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Tanshinone production in Ti-transformed *Salvia miltiorrhiza* cell suspension cultures

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Abstract

Transformed cell cultures of *Salvia miltiorrhiza* were established by infecting sterile plantlets with *Agrobacterium tumefaciens* strain C58. The transformed cells in suspension formed macroscopic clumps of cell aggregates up to 2–3 cm in size rather than homogeneous cell suspensions. These transformed cells grew well in hormone-free media. It was found that the B5 medium supported the best growth while the 6,7-V medium promoted tanshinone production in the transformed cell suspension cultures. The effect of initial sucrose concentrations on cell growth was also studied. The best growth was observed when cells were cultivated in the B5 medium containing 30 g l^{-1} sucrose. Although low levels of tanshinones were produced in fast growing cell cultures, there existed a rapid increase in tanshinone production when the cell aggregates were transferred to the fresh yeast-extract-containing medium. By this two-stage culture method, about 22 mg tanshinones were produced in 1 liter of medium. Green cell aggregates were formed when cells were cultured under illumination. Light was found to have an inhibitory effect on tanshinone biosynthesis. A sensitive high-performance liquid chromatographic method was developed for the measurement of tanshinones. Cryptotanshinone, tanshinone I and tanshinone IIA were identified from the transformed cultures. © 1997 Elsevier Science B.V.

Keywords: *Agrobacterium tumefaciens*; Elicitation; *Salvia miltiorrhiza*; Tanshinone; Transformed cell culture; Yeast extract

1. Introduction

Salvia miltiorrhiza Bunge (Lamiaceae) is a famous traditional Chinese medicinal plant. Its roots, called Dan-shen or Tan-shen, have a reputation for being a sedative, bactericide, and car-

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diac stimulant, and are used, among other things, to treat certain cardiac disorders (Bruneton, 1995). The effective components of the roots have been studied extensively and well documented. The major active principles of the organic extract of Dan-shen are tanshinones, the quinoid diterpenes, which impart the reddish-brown color to the roots. For example, tanshinone I and cryptotanshinone prevent the complications of myocardial ischemia; tanshinone IIA has undergone successful clinical trials for the treatment of angina pectoris in China (Bruneton, 1995).

Plant cell and organ culture technology offers an alternative means of producing these secondary metabolites. Studies on untransformed cell suspension cultures and immobilized cells of this plant have been carried out and reviewed (Miyasaka et al., 1989). However, the main product was ferruginol, and the production of tanshinones was not stable and even decreased gradually in successive subcultures. The high-yield production of tanshinones in untransformed root cultures has been reported, but the period of cultivation was long (typically about 8 weeks) (Shimomura et al., 1991). Diterpenoid production in Ri transformed root or hairy root cultures of *S. miltiorrhiza* has also been reported (Hu and Alfermann, 1993). In these cultures, although fast growth as well as relatively high tanshinone production was achieved, the morphological characteristics of the hairy roots require special bioreactors for the cultivation, which has hindered the scale-up of such processes.

Transformed cell cultures may have several advantages over normal cell cultures including no requirement for exogenous phytohormones, fast growth rates and the introduction of foreign genetic materials into the plant cells which may favor the formation of secondary metabolites (Towers and Ellis, 1993). Transformed cell cultures have shown the ability to produce secondary compounds which are otherwise difficult to obtain in normal cell cultures (Norton and Towers, 1985, Cosio et al., 1986, Moldenhauer et al., 1990). The aim of the present work is to explore the tanshinone production potential of Ti transformed *S. miltiorrhiza* cell suspension cultures.

2. Materials and methods

2.1. Plant material

Seeds of *S. miltiorrhiza* were collected from the cultivated plants at the botanical garden of the Institute of Medicinal Plant Development, Chinese Academy of Medicinal Sciences, Beijing, PR China. After being flushed with tap water for 24 h, the seeds were surface sterilized with 0.1% mercuric chloride for 10 min. Then, the seeds were washed with sterile distilled water for three times and germinated on 1/2 MS medium (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose and solidified with 8 g l^{-1} agar. The cultures were incubated at 25°C under illumination by fluorescent lamps (photoperiod 16 h with an irradiance of 3000 lux) for 1 month before the shoot tips of seedlings were cut off and transferred onto the same fresh medium for subsequent sterile plantlet subculture.

Commercial Dan-shen, the dried root chips of *S. miltiorrhiza*, was purchased from the Hong Kong market.

