Comparison of Phytochemical and Antioxidant Activities in Micropropagated and Seed-derived *Salvia miltiorrhiza* Plants

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Abstract. *Salvia miltiorrhiza* (commonly known in China as Danshen) is widely used in traditional Chinese medicine, and it is applied in the treatment of many diseases, particularly cardiovascular disease. Commercial propagation of Danshen is carried out either through seed germination or in vitro regeneration (micropropagation). However, it is not clear if the different propagation methods affect the chemical properties of the derived plants. In the present study, we first established a highly efficient tissue culture system for Danshen propagation. The addition of 1.0 mg·L⁻¹ 6-benzyladenine (BA) and 0.1 mg·L⁻¹ α-naphthalene acetic acid (NAA) to Murashige and Skoog (MS) medium was optimal for inducing adventitious shoots; the highest rate of rooting was recorded on MS medium with 0.2 mg·L⁻¹ NAA, on which the survival rate of transplanted plantlets was 95%. Next, we assessed antioxidant properties in the different tissues of plants of the same age, derived from micropropagation or seed germination, and measured tanshinone, total phenol, and total flavonoid contents. Our results showed that tissues of micropropagated plantlets had higher antioxidant activities than tissues of seed-derived plantlets; the micropropagated plantlets also had higher tanshinone contents in their roots. Thus, a rapid and efficient micropropagation system was established for Danshen, and it can be used for cultivating this plant to obtain therapeutic compounds.

The genus *Salvia* (family Lamiaceae) consists of nearly 1000 species (Claßen-Bockhoff et al., 2004); many of them are used as herbs or traditional medicines because they contain active compounds that function against tumors, inflammation, and cardiovascular disease (Li et al., 2015; Wang et al., 2013). Species within the genus *Salvia* are also exploited for food, spices, cosmetics, and ornamental purposes (Neugebauerová et al., 2015; Wang et al., 2011). The dried roots of *S. miltiorrhiza*, known as Danshen in China, contains the pharmaceutically important secondary metabolites tanshinone and methyltanshinone, and are widely used in coronary heart disease therapeutics, especially for treating angina pectoris and myocardial infarction in China, Japan, Korea, