L [35]. Although cryptotanshinone was poorly released into medium, the immobilization of the cells resulted in lower diterpene production contrary to free cells [34]. The application of two-stage method (mass cultivation of tissues on growth medium and transfer to production medium without 2,4-D) improved cryptotanshinone and ferrruginol production at a level of 0.8–4% and 0.7–14%, respectively [36]. The content of diterpenes was higher that in the intact plants, although, the production was not stable in spite of cell lines selection.

The subsequent studies on *S. miltiorrhiza* cultures concentrated mainly on transformation with *A. tumefaciens* and *A. rhizogenes* and enhanced production of secondary metabolites as a result of the elicitation treatment.

More recent studies on the callus cultures were described by Wu [37] and Krajewska-Patan [38]. The elimination of 2.4-D from the medium composition caused the rapid increase of cryptotanshinone synthesis from 0.26 mg/g to 4.59 mg/g in d.w. of callus tissues [37]. This report confirmed the inhibition effect of 2.4-D on diterpene production made by Miyasaka in the earlier studies [33, 36]. The callus tissues cultivated on solid and liquid medium including bioreactor system was reported by Krajewska–Patan *et al*. [38]. The obtained cultures did not synthesize the tanshinones but produced phenolic compounds (2.85–5.72% in d.w.).

Such high concentration of the phenolic compounds was comparable to the intact plants. The elicitation with yeast extract (YE) slightly increased the diterpene production but the decrease of rosmarinic acid content was noted. The biomass cultivated (compact callus aggregates) in stirred bioreactor (5 L, New Brunswick Scientific) was enabled to produce tanshinones (mainly dihydrotanshinone) at a level of 0.41% in d.w.

One of the latest work concerning the suspension culture presents the effect of biotic and abiotic elicitors usage on the tanshinone production [39]. The elicitors were administered to the shake flask culture before reaching the stationary phase (day 18 of culture). Different elicitors: YE (polysaccharide fraction), chitosan, salicylic acid – SA, methyl jasmonate – MJ, sorbitol and heavy metals ions (cobalt chloride, silver nitrate and cadmium chloride) in the experiments were used. The most elicitors (with exception of Co^{2+}, sorbitol, SA and MJ) increased total tanshinones content. The strongest stimulating effect was observed after Ag (dose: 25 μM), Cd (25 μM) and YE (100 mg/L^{-1}) treatment. The enhancement of tanshinone production was 2.3 mg/g d.w. – about 11.5-fold as compared to the control. Other elicitors had a weaker effect. The cryptotanshinone biosynthesis was stimulated most significantly, the highest concentration (2 mg/g in cells) was achieved: it means about 31–34-fold more than control. Tanshinone – T-IIA was stimulated by most of elicitors, while T-I only by chitosan. All three elicitors caused the decrease of the biomass (up to 40%) as a side effect. The treatment with YE and ions (cadmium and silver) together resulted in the highest increase of diterpene production (nearly 40%) but it caused the inhibition of biomass growth – up to 50%.

THE TRANSFORMED *S. MILTIORRHIZA* CULTURES

The Ti-plasmid transformed *S. miltiorrhiza* cell cultures

The transformed cultures are characterized with rapid growth (free hormone medium) in contrary to undifferentiated cultures. Moreover, the transformed cultures are usually capable of stable metabolites production in the scaled-up conditions during long time.

The studies on Ti-transformed *S. miltiorrhiza* cultures have been initiated by Zhang [40]. The *A. tumefaciens* C58 stain was used for the transformation and the crown gall culture producing tanshinones was obtain on 6,7 V medium. The works have been continued by Chen and his group [41-47]. Two lines have been chosen for studding: line A – producing mainly phenolic compound on B5 medium and line B – synthesizing tanshinones on 6,7 V medium [41]. The suspension culture (line B) produced cryptotanshinone at average level of 1% in d. w. while T-I and T-IIA – slightly higher than 0.5% [44]. The presence of rosmarinic acid (in maximum content of 5.5%) and litospermic B (2.2%) was noted in the medium. The diterpenes were accumulated in cells and also released into medium (nearly 40%). The

content of CT was variable during cultivation – from 0.47% up to 1.31% [44]. The incubation with YE (polysaccharide fraction) increased the total content of tanshinones up to 22 mg/L [41]. The positive results of the elicitation promoted subsequent research on the elicitation treatment. The fractioned YE improved cryptotanshinone biosynthesis to about 100% (from 0.24% in control to 0.54% in d.w. of the cells) in line B [46]. The maximum noted value of cryptotanshinone content was 33mg/L at day 7 after YE elicitation (dose 0.1–0.5 ml/flask). The elicited line A (that normally produced only trace amount of diterpenes) started to synthesize cryptotanshinone although the content was lower that in line B [43,45]. Another tested elicitors (JM, SA, ibuprofen) were less effective when tested separately, whereas SA applied together with fractioned YE strongly stimulated tanshinones production [43]. In the elicited cultures the inhibition of biomass growth and rosmarinic acid production was observed [43, 45, 46]. The studies proved that cryptotanshinone is a phytoalexin and its biosynthesis is stimulated by fungus pathogens [45].

At the same time the studies on the phenolic acids production and scale up of the cultivation have been carried out [42]. The suspension culture (line A) cultivated in stirred bioreactor (1.5 L) produced: rosmarinic acid, lithospermic acid B and trace amounts of caffeic acid and protocatechuic aldehyde. The content of them and the biomass growth ratio were lower than in flasks on the shakers [42]. According to authors, the lower biomass growth was caused of insufficient mixing and enlarged evaporation. Similar works were continued by Zhong [48] in the bigger bioreactors (2.4 L) with different size of the turbine impellers. The production of rosmarinic acid and lithospermic acid was higher than in flasks and the maximal values were noted: 1126 mg/L and 149.1 mg/L, respectively. The total tanshinone content was also higher – 5.52 mg/L in bioreactor *vs* 5.3 mg/L in flasks. The better cultivation parameters were achieved in the large impeller bioreactor due to more beneficial culture environment and lower shear stress. Although the transfer from flasks to bioreactors system was succeed, the production and content of the metabolites was not sufficient for the economic production in the large scale.

Hairy roots cultures

Hairy roots cultures is a suitable *in vitro* system for producing secondary metabolites due to lower costs, hormone-independent growth, lack of geotropism and branching and the most important – genetic stability.

The first studies on *S. miltiorrhiza* transformation were published by Hu and Alferman [49]. Five *A. rhizogenes* strains were used but LBA9402 was the most effective in the transformation process. The obtained hairy roots produced seven diterpenoids: CT, T-I, T-IIA, dihydrotanshinone, T-V, T-VI and ferruginol. The main compound was CT and comprised 60% of all tanshinones. The total sum of tanshinones achieved the level of 0.5–1.9%.

The hairy roots cultures of *Salvia miltiorrhiza* have been obtained and intensively studied in many laboratories. The sum of tanshinones (TI, TIIA, CT, dihydrotanshinon) produced by hairy roots obtained by Wysokińska was 18 mg/g d.w. – it was higher than in untransformed cultures and close to diterpene content in the intact plants [50]. The main diterpenoid, CT, achieved level of 5.8 mg/g d.w. – twice as high as in the raw material obtained from field culture [50].

The hairy roots cultures obtained by Chen and his group produced the significant amounts of phenolic acids: romarinic at stable level – 0.48% and lithospermic B – varying from 0.73 to 1.6% [51]. The tanshinones were synthesized in trace amounts. The elicitation with polysaccharide fraction of YE increased rosmarinic acid content (from 1.24% to 2.97%) and biomass growth in contrary to the studies on crown gall cultures [52]. The content of tanshinones was enhanced too, but it was still a trace amount. The authors attributed the enhancement of rosmarinic acid production (instead of decrease noted in the crown gall cultures) to the increase of the whole biomass, although, they admitted that such explanation was not satisfactory.