2.2. Chemicals and standards

Cryptotanshinone, tanshinone I, tanshinone IIA, tanshinone IIB and dihydrotanshinone I were obtained from Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, PR China. All other chemicals used throughout this research were purchased from Sigma (St. Louis, USA).

2.3. Initiation and maintenance of transformed cell cultures

When the sterile plantlets grew to about 6 cm high, all of the leaves were removed except the shoot tips and three top leaves. The nopaline-type *Agrobacterium tumefaciens* strain C58, grown in YMB medium (Hooykaas et al., 1977) for 48 h was inoculated into the nodes of the plantlets by a syringe. The infected plantlets were incubated in the cultivation room at 25°C under illumination (photoperiod 16 h with an irradiance of 3000 lux). Six days after the inoculation, tumefaction was

observed at the site of infection and crown galls appeared thereafter. When the crown galls grew to about 5 mm^3 in size, they were cut off from the plantlets and transferred to MS medium (supplemented with 30 g l^{-1} sucrose and solidified with 8 g l^{-1} agar) containing carbenicillin (0.5 g l^{-1}) without plant growth regulators. Subcultures in that medium were carried out five times over a period of 70 days at 25°C in darkness until axenic clones were obtained. Axenic cultures were maintained by subculturing every 14 days on antibiotic-free MS medium containing 30 g l^{-1} sucrose and 8 g l^{-1} agar under the same conditions.

Transformation was confirmed by the method of nopaline assay (Otten and Schilperoort, 1978, Aerts et al., 1979). Nopaline was separated by paper electrophoresis and detected by treatment with phenanthrene quinone.

2.4. Culture experiments

The effects of different basal media on the growth rate and tanshinone production of the cell suspension culture were investigated by employing the MS, 1/2 MS, B5 (Gamborg et al., 1968) and 6,7-V (Veliky and Martin, 1970) media. Cells were cultivated in 250-ml shake flasks containing 100 ml of the hormone-free liquid basal medium supplemented with 30 g l^{-1} sucrose. Cultivation was performed on an orbital shaker at 140 rpm in darkness at 25°C for 16 days. The inoculation size was controlled at about 3 g fresh weight biomass for each flask. At the end of the culture period, the growth rate and tanshinone content of each culture were measured.

The effect of initial sucrose concentrations (i.e. 20, 30, 40, 60, 80 and 100 g l^{-1}) on the cell growth was examined using the B5 medium. After 16 days of culture, the flasks were harvested and analyzed for biomass and tanshinone production.

The elicitation effect of yeast extract on tanshinone production of cell suspension culture was studied by transferring the cell aggregates grown at stationary stage to the fresh B5 or 6,7-V medium containing yeast extract (4 g l^{-1}) and to the fresh B5 medium without adding yeast extract which was used as a control. Cultures were harvested after 8 days and the tanshinone contents were analyzed.

The effect of light irradiation was determined by incubating the shake flask cultures on a rotary shaker (120 rpm) at 25°C under continuous illumination at an intensity of 4000 lux supplied by ordinary fluorescent lamps.

2.5. Analyses

Fresh weight (FW) was measured after separating the medium from the cell aggregates on Whatman no. 1 filter paper under vacuum. Dry weight (DW) was determined by heating the biomass in a vacuum oven at 60°C overnight.

The growth index (GI) is defined as the final biomass harvested (FW) divided by the inoculum (FW).

Quantitative analysis of tanshinones was carried out by high-performance liquid chromatography (HPLC). About 50 mg dried cells were extracted with 5 ml of MeOH–CH_2Cl_2 (3:1, v/v) twice at room temperature. HPLC was conducted on a Waters liquid chromatograph equipped with two 510 pumps and a 996 photodiode array detector. The MeOH–CH_2Cl_2 extract was filtered through 0.45 μm filters and separated by using a Resolve ™ C_{18} column (Waters, 300 mm × 3.9 mm, 5 μm) at 25°C. The mobile phase consisted of acetonitrile (65%) and water (35%). The flow rate was set at 1.0 ml min^{-1}. The filtered extracts were injected with a Rheodyne 7725 valve with a 20 μl loop. Peaks were measured at a wavelength of 270 nm to facilitate the simultaneous detection of tanshinones. Chromatographic peaks were identified by comparing retention times and spectra against known standards.

Chlorophylls and lutein were determined by HPLC. Fresh cell aggregates were ground by means of a pestle and extracted with 10 ml of MeOH–CH_2Cl_2 (3:1, v/v) for 16 h at room temperature in the dark. The extract was filtered through a 0.45 μm filter and subjected to HPLC. A Beckman Ultrasphere C_{18} (250 mm × 4.6 mm, 5 μm) column was used. The mobile phase consisted of dichloromethane (17%), acetonitrile (11.5%), methanol (69%) and water (2.5%). The flow rate was set at 1.0 ml min^{-1} and the peaks were detected at a wavelength of 450 nm to facilitate the simultaneous detection of chloro-

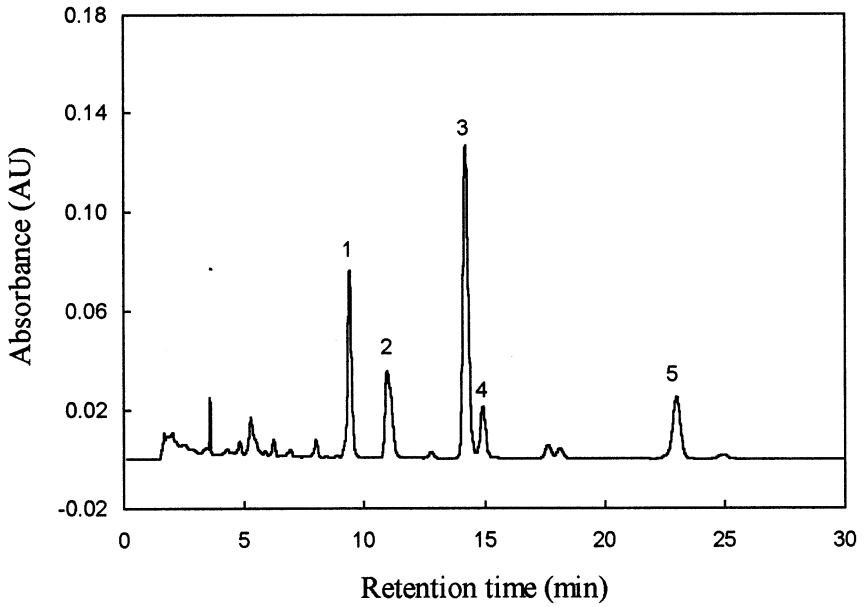

Fig. 1. Separation of tanshinones in the transformed cell aggregates of *S. miltiorrhiza*. HPLC was conducted on a Waters liquid chromatograph equipped with two 510 pumps and a 996 photodiode array detector as described in Section 2. Peaks: 1 and 2, unknown; 3, cryptotanshinone; 4, tanshinone I; 5, tanshinone IIA.

phylls and carotenoids (Yuan et al., 1996). The retention times were 3.8 min for lutein, 8.9 min for chlorophyll *b* and 14.2 min for chlorophyll *a*.

3. Results and discussion

3.1. Development of transformed cell cultures

In the establishment of transformed cell cultures, it was observed that the infection of *S. mitiorrhiza* nodes by *A. tumefaciens* C58 produced crown galls in about 10 d after inoculation. Different clones were obtained by excision of crown galls which were transferred to the hormone-free MS medium containing carbenicillin (0.5 g l^{-1}), and several subcultures were carried out until axenicity. The clones obtained were maintained as described in the Section 2. The cultures originated from different crown galls had different characteristics such as morphology, growth rate and color. Strain A (with fast-growing characteristics) and strain B (with red-spotted pigment) were selected from crown gall-derived cultures. Crown gall cells in suspension formed macroscopic clumps of cell aggregates rather than a homogeneous cell suspension. This culture exhibited similarity to that of self-immobilized *Solanum aviculare* cells (Tsoulpha and Doran, 1991). Self-immobilized cell cultures may have several advantages over dispersed cell cultures such as lower liquid viscosities, better mixing and improved liquid-phase mass transfer. In addition, aggregation of tissues could encourage differentiation and improve secondary syntheses (Tsoulpha and Doran, 1991). However, the formation of cell aggregates made continuous sampling from a single flask for biomass estimation impossible and plenty of flasks had to be used for studying the time course of cell growth and the accumulation of secondary metabolites.

3.2. HPLC profile of tanshinone production in transformed cell aggregates

The HPLC profile of tanshinone production in the transformed cell aggregates of *S. miltiorrhiza* is shown in Fig. 1. By comparing retention times and spectra against known standards, peaks 3, 4,

and 5 were identified as cryptotanshinone, tanshinone I and tanshinone IIA, respectively, while peaks 1 and 2 remain to be identified. In normal cell suspension culture systems of *S. miltiorrhiza*, only cryptotanshinone and ferruginol were identified (Miyasaka et al., 1989). In Ri transformed root or hairy root culture systems, seven tanshinones and ferruginol were identified and cryptotanshinone was found to be the main constituent (Hu and Alfermann, 1993). Our results also showed that cryptotanshinone was the main constituent. In this paper, total tanshinone production is expressed as the sum of cryptotanshinone, tanshinone I and tanshinone IIA.

3.3. Effects of different basal media on cell aggregate suspension culture of strain A

As shown in Fig. 2 and Table 1, of the four basal media tested, the B5 medium supported the best growth while the 6,7-V medium promoted tanshinone production in transformed cell suspension cultures. The growth index of the transformed cell culture in the B5 medium after 16 days of cultivation (GI 12.4 with a harvest of about 360 g FW or 13.5 g DW cell aggregates per liter) was obviously higher than that of the normal *S. miltiorrhiza* cell suspension culture of the ferruginol-producing strain (GI 3.5 with a biomass of about 11.6 g DW per liter after 18 days of cultivation) as reported by Miyasaka et al. (1989). The growth rate of their cryptotanshinone-producing strain was not reported (Miyasaka et al., 1989). As for tanshinone production, though relatively higher tanshinone production in the transformed cell suspension culture was achieved in the 6,7-V medium than in the others (Fig. 2B), the productivity was still low, which raised challenges for other approaches to promote the tanshinone productivity in this fast-growing strain.

The above results suggested the possibility of using a two-stage culture method for tanshinone production in the transformed cell suspension cultures, namely the proliferation of cells in one medium, which was the first stage and the production of tanshinone from the resulting cells in another medium, which was the second stage. This was similar to the well-known case of *Lithospermum erythrorhizon* (Fujita, 1988). A two-stage culture method was also employed in the cultivation of normal cell suspension of *S. mitiorrhiza* for the production of ferruginol and crytotanshinone (Miyasaka et al., 1989).

3.4. Effect of initial sucrose concentration on cell aggregate suspension culture of strain A

The initial sucrose concentrations tested were

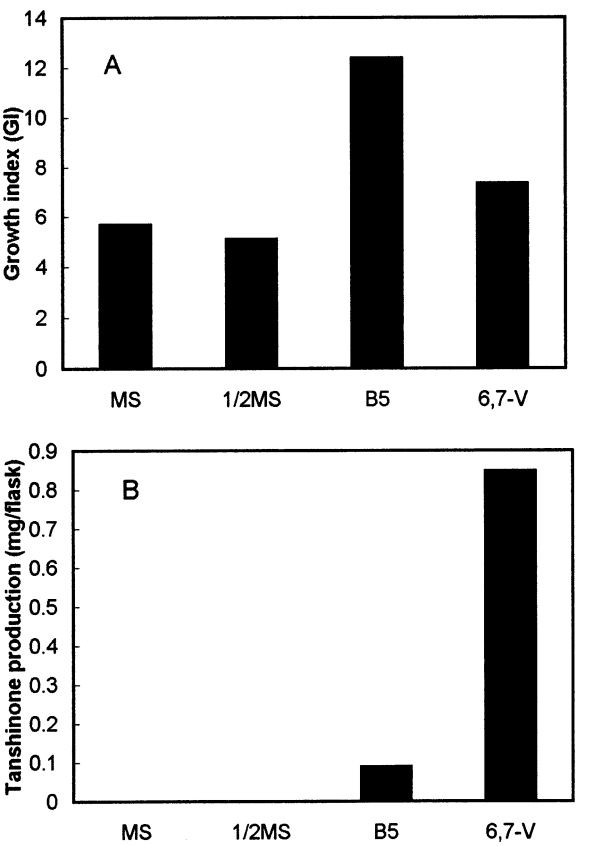

Fig. 2. Effects of different basal media on the growth (A) and tanshinone production (B) of transformed cell suspension culture of *S. miltiorrhiza* strain A. Cells were cultivated in 250-ml shake flasks containing 100 ml of various hormone-free liquid basal media supplemented with 30 g l^{-1} sucrose. Cultivation was performed on an orbital shaker at 140 rpm in darkness at 25°C for 16 days. The inoculation size was controlled at about 3 g FW biomass in each flask. At the end of culture period, the growth index and tanshinone content of each culture were measured.

