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Ultrahigh diterpenoid tanshinone production through repeated osmotic stress and elicitor stimulation in fed-batch culture of *Salvia miltiorrhiza* hairy roots

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Abstract Hyperosmotic stress (OS, created with 50 g/L sorbitol) and a yeast elicitor (YE, polysaccharide fraction of yeast extract) applied to *Salvia miltiorrhiza* hairy root cultures had a synergistic effect on the diterpenoid tanshinone production. With a single OS+YE treatment and nutrient feeding, the total tanshinone content of roots was increased by sevenfold (from 0.2 to 1.6 mg/g dry weight (dw)) and the volumetric yield by 13-fold (from 1.95 to 27.4 mg/L) compared to the batch control culture. With repeated feeding of OS and nutrient medium in an extended fed-batch culture process (i.e., 10 mL fresh medium with 50 g/L sorbitol 25 mg/L YE, every 5 days from day 21 to day 60), the total tanshinone content of roots was increased to 18.1 mg/g dw (or 1.8 wt.%) and the volumetric tanshinone yield to 145 mg/L, which were about 100-fold and 70-fold of those, respectively, in the batch control. Another interesting finding was the presence of root fragments (fine particles) with extremely high tanshinone content in the OS+YE treated cultures. It was also possible to reuse the sorbitol medium for the hairy root growth and tanshinone production to reduce the medium expenses.

Keywords *Salvia miltiorrhiza* · Hairy root · Tanshinones · Osmotic stress · Yeast elicitor · Fed-batch culture

Introduction

Plant hairy root culture is a valuable tool of plant biotechnology and a promising tissue culture method for mass production of useful plant secondary metabolites (Giri and Narasu 2000). Compared with the normal plant tissue and cell suspension cultures, the transformed hairy root culture has the advantages of high genetic stability, rapid and hormone-free growth. Plant hairy root culture has been widely explored for the production of medicinal plants and their bioactive principles (Guillon et al. 2006; Zhou and Wu 2006). *Salvia miltiorrhiza* Bunge (Lamiaceae) root or Danshen in Chinese, is a well-known Chinese herb, which is widely used in modern and traditional medicine for the treatment of menstrual disorders and blood circulation diseases, and for the prevention of inflammation. A major class of bioactive ingredients of Danshen is ascribed to the lipophilic diterpene pigments generally known as tanshinones (Tang and Eisenbrand 1992). The hairy root culture of *S. miltiorrhiza* has been established as an alternative to farm growth of the whole plants for more efficient production of tanshinones (Hu and Alfermann 1993; Chen et al. 1999).

Elicitation or elicitor treatment is one of the most effective means for improving secondary metabolite production in plant tissue and cell cultures including hairy root cultures (Zhao et al. 2005; Zhou and Wu 2006). Among the most common elicitors used in previous studies are fungal carbohydrates or polysaccharides, jasmonic acid or methyl jasmonate, chitosan, and heavy metal ions. In addition to these chemical and biochemical agents, abiotic stress such as hyperosmotic stress has been shown effective for stimulating the production of various secondary metabolites in plant cell cultures, such as anthocyanins in grape cells (Do and Cormier 1990), saponins in ginseng cells (Wu et al. 2005), and paclitaxel in *T. chinensis* (Kim et al. 2001). Although single elicitors have been used in most previous studies, the combined dose of two different elicitors has been shown more effective due to a synergistic effect (Linden and Phisalaphong 2000; Zhao et al. 2001).

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The effect of elicitation on the secondary metabolite production in plant tissue cultures is limited to the increase of the product content or yield per unit mass of cells; however, the overall productivity also depends on the biomass growth rate and biomass concentration in the culture. In this regard, much higher productivity may be achieved by the integration of elicitation with a nutrient feeding or fed-batch process strategy which can sustain and promote the biomass growth. Nutrient feeding during elicitation may also overcome the growth inhibiting effect of some elicitors such as heavy metal ions and abiotic stress (Furze et al. 1991; Pitta-Alvarez et al. 2000). Moreover, nutrient feeding allows for the application of multiple elicitor doses or repeated elicitor treatments over a longer culture period, which has been shown to be more effective for the production of taxanes in *Taxus* cell cultures (Qian et al. 2005). Our previous study has shown that the integration of elicitor treatment (elicitation) and nutrient feeding (or medium renewal) is an effective strategy for improving the tanshinone production in *S. miltiorrhiza* hairy root cultures (Zhang et al. 2004).

Our recent study (Shi et al. 2007) has shown that the combination of hyperosmotic stress (OS, by adding sorbitol as an osmoticum) and a yeast elicitor (YE) can enhance the tanshinone production in *S. miltiorrhiza* hairy root cultures more significantly than the two used separately. This study was conducted to explore more substantial enhancement of the tanshinone production by applying the combined OS stimulation to a fed-batch culture process with multiple or repeated nutrient feeding. Moreover, to overcome the drawback of high sorbitol consumption with the OS stimulation measure, we exercised a medium recycle process to reuse the sorbitol medium for the hairy root growth and tanshinone production.

Materials and methods

Hairy root culture

The *S. miltiorrhiza* hairy root culture used in this work was derived after the infection of plantlets with a Ri T-DNA bearing *Agrobacterium rhizogenes* (ATCC15834), and maintained on hormone-free MS medium with 30 g/L sucrose but without ammonium nitrate at 25°C in the dark (Chen et al. 1999). All experiments in this study were carried out in shake-flask cultures on an orbital shaker at 110–120 rpm. Unless stated otherwise, 125-mL Erlenmeyer flasks were used, each filled with 25 mL liquid MS medium and inoculated with 0.2 g fresh weight (fw) of roots from 21-day-old shake-flask cultures. All treatments were performed in triplicate and repeated at least once, and the results were averaged.

Application of osmotic stress and yeast elicitor to hairy root cultures

As described previously (Shi et al. 2007), hyperosmotic stress (OS) on the hairy roots was created by adding sorbitol to the culture medium, and the yeast elicitor (YE) was the polysaccharide fraction of yeast extract isolated by ethanol precipitation. In this study, sorbitol was fixed at 50 g/L (raising the medium osmolality nearly twofold, from 260 to 500 mmol/kg) and YE fixed at 25 mg/L (represented by the total sugar content measured by anthrone test using glucose as a reference), which were the most effective doses found from preliminary tests. As in our previous study, OS and YE were applied to the hairy root cultures on day 21, by removing the used liquid medium in the culture flasks and adding an equal volume of fresh medium with 50 g/L sorbitol (for OS-treatment cultures) or without (for the control). The yeast elicitor for the YE- or OS+YE treated cultures was added to the culture flasks immediately after the medium exchange.

Medium recycle culture experiments

The recycled medium containing 50 g/L sorbitol was collected from hairy root culture at day 9 after the sorbitol addition and medium exchange on day 21 (stage 1 culture, overall period 21+9=30 days). After removal of the hairy roots, the medium was centrifuged at 6,000 rpm for 5 min to remove suspended solids, and then filter-sterilized. The recycled medium was applied with fresh medium at 25–75% (*v*/*v*) to the next culture cycle (stage 2). For the stage 2 culture, the recycled medium was also applied to 21-day-old hairy roots in shake-flasks and run for another 9 days before harvesting, so that the overall culture period including the two stages was 60 days.

Fed-batch culture with repeated nutrient feeding

In this set of experiments, 200-mL (instead of 125-mL) Erlenmeyer flasks were used to accommodate the relatively large volume of medium due to repeated addition. Two feeding schemes were exercised, 10 mL fresh medium every 5 days, and 20 mL fresh medium every 10 days, starting on day 30 (at the end of the first medium renewal and elicitor treatment) and ending on day 60 when the cultures were harvested for analysis. In all experiments, sorbitol and YE in the medium were maintained at 50 g/L and 25 mg/L, respectively.

Measurement of root weight and tanshinone content

The hairy roots were collected from the culture flask with a pare of forceps and rinsed thoroughly with distilled water,

and then dried at 45–50°C in an oven until constant dry weight (dw). The dry root mass was extracted with 4:1 methanol/dichloromethane and the tanshinone content was determined quantitatively by high-performance liquid chromatography (HPLC; C18 column, 55:45 acetonitrile/water as mobile phase, and UV detection at 275 nm), as described previously (Shi et al. 2007). Three tanshinone species (Fig. 1), cryptotanshinone (CT), tanshinone-I (T-I), and tanshinone-IIA (T-IIA) were quantified, and their sum was taken as the total tanshinone (TT) content of roots. Fine particles (root fragments) suspended in the medium were recovered by centrifugation, dried, and then extracted for tanshinones as with the hairy roots. Tanshinone content (dissolved) in the medium was negligible and not determined (as tanshinones are lipophilic and barely soluble in an aqueous medium).

Results

Effects of osmotic stress and YE on root growth and tanshinone production

The total tanshinone content of roots was increased (three to fourfold) by both osmotic stress (OS) and yeast elicitor (YE) treatment, but more dramatically by their combination (1.57 vs 0.20 mg/g dw, about eightfold) (Fig. 2a and Table 1), compared with the untreated batch control. The enhancing effect of OS and YE on tanshinone production could be observed within 3 days of treatment, approaching a plateau in 9 days. In addition to the enhanced tanshinone production, both OS- and OS+YE treated hairy root cultures showed a more rapid growth of root biomass and gained a much higher root dry weight at the end than the control or YE-treated culture (Fig. 2b). The result suggests that the high medium osmotic pressure caused by the addition of sorbitol was beneficial to the hairy root biomass accumulation. The increases in the TT content and the root dry weight together led to a larger increase in the volumetric tanshinone yield in the OS+YE treated culture, to about 13.4-fold of that in the control culture (23.6 mg/L vs 1.76 mg/L; Table 1).

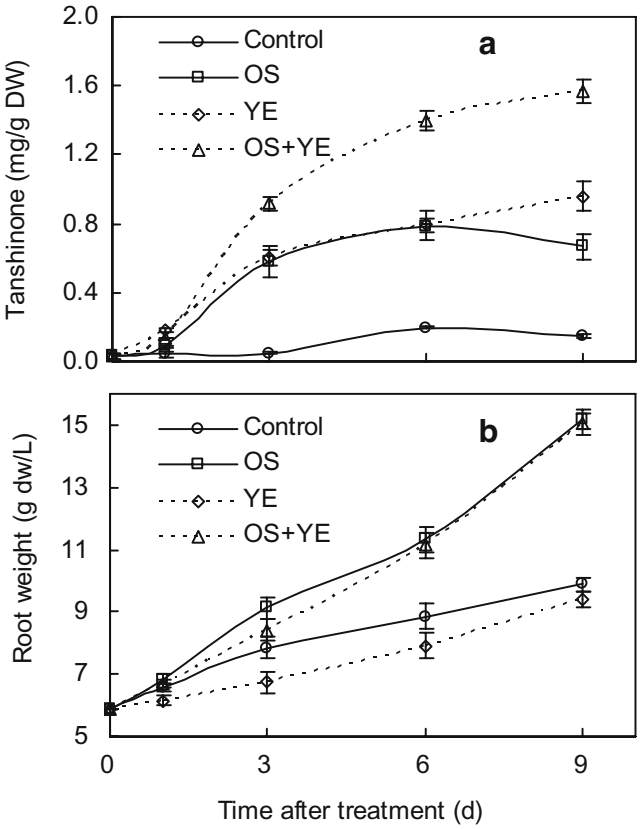

Fig. 2 Time courses of root biomass (**a**) and total tanshinone (TT) content (**b**) after medium renewal, and OS and YE treatments (from day 21 to day 30) (control culture was also subjected to medium renewal on day 21 but not to OS or YE treatment). *Error bars* for standard deviation, $n=3$

Tanshinone content of root fragments in the culture medium

In the course of hairy root culture experiments, many small dark-brown solid particles were noticed suspended in the culture medium, especially in the OS+YE treated culture flasks and during the later days of culture. As observed under the microscope, these suspended particles were actually root fragments with a maximum dimension no more than 1 mm (Fig. 3). Most notable is that these root fragments had very high tanshinone content, 10–20 times higher than in the roots (Table 1). In the OS+YE treated culture, for example, the tanshinone yield from the root

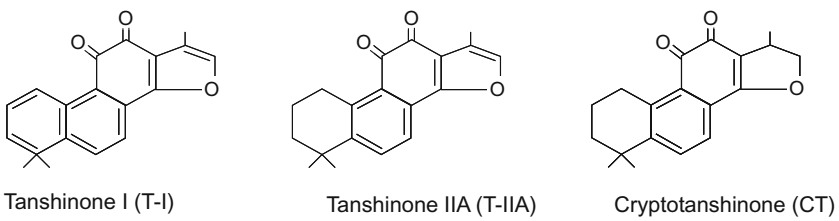

Fig. 1 Chemical structures of the three major tanshinones in *S. miltiorrhiza* hairy roots determined in this study

Table 1 Mass and tanshinone content of roots and root fragments (all treatments applied to hairy root cultures on day 21 post-inoculation; measurements taken 9 days later)

Treatment	Roots[a]		Root fragments		TT yield (mg/L)
	Weight (g dw/L)	TT content (mg/g DW)	Weight (g dw/L)	TT content (mg/g dw)	
Control	8.85±0.55	0.20±0.01	0.024	8.04	1.96
OS	15.2±0.85	0.67±0.07	0.032	10.0	10.5
YE	9.39±0.45	0.96±0.08	0.084	10.8	9.92
OS+YE	15.3±0.31	1.57±0.17	0.18	21.1	27.8

[a] Root weight and TT content are represented by mean ± standard deviation, $n=3$.

fragments (3.8 mg/L) accounted for 14% the volumetric tanshinone yield (27.6 mg/L), though the total mass of root fragments was only 1–2% of the total root mass. The volumetric tanshinone yield in the hairy root culture (content in roots+content in root fragments) in the OS+YE treated culture was 14-fold of that in the control (1.95 mg/L).

Medium recycle for root growth and TT production

In the culture stage using recycled medium (stage 2), the root weight and tanshinone content showed a gradual decrease with the volume fraction of recycled (old) medium in the culture (Table 2). Nevertheless, the hairy roots still grew fairy well, and even with 75% recycled medium in the culture, the root weight was only 15% lower than in the fresh medium. The tanshinone yields with 25–75% used medium in the culture were all much higher than that in the batch control culture. Moreover, the overall tanshinone yield on per volume of fresh medium in the two-stage process (a stage on fresh medium plus a stage on a portion of recycled medium) increased with the proportion of recycled medium, which means a net reduction of the medium and sorbitol expenses.

Tanshinone production in fed-batch process with repeated OS+YE and nutrient feeding

As shown by the culture time courses with one-time medium renewal and elicitor treatment (Fig. 2), the root biomass was still on the rise and the tanshinone content was at the highest level at the end of the culture period (9 days after treatment). If the culture period was extended from this point, much higher biomass weight and tanshinone yield could be achieved. Therefore, we exercised a fed-batch culture process with multiple nutrient feeding and OS+YE treatment for an overall period of 60 days, with the first feeding on day 21 as for the one-time-feeding culture.

Table 3 shows the total amounts of medium and sorbitol used, and the root biomass and tanshinone yield at the end of this multiple-feeding process. The root biomass concentrations (grams dry weight per liter) did not increase during the fed-batch culture process or were even lower at the end on day 60 than the initial concentrations (as those shown in Table 1), due mainly to the dilution effect of increasing medium volume. The total root mass per flask from the 60-day fed-batch cultures (0.61–1.16 g dw) was much higher than that in the 30-day cultures subjected to the same treatment (0.22–0.38 g dw with the root dry weight per liter in Table 1 times the 25 mL medium volume). Therefore, the roots were still growing during the prolonged fed-batch process, though the growth rate was much slower than that in the batch or shorter period fed-batch cultures. The lower average growth rate of roots over the 60-day fed-batch period may be partially attributed to the larger liquid volume in the culture flasks which caused oxygen transfer limitation.

Most notable was the ultrahigh tanshinone content attained in the OS+YE treated culture, 18.1 mg/g dw (equivalent to 1.8 wt.%), about 11.5-fold of that in the

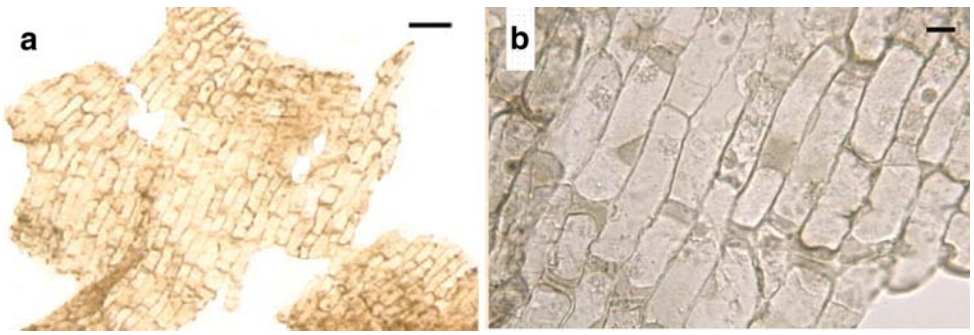

Fig. 3 Microscopic photos of root fragments in the culture medium (bar=50 μm (**a**) and 10 μm (**b**))

Table 2 Tanshinone yields vs medium and sorbitol expenses in two-stage culture process with medium recycle

Culture stage[a]	Recycled medium (vol%)[b]	Medium use (mL)	Sorbitol use (g/L)	Root weight[c] (g dw/L)	TT yield[c] (mg/L)
Stage 1 (30 days)	0	25	50	15.0±0.31	23.6±0.58
Stage 2 (30 days)	0	25	50	14.7±0.84	24.2±2.30
	25	18.75	37.5	13.3±1.17	21.1±1.17
	50	12.5	25	13.0±0.39	17.7±1.75
	75	6.25	12.5	12.6±0.70	15.9±1.34
Overall (60 days)	0	50	50	14.86	23.9[d]
	25	43.75	43.75	16.39	25.5
	50	37.5	37.5	20.47	27.5
	75	31.25	31.25	32.75	31.6

[a] In both stages, hairy root cultures were initially maintained on MS medium for 21 days, and then replaced with fresh medium containing 50 g/L sorbitol 25 mg/L YE, and cultured for another 9 days.
[b] Recycled medium was collected from stage 1 and applied to stage 2 at the given vol%.
[c] Root weight and TT yield are represented by mean ± standard deviation, $n=3$.
[d] Overall root weight or TT yield (stage 1 2) equal to (weight or yield in stage 1 yield in stage 2)×25/total medium use.

process with a single treatment (1.57 mg/g dw), and nearly 100-fold of that without any elicitor treatment (0.2 mg/g dw). Moreover, the root fragments released from the roots in the OS+YE treated culture had an even higher tanshinone content, 110 mg/g dw or 11 wt.%. A decrease of the feeding frequency from 10 mL every 5 days to 20 mL every 10 days had no significant effect on the tanshinone content but resulted in lower root biomass growth and a lower volumetric tanshinone yield.

Figure 4 presents the typical chromatograms of tanshinones extracted from the hairy roots in the control and elicitor-treated hairy root cultures. Note that the chromatogram for OS+YE treated roots was produced with the sample extract being diluted 40 times before the HPLC analysis because of the extremely high tanshinone content. Among the three detected tanshinone species, cryptotanshinone (CT) was mainly increased with the repeated OS+YE treatment and nutrient feeding, and the other two were only slightly increased.