Later studies on hairy roots concentrated on synergistic effect of the combined elicitors such as: yeast extract, ions metals, sorbitol, BABA (β-aminobutyric acid) and methods of cultivations (co-culture, immobilization, semi–continuous) on secondary metabolites production.

Zhang [53] used silver ions ($Ag_2S_2O_3$) that stimulated the enhanced (2-fold) tanshinones production – CT, T-I, T-IIA. The effect was dose and time depended and the inhibition of growth was noted. The application of the combined method – silver ions, sucrose feeding and medium renewal before elicitor treatment caused tremendous increase of the tanshinones concentration (55.7 mg/L *vs* 7.3 mg/L in control) in biomass. Similar method (with use of silver ion and YE) was described by Ge and Wu [54]. The Ag^+ treatment before YE (fractioned) administration resulted in the enhanced production of diterpenes (mainly cryptotanshinone), although, when YE was administered separately the increase was higher. Other experiments with JM and BABA elicitors demonstrated stronger stimulating activity of BABA. The effect was more significant – 9-fold increase of tanshinones (2.26%) when elicitor was applied three days before YE treatment [55].

The silvers ions and YE were also used for the enhanced production of phenolic acids [56]. Both elicitors stimulated rosmarinic acid production, although YE (at a dose of 200 mg/L) was more effective than Ag^+ and high content (74.1 mg/g) of rosmarinic acid was achieved. The studies demonstrated the suppression of PAL (Phenylalanine Ammonia-lyase) and enhanced TAT (Tyrosine Aminotransferase) activity during elicitation treatment. The suppressed PAL activity was in the contrast to earlier studies. The authors suggested that elicitors stimulated rosmarinic acid biosynthesis *via* tyrosine-derived pathway.

More recent study on elicitation with silver ions revealed the strong responsive reaction that resulted in lithospermic acid B (from 5.4% to 18.4%) but not rosmarinic acid accumulation [57]. The profiling analysis of genes and RA (ros-

marinic acid) intermediates showed the temporal changes in gene transcripts. Thus the putative route from RA to LAB (lithospermic acid B) was suggested [57]. The earlier work of Xiao et al. [58] demonstrated the enhanced production of rosmarinic acid and lithospermic acid B (from 3.25% to 6.02% and from 2.95 to 19.3%, respectively) as a response to MJ treatment. The induction of the rosmarinic acid biosynthetic gene transcripts (including PAL and TAT) displaying rapid increase was noted.

The combination of the immobilization on hydrophobic polymeric resin, repeated elicitation and semi-continuous culture was applied by Yan and coworkers [59]. The fractioned YE increased the total sum of tanshinones about two-fold (from 0.46 mg/g to 1.37 mg/g) and the adsorption *in situ* improved the recovery of them. The medium renewal (with addition of fresh elicitor and resin) allowed to prolong the duration of the cultivation up to 60 days without decrease of the growth parameters. The repeated small dose (100 ml) of the elicitor was more effective than a use of the single large dose (200 ml). The integration of all methods resulted in the biomass increase and tanshinones accumulation – 87.5 mg/L (15-fold).

The application of sorbitol and the fractioned YE caused the increase of the tanshinones content (10-fold) and demonstrated the strong tolerance of hairy roots to hyperosmotic stress [60]. The elaboration of the improved method (repeated osmotic stress, elicitation and fed-batch culture) effected the high diterpene content (1.8% in d.w.) [61]. The addition of the fresh medium with sorbitol and YE every five days increased over 100-fold the tanshinones yield (145 mg/L). Moreover, the ultra high diterpene fraction of the roots was obtained – 100 mg/g d.w. (11%), although, it constituted only 1–2% of whole biomass.