Table 1
Profiles of tanshinone production in the transformed *S. miltiorrhiza* cell suspension cultures of strain A when cultivated in different basal media for 16 days

Basal media	Tanshinone production (mg/flask)						
	In medium			In cells			Total
	Cryptotanshinone	Tanshinone I	Tanshinone IIA	Cryptotanshinone	Tanshinone I	Tanshinone IIA	
MS	0	0	0	0	0	0	0
1/2MS	0	0	0	0	0	0	0
B5	0.036	0.0047	0.026	0.014	0.005	0.003	0.089
6,7-V	0.039	0.051	0.115	0.108	0.333	0.201	0.847

between 20 and 100 g l^{-1}. Results for biomass and tanshinone production after 16 days of culture are shown in Fig. 3. Initial sucrose concentration had a significant influence on the growth of transformed cell cultures; growth was the fastest in medium containing 30 g l^{-1} sucrose, and the growth was obviously reduced at 60 g l^{-1} sucrose and totally inhibited at 100 g l^{-1} sucrose (Fig. 3A). To distinguish the effects between sucrose concentration and osmotic pressure, 17 g l^{-1} mannitol was added to the medium containing 30 g l^{-1} sucrose so that the initial osmolality was approximately equal to that of the medium containing 60 g l^{-1} sucrose (7.8 atm) (Yu et al., 1996). The growth index of the cell aggregates cultivated on this medium was 5.4 which was comparable to that (5.0) achieved on the medium containing 60 g l^{-1} sucrose. This result indicated the sensitivity of the cell growth to the osmotic pressure.

The tanshinone production under these culture conditions was also determined as shown in Fig. 3B. The tanshinone production was better in the 30 g l^{-1} sucrose medium than that in the 20 g l^{-1} or 40 g l^{-1} sucrose medium. However, much higher tanshinone production was observed in the 100 g l^{-1} sucrose medium although the growth of cells was almost completely inhibited under the conditions. This result was in agreement with that of normal cell suspensions of *S. mitiorrhiza*, in which cryptotanshinone production was stimulated only after cell growth was suppressed (Miyasaka et al., 1989).

3.5. Effect of yeast extract on cell aggregate suspension culture of strain A

Yeast or yeast extract is one of the most commonly used elicitors for the induction or enhancement of secondary metabolite formation. With yeast extract, the production of berberine by *Thalictrum rugosum* cells was increased up to four times and the production of alkaloids by *Eschscholtzia californica* was increased about 30 times (Brodelius et al., 1989). Earlier work has shown that a carbohydrate fraction isolated from yeast extract was the active compound (Hahn and Albersheim, 1978). In our case as shown in Table 2, by transferring cell aggregates of strain A to the yeast-extract-containing fresh medium, the production of tanshinones increased substantially (2.01 mg/flask in the yeast-extract-containing B5 medium and 2.22 mg/flask in the yeast-extract-containing 6,7-V medium, respectively). These were much higher than those obtained without yeast extract (Table 1). The productivity of 22.2 mg l^{-1} (i.e. 2.22 mg/flask) was comparable to the immobilized normal cell suspensions of *S. mitiorrhiza* (i.e. 27 mg l^{-1} cryptotanshinone), in which case the selected cell line with the high-cryptotanshinone-production characteristic was used (Miyasaka et al., 1989). Among the elicited tanshinones, no tanshinone I was detected, which suggested that changes not only in tanshinone production but also in tanshinone profiles took place after the elicitation. Work is in progress in our laboratory on the optimization of elicitation