Discussion

In the above experiments, an ultrahigh tanshinone yield was achieved through repeated OS+YE stimulation and nutrient feeding operation in the *S. miltiorrhiza* hairy root cultures. Such a high tanshinone content of the hairy roots (nearly 2 wt.%) has never been reported before either with Danshen roots from plant tissue culture or with field-grown plant roots (mostly ≤1.0%) (Chen 1991; Tang and Eisenbrand 1992; Chen and Zhu 2004). Both a frequent elicitor challenge and a sufficient nutrient supply to the hairy roots were essential for maintaining the secondary metabolite production through the extended fed-batch process. Elicitation (as with OS+YE treatment in this study) and nutrient feeding are two complementary strategies for the promotion of secondary metabolite production in plant tissue cultures, elicitation stimulating the secondary metabolite biosynthesis to higher levels and nutrient feeding sustaining the growth and biosynthetic activity of the plant cells, together

Table 3 Root biomass and tanshinone yields (on day 60) in fed-batch culture with repeated nutrient feeding and elicitor exposure

Treatment	Feeding scheme[a]	Root weight (g dw/L)	Root mass per flask (g dw)	TT content (mg/g dw)	TT yield[b] (mg/L)
Control	Sch 1	8.29±0.82	0.788	0.36±0.03	3.01
	Sch 2	8.72±0.72	0.741	0.36±0.03	3.10
OS	Sch 1	12.3±0.01	1.164	3.22±0.01	39.4
	Sch 2	13.3±1.04	1.133	3.20±0.02	42.6
OS+YE	Sch 1	7.94±0.78	0.754	18.1±0.36	143.6
	Sch 2	7.19±0.65	0.611	17.9±1.35	129.0

[a] Sch 1: 10 mL fresh medium every 5 days; final liquid volume 95 mL in flask. Sch 2: 20 mL fresh medium every 10 days, final liquid volume 85 mL in flask. In both schemes, feeding began on day 21 from an initial liquid volume 25 mL in each flask (average root weight 5.88±0.43 g dw/L). Sorbitol was maintained at 50 g/L in OS and OS+YE treated cultures, and YE at 25 mg/L for OS+YE treated culture.
[b] Not including that of the root fragments. In OS+YE treated cultures, the total mass of root fragments was 0.065 g/L and the TT content was 110.7 mg/g (in Sch 1). Not determined in other cultures.