Numerous studies demonstrated the favorable effect of elicitation with YE or heavy metal ions on secondary metabolites production in hairy root cultures. The novel idea of co-culture was developed by Wu [62]. The hairy roots were inoculated and co-cultured with live *Bacillus cereus* bacteria. Although the growth of hairy roots was significantly inhibited the tanshinones production was stimulated, leading to their accumulation (2.67 mg/g). The effect was probably caused by some elicitor compounds released by bacteria and stress conditions like pH drop or the competition for the nutrients. The inhibition of the bacteria growth was noted too, mainly due to the antibacterial activity of diterpenes and nutrient competition. Although the co-culture method is a promising idea, it still needs the elaboration, particularly the elimination of the biomass inhibition during the cultivation.

CONCLUSIONS

Both undifferentiated and transformed cultures are able to synthesize the same active compounds like the intact plants but the content of them are relatively low. The production of metabolites is variable and may be suppressed in the subse-

quent passages particularly in undifferentiated cultures. The elicitation treatment effectively enhances the metabolites content at a level close or higher than the intact plants as well as in the hairy root and in the callus cultures. The induction effect depends on the type of culture and its susceptibility, the kind and dose of an elicitor, time and ways of the administration, the growth state of the tissues etc. The elicitors usually caused the inhibition of the biomass growth as a side effect. This unfavorable effect may be reduced or completely eliminated by the combination of integrated methods: elicitation, fed-batch culture or the medium renewal. The yeast and some heavy metal ions effectively induced tanshinones biosynthesis, such as cryptotanshinone, whereas MJ stimulated mainly phenolic compounds (lithospermic acid B) and demonstrated little effect on diterpenes accumulation. The others elicitors like SA or chitosan had a minor or no effect on the metabolites accumulation. The yeast elicitor allowed not only to significant increase the content of tanshinones but also stimulated the production *de novo* [38, 43, 45]. This fact confirmed that tanshinones (mainly CT) are phytoalexins and their biosynthesis is induced by fungus pathogens. Different effect of MJ and fungal elicitors may result from different signal transduction pathways and from cross-talks among multiple signaling pathways. In defensive process usually more than one signaling pathway is involved [63].

Phenolic acids are synthesized and accumulated in the cells of many unrelated plant species. Rosmarinic acid can exceed up to 10% of the biomass yield and 6g/L of medium in the cultures generated from *Salvia officinalis* or *Coleus blumei* [64]. The comparable results have been obtained in *S. miltiorrhiza in vitro* cultures. Other phenolic acid – LAB – was produced by *S. miltiorrhiza* elicited hairy roots cultures in the extremely high content – up to 18–19% [57, 58]. Previous studies on rosmarinic acid biosynthesis in other species proved that it was derived from phenylopropanoid pathway [65, 66]. Nowadays, two different pathways: phenylopropanoid pathway (PAL) and tyrosine-derived pathway (TAT) are considered to be involved in RA formation in *S. miltiorrhiza* plants. Both PAL and TAT pathways are joined by RAS (Rosmarinic Acid Synthase) that catalyzes RA formation from 4-coumaroyl-CoA (derived from PAL pathway) and 4-hydroxyphenyllactic acid (TAT pathway) [66]. The TAT side-branch pathway leading to tocopheroles may influence the RA production competing for the same substrate (4-hydroxyphenyruvic acid) [67]. The studies on PAL genes confirmed its key role in the RA biosynthesis in *S. miltiorrhiza* species and also proved the inducible expression as a response to elicitors: MJ, SA and GA_3 [68]. The formation of LAB from RA in PAL pathways has been also suggested [57]. Further works concerning the correlation analysis of gene-to-metabolite networks will allow to indicate the genes and enzymes links to phenolic acids.