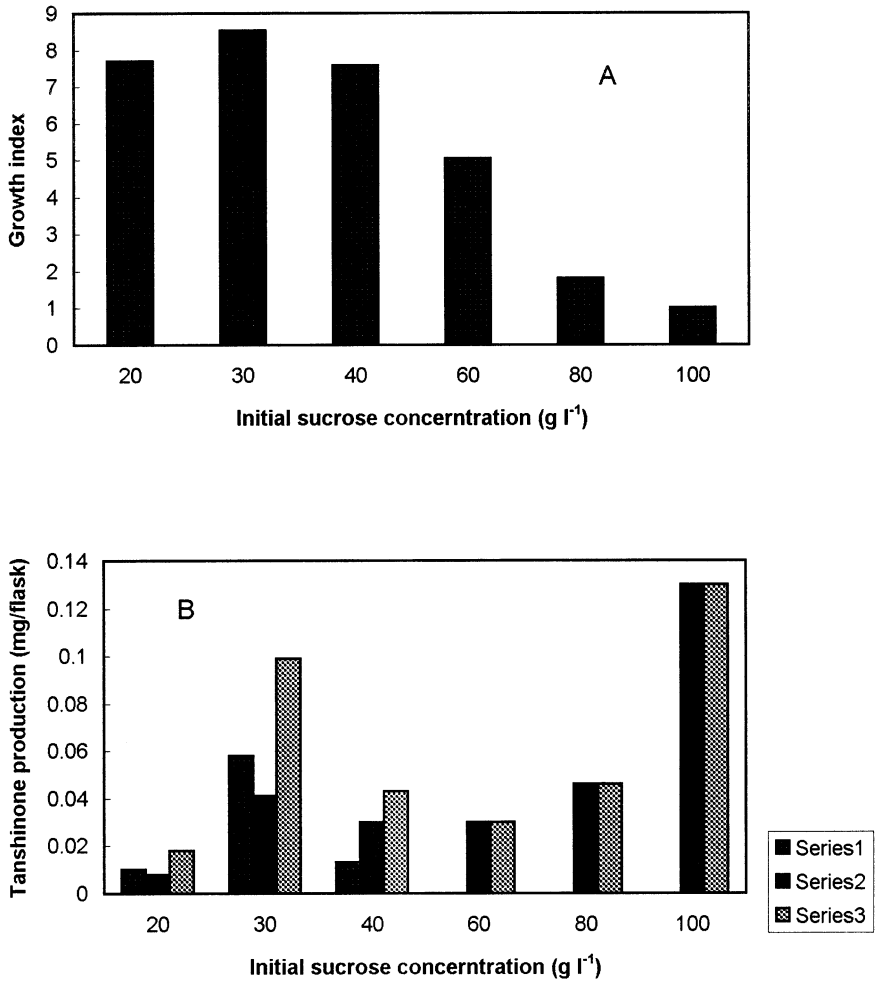

Fig. 3. Effects of initial sucrose concentrations on the growth (A) and tanshinone production (B) of transformed cell suspension cultures of *S. miltiorrhiza* strain A. Cells were cultivated in 250-ml shake flasks containing 100-ml of hormone-free liquid B5 medium supplemented with different concentrations of sucrose. Cultivation was performed on an orbital shaker at 140 rpm in darkness at 25°C for 16 days. The inoculation size was controlled at about 4.5 g FW biomass in each flask. At the end of culture period, the growth index and tanshinone content of each culture were measured. In (B): series 1, tanshinones in medium; series 2, tanshinones in cells; series 3, total tanshinones per flask.

(such as elicitor concentrations, the incubation time for the elicitor treatment, and physiological stages of cell aggregates) with yeast extract as well as other elicitors.

3.6. Effect of light irradiation on cell aggregate suspension culture of strain A

When these transformed cell aggregates were cultivated in the light, they turned green (so-called green callus) and a conspicuous morphological change of cell aggregates was observed with shooty teratoma derived from the undifferentiated cells. The ability of these cultures to produce tanshinones as well as chlorophylls and lutein under different conditions was also examined (Table 3). No detectable tanshinones were produced in green callus when cultured in the B5 medium. The effect of yeast extract on tanshinone production in these green cell aggregates also

Table 2
Effect of yeast extract on tanshinone production in the transformed *S. miltiorrhiza* cell suspension cultures of strain A

Medium	Growth rate		Tanshinone production (mg/flask)				
	Inoculum FW (g/flask)	Harvest FW (g/flask)	In medium		In cells		Total
			Cryptotanshinone	Tanshinone IIA	Cryptotanshinone	Tanshinone IIA	
B5 (control)[a]	32.7	47.78	Trace	Trace	Trace	Trace	Trace
B5+y[b]	32.7	33.48	0.23	0.014	1.69	0.075	2.01
6,7-V+y[c]	32.7	34.19	0.19	0.011	1.91	0.107	2.22

Cells with an inoculum of approx. 2.5 g FW were first cultivated in several 250-ml shake flasks containing 100 ml B5 medium with 20 g l^{-1} sucrose on an orbital shaker at 140 rpm in darkness at 25°C for 16 days. The cell aggregates were then transferred to fresh media by decanting the old medium, and further cultivated for 8 days under the same conditions.
[a] Fresh B5 medium containing 20 g l^{-1} sucrose without yeast extract.
[b] Fresh B5 medium containing 20 g l^{-1} sucrose with 4 g l^{-1} yeast extract.
[c] Fresh 6,7-V medium containing 20 g l^{-1} sucrose with 4 g l^{-1} yeast extract.