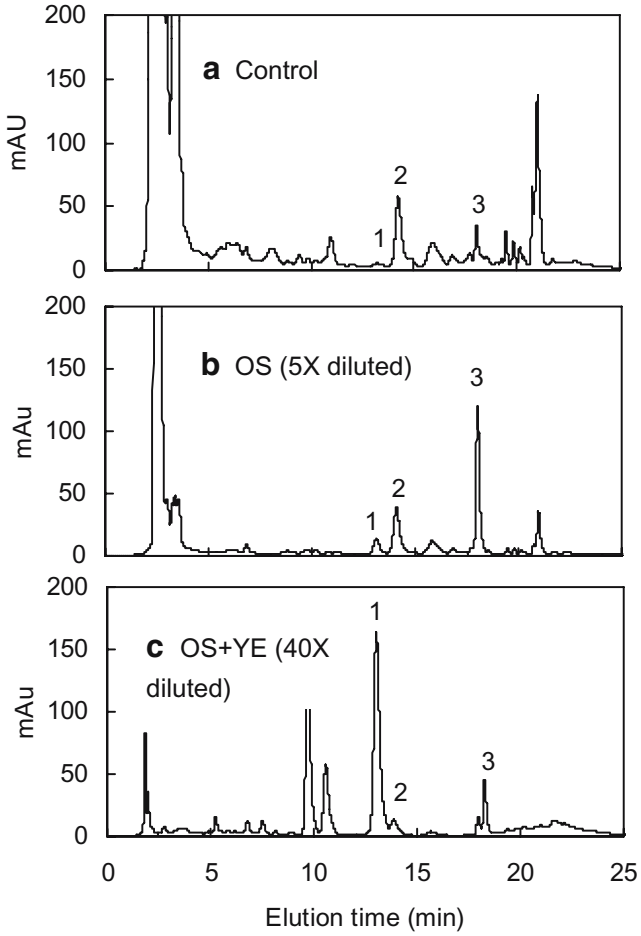

Fig. 4 Chromatograms of tanshinones in the hairy roots from **a** control culture, **b** OS-treated culture (with 50 g/L sorbitol), and **c** OStreated culture, all in fed-batch process (10 mL fresh medium every 5 days from day 21 to day 60, samples taken on day 60; tanshinone peaks, 1 for CT, 2 for T-I and 3 for T-IIA)

leading to the high product yield in the culture. In our present study, for example, OS+YE stimulated the tanshinone accumulation to a high content in the roots (in milligram per gram dry weight), and the nutrient feeding maintained the root growth at a high density (in gram dry weight per liter), both contributing to the high volumetric tanshinone yield (milligram per liter) in the *S. miltiorrhiza* hairy root cultures.

The selection of sorbitol as the osmoticum in our present and previous studies (Shi et al. 2007) was mainly because of its strong stimulation of the tanshinone production and few or no adverse effects on the hairy root growth. In addition to sorbitol, polyethylene glycol (PEG, molecular weight 8,000) was also found effective in stimulating the tanshinone production in our preliminary tests (data not shown). PEG-8000 added to the culture medium at 170 g/L raised the medium osmolality to a level approximately equal to that with 50 g/L sorbitol, increasing the tanshinone content by about twofold. PEG is a non-permeating and non-ionic osmoticum which is widely applied in experimental plant morphogenesis (Bozhkov and von Arnold 1998). Compared with sorbitol, however, PEG is less effective because a much higher concentration is required to achieve the same osmolality. Such a high concentration of PEG causes a significant increase in the medium viscosity and is not desirable for large-scale processes. NaCl is an effective salt for increasing medium osmolality but unfavorable for plant tissue cultures owing to its strong salinity toxicity and inhibitory effect on plant cell growth (Wu et al. 2005).

We believe that the enhancement of tanshinone accumulation by sorbitol in the *S. miltiorrhiza* hairy root cultures was mainly a result of hyperosmotic stress-induced response of the hairy roots. This means that sorbitol in the hairy root culture mainly acted as an osmotic stimulus. The stimulation of tanshinone production by PEG provides further support for a hyperosmotic stress-induced tanshinone accumulation as PEG is not permeable to the cell membrane and cannot be used by the hairy root cells. We rule out the possibility of sorbitol being utilized as a nutrient (alternative carbon source) for increasing the tanshinone production or the hairy root growth (increase in root weight) based on the following two facts. First of all, there was plenty of sucrose in the culture during the days of sorbitol treatment for the hairy root medium had been replaced with fresh medium containing 30 g/L sucrose prior to sorbitol addition, and sucrose is a more favorable carbon source for the roots than sorbitol. Secondly, the high medium osmolality created by sorbitol did not show any decrease from sorbitol addition (day 21) to hairy root harvesting (day 30; data not shown), implying that the osmoticum was not consumed by the root cells during the culture period.