The tanshinones, in contrary to the phenolic acids, are specific compounds produced only by *S. miltiorrhiza* and related species – *S. przewalskii* [70]. Nowadays, the chemical and biological properties as well as the *in vitro* cultures of this species have been intensively studied [5, 69-71]. The tanshinones are considered to be derived *via* at least two different pathways: the mevalonate (MVA) located in

cytosol and non-MVA – DXP (1-deoxy-D-xylulose 5-phospate) pathway in plastids [72]. Although non-MVA pathway is believed as the main diterpene pathway, there are evidences of the cross-talks between isoprenoids and others pathways [72].

Recently much efforts have been paid to the studying of the biosynthetic pathways and involved enzymes, signal transduction pathways, cloning of the secondary metabolite biosynthetic genes, transcription factors. It is expected that results of these studies will facilitate better understanding of the biosynthetic regulation mechanism. As long as the biosynthetic genes and particularly signal transduction pathways are not revealed, the success in the metabolic engineering of the plant secondary metabolites will be limited. In spite of the successful attempts of the cultivation in the bioreactor systems, the technology is still less economically effective and competitive for the industrial production.

Although field cultivations are still a major source of *S. miltiorrhiza* raw material, the tissue cultures are the most promising and useful systems for examining and exploring the factors affecting the active compound biosynthesis.

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PRODUKCJA METABOLITÓW WTÓRNYCH W KULTURACH *IN VITRO* SZAŁWII CZERWONOKORZENIOWEJ (*SALVIA MILTIORRHIZA* BUNGE)

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

Szałwia czerwonokorzeniowa (*Salvia miltiorrhiza* Bunge; rodzina: Lamiaceae) należy do najbardziej znanych ziół w chińskiej medycynie ludowej (Danshen) i od wieków używanych głównie w leczeniu chorób i schorzeń układu krążenia. Liczne badania farmakologiczne potwierdzają szerokie spektrum działania, z których najważniejsze to protekcyjne działanie na układ sercowo-naczyniowy, aktywność antyoksydacyjna i przeciwzapalna, a także neuroprotekcyjna, przeciwdrobnoustrojowa i antynowotworowa. Korzenie tego gatunku zawierają dwie główne grupy związków: związki fenolowe i tanszinony (diterpeny typu abietanu). Badania nad produkcją metabolitów wtórnych w kulturach *in vitro Salvia miltiorrhiza* prowadzone są od ponad dwudziestu lat. Stwierdzono, że zarówno kultury niezróżnicowanych tkanek, jak i kultury transformowane zdolne są do produkcji związków biologicznie czynnych, choć ich zawartość jest zazwyczaj niska. Zastosowanie elicytorów zwiększa zawartość metabolitów do poziomu zbliżonego lub przewyższającego zawartość w roślinach z gruntu. Efekt elicytacji zależny jest od wielu czynników: rodzaju kultury i jej podatności na działanie elicytora, rodzaju i dawki elicytora oraz terminu i sposobu jego podania, a także fazy wzrostu kultury *etc.* Stwierdzono, że wyciąg drożdżowy i niektóre jony metali ciężkich stymulują biosyntezę tanszinonów (głównie kryptotanszinonu), natomiast jasmonian metylu – kwasów fenolowych, bez większego wpływu na syntezę diterpenów. Obecnie badania skupiają się głównie na studiowaniu szlaków metabolicznych i enzymów oraz odpowiedzialnych genów. Dokładne poznanie genów odpowiedzialnych za biosyntezę metabolitów i jej regulację (transdukcja sygnałów, czynniki transkrypcyjne) przyczyni się nie tylko do lepszego zrozumienia mechanizmów regulujących i kontrolujących wytwarzanie produktów przemiany materii, ale także do wydajniejszej ich produkcji w kulturach *in vitro*, a docelowo do zastosowania w produkcji dla potrzeb przemysłu farmaceutycznego.

Słowa kluczowe: *Salvia miltiorrhiza, elicytor, kultury korzeni transformowanych, crown gall culture, tanszinony, PAL (amoniako-liaza L-fenyloalaniny), MVA (kwas mewalonowy)*