showed positive results. The green cell aggregates transferred to the fresh B5 medium containing 4 g l^{-1} yeast extract and cultivated under darkness produced more tanshinones than their counterparts cultivated under continuous illumination, which suggested the inhibitory effect of light on tanshinone production in the cell cultures. The use of green callus or green hairy root culture systems for the production of plant secondary metabolites has been reported by several other authors. For example, the green hairy root cultures of *Lobelia sessiliforlia* cultured in the light showed superiority in terms of both growth rate and production of secondary metabolites (Ishimaru et al., 1994). The growth rates of the green hairy roots of *Lobelia chinensis* cultivated in the light were superior to those in the dark. However, larger amounts of polyacetylenes appeared in the latter than in the former (Tada et al., 1995). In our case of *S. miltiorrhiza*, both fast growth (with an inoculum of about 0.8 g fresh weight cell aggregates in 100 ml B5 medium containing 20 g l^{-1} sucrose, about 24 g fresh weight cells were harvested after 24 days of culture) and differentiation were observed in the green cell cultures as well as the inhibitory effect of light on tanshinone production.

3.7. Tanshinone production in transformed cell aggregates of strain B

During continuous subculturing on the solidified 6,7-V medium containing 20 g l^{-1} sucrose and 8 g l^{-1} agar, some red-pigmented cells derived from the light yellowish callus. Every month, the red-pigmented cell aggregates were picked out and transferred onto the fresh solidified 6,7-V medium and cultivated at 25°C in darkness. Using this cell-aggregate selection method, cell strain B was established after 12 months. The tanshinone content of this cell strain was also determined and compared with that of commercial Dan-shen (Table 4). The results showed that tanshinone content of the cell aggregates was approximately ten times that of the commercial Dan-shen on a dry weight basis. Cryptotanshione was the main constituent in the cell cultures while tanshinone IIA was the main constituent in the commercial Dan-shen. The growth characteristics of this cell strain are still under investigation.

4. Conclusions

The Ti transformed cell suspension culture system was successfully established. Both high

Table 3
Effects of light irradiation on the growth and tanshinone production of *S. miltiorrhiza* cell suspension cultures of strain A

Culture mode	Chl. *a* (mg g^{-1} FW)	Chl. *b* (mg g^{-1} FW)	Lutein (mg g^{-1} FW)	Tanshinone contents (mg/flask)		
				In medium	In cells	Total
B5+L[a]	0.04	0.012	0.0074	0	0	0
B5+y+L[b]	0.016	0.0048	0.0047	0.0254	0.167	0.192
B5+y+D[c]	0.022	0.0088	0.0061	0.093	0.783	0.876

The cell aggregates were cultivated in shake flasks containing 100 ml B5 medium on a rotary shaker (120 rpm) at 25°C with or without continuous irradiation at an intensity of 4000 lux.
[a] Cells were cultivated under continuous illumination.
[b] Green cell aggregates (about 26 g FW) were transferred to fresh B5 medium containing 4 g l^{-1} yeast extract and continued to be cultivated in the light for 8 days.
[c] Green cell aggregates (about 21 g FW) were transferred to fresh B5 medium containing 4 g l^{-1} yeast extract and continued to be cultivated in darkness for 8 days.

Table 4
Tanshinone contents in the transformed *S. miltiorrhiza* cell aggregates of strain B cultivated on solidified 6,7-V medium containing 20 g l^{-1} sucrose and 8 g l^{-1} agar and commercial Dan-shen

Materials	Tanshinone content (mg g^{-1} DW)			
	Cryptotanshinone	Tanshinone I	Tanshinone IIA	Total
Cell strain B	5.57	1.47	3.81	10.85
Commercial Dan-shen	0.145	0.293	0.776	1.21

growth rate and high elicited tanshinone production was observed in cell strain A. Through continuous cell aggregate selection, cell strain B with higher tanshinone content was also obtained. With the combination of strain selection, medium optimization and elicitation, the transformed *S. miltiorrhiza* cell suspension culture system would most likely be employed for the commercial production of tanshinones.

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References

Aerts, M., Jacobs, M., Hernalsteens, J.P., Van Montagu, M., Schell, J., 1979. Induction and in vitro culture of *Arabidopsis thaliana* crown gall tumours. Plant Sci. Lett. 17, 43–50.