Secondary metabolite biosynthesis in plants is an important element of plant response to biotic and abiotic stresses. Plants use multiple signal pathways to mediate their responses to different stresses, and the signal pathways in response to different stresses can have cross-talk or converge at a certain point, leading to common cellular processes (Zhao et al. 2005; Fujita et al. 2006). The convergence and cross-talk among different stress signal pathways has been suggested to contribute to the synergistic effect of multiple elicitors on a common set of responses (Dombrowski 2003). The results also showed that the YE treatment stimulated the cryptotanshinone accumulation more significantly than the other two tanshinones in the hairy roots. This implies that the elicitor treatments can alter the relative abundance or the distribution of chemical constituents of the roots. Such a change may affect the therapeutic effect and potency of the roots for medicinal uses. The differential stimulation of secondary metabolite relatives by biotic and abiotic elicitors has also been

observed in other plant hairy root cultures, such as sesquiterpenes in *Hyoscyamus muticus* by methyl jasmonate and a fungal elicitor (Singh et al. 1998), and tropane alkaloids in *Brugmansia candida* by heavy metal ions and yeast extract (Pitta-Alvarez et al. 2000). Likewise, the phenomenon also occurred in cell suspension cultures such as alkaloids in *Catharanthus roseus* cell stimulated by biotic and biotic elicitors in combination (Zhao et al. 2001), and diterpenoid taxanes in *Taxus* cells by methyl jamonate and triterpenoid saponins in *Panax* cells (Zhong and Yue 2005). Although the physiological mechanism for this phenomenon is not well understood, it may be associated with the shifting of metabolic fluxes toward different secondary metabolites which can be induced by elicitation (Zhao et al. 2006). A better understanding of the mechanisms is essential for effective control and manipulation of the metabolite profile so as to retain the desired medicinal properties and bioactivities of the culture products.

Another new phenomenon found in our experiments was the presence of small root fragments in the culture medium which had very high tanshinone content. To the best of our knowledge, the presence of tissue fragments with high content of secondary products in the liquid medium has never been reported previously on the *S. miltiorrhiza* hairy root cultures or any other plant tissue and organ cultures. These small root fragments were probably the surface tissues of old roots, and the old root tissues usually had much higher tanshinone content than the fresh and newly grown root tissues, as with the natural or farm-grown plant roots (Chen 1991). When these old root tissues (perhaps mostly dead) were released from the growing roots into the culture medium, they may act as the sorbent to adsorb the lipophilic tanshinones from the roots to gain even higher tanshinone content. We also tested on in situ adsorption of tanshinones in the hairy root culture using the same polymeric resin (macroporous polystyrene resin X-5) as in our previous study (Yan et al. 2005). It was found that the placement of 2 g resin (wrapped within nylon cloth) in each flask could completely recover the root fragments from the culture medium, and also adsorbed much of the tanshinones from the hairy roots (data not shown). The total amount of tanshinone adsorbed in the resin accounted for more than 70% of the volumetric tanshinone yield in the whole culture. However, the in situ adsorption scheme did not significantly increase the overall or volumetric tanshinone yield in the culture flask.

A major drawback with the addition of sorbitol and the repeated nutrient feeding is the high expenses of sorbitol and nutrient medium. This can be reduced with the medium recycle operation as shown in our experiments. As this medium and sorbitol saving required additional operations such as medium separation and sterilization, however, it may not lead to a net saving of the production cost. To cut down the medium expenses more effectively, we may replace the complete medium renewal by feeding a concentrated solution of selected nutrient components which have been exhausted. To minimize sorbitol use, we may reuse the sorbitol medium more than once. We may also seek other more economical and feasible means rather than adding an osmoticum to create the hyperosmotic stress, especially the use of gas-phase (instead of submerged) tissue culture bioreactors such as trickle bed and mist reactors (Kim et al. 2002; Ramakrishnan and Curtis 2004). Nevertheless, the remarkable effect of hyperosmotic stress plus yeast elicitor on the tanshinone production is very attractive for large-scale application, especially when more economical means for the creation of osmotic stress can be adopted.

The achievement of an ultrahigh secondary metabolite yield in our study is an exemplification for the ultimate process enhancement through the integration of multiple elicitor treatment and multiple nutrient feeding strategies. A synergy among various strategies is a key to the success of this approach. The low and unstable productivity of secondary metabolites in most plant tissue cultures has been a bottleneck for the commercial realization of their attractive potential. Further development of novel and efficient process strategies will lead to new breakthroughs for plant tissue culture bioprocess technology.

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