Brodelius, P., Collinge, M.A., Funk, C., Gugler, K., Marques, I., 1989. Studies on alkaloid formation in plant cell cultures after treatment with a yeast elicitor. In: Kurz, W.G.W. (Ed.), Primary and Secondary Metabolism of Plant Cell Cultures II. Springer-Verlag, Berlin, pp. 191–199.

Bruneton, J. (1995) Pharmacognosy, Phytochemistry, Medicinal Plants. Intercept, UK, 522 pp.

Cosio, E.G., Norton, R.A., Towers, E., Finlayson, A.J., Rodriguez, E., Towers, G.H.N., 1986. Production of antibiotic thiarubrines by a crown gall tumor line of *Chaenatis douglasii*. J. Plant Physiol. 124, 155–164.

Fujita, Y., 1988. Shikonin: production by plant (*Lithospermum erythrorhizon*) cell cultures. In: Bajaj, Y.P.S. (Ed.), Biotechnology in Agriculture and Forestry, Vol. 4, Medicinal and Aromatic Plants I. Springer-Verlag, Berlin, pp. 225–236.

Gamborg, O.L., Miller, R.A., Ojima, K., 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50, 151–158.

Hahn, M.G., Albersheim, P., 1978. Host–pathogen interactions. XIV. Isolation and partial characterization of an elicitor from yeast extract. Plant Physiol. 62, 107–111.

Hooykaas, P.J.J., Klapwijk, P.M., Nuti, M.P., Schilperoort, R.A., Rörsch, A., 1977. Transfer of the *Agrobacterium tumefaciens* Ti plasmid to avirulent Agrobacteria and to Rhizobium ex planta. J. Gen. Microbiol. 98, 477–484.

Hu, Z.B., Alfermann, A.W., 1993. Diterpenoid production in hairy root cultures of *Salvia miltiorrhiza*. Phytochemistry 32, 99–703.

Ishimaru, K., Arakawa, H., Yamanaka, M., Shimomura, K., 1994. Polyacetylenes in *Lobelia sessilifollia* hairy roots. Phytochemistry 35, 365–369.

Miyasaka, H., Nasu, M., Yoneda, K., 1989. *Salvia mitiorrhiza*: in vitro production of cryptotanshinone and ferruginol. In: Bajaj, Y.P.S. (Ed.), Biotechnology in Agriculture and Forestry, Vol. 7, Medicinal and Aromatic Plants II. Springer-Verlag, Berlin, pp. 417–430.

Moldenhauer, D., Fürst, B., Diettich, B., Luckner, M., 1990. Cardenolides in *Digitallis lanata* cell transformed with Ti-plasmids. Planta Med. 56, 435–438.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15, 473–497.

Norton, R.A., Towers, G.H.N., 1985. Synthesis of polyacetylenes in tumor callus of *Bidens alba*. J. Plant Physiol. 120, 273–283.

Otten, L.A.B.M., Schilperoort, R.A., 1978. A rapid microscale method for the detection of lysopine and nopline dehydrogenase activities. Biochim. Biophys. Acta 527, 497–500.

Shimomura, K., Kitazawa, T., Okamura, N., Yagi, A., 1991. Tanshinone production in adventitious roots and regenerates of *Salvia miltiorrhiza*. J. Nat. Prod. 54, 1583.

Tada, H., Shimomura, K., Ishimaru, K., 1995. Polyacetylenes in hairy root cultures of *Lobelia chinensis*. J. Plant Physiol. 146, 199–202.

Towers, G.H.N., Ellis, S., 1993. Secondary metabolism in plant tissue cultures transformed with *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. In: Kinghorn, A.D., Balandrin, M.F. (Eds.), Human Medicinal Agents from Plants. American Chemical Society, Washington, DC, pp. 56–79.

Tsoulpha, P., Doran, P.M., 1991. Solasodine production from self-immobilized *Solanum aviculare* cells. J. Biotechnol. 19, 99–110.

Veliky, I.A., Martin, S.M., 1970. A fermenter for plant cell suspension cultures. Can. J. Microbiol. 16, 223–226.

Yu, S., Kwok, K.H., Doran, P.M., 1996. Effect of sucrose, exogenous product concentration, and other culture conditions on growth and steroidal alkaloid production by *Solanum aviculare* hairy roots. Enzyme Microb. Technol. 18, 238–243.

Yuan, J.P., Gong, X.D., Chen, F., 1996. Separation and identification of astaxanthin esters and chlorophylls in *Haematococcus lacustris* by HPLC. Biotechnol. Tech. 10, 655–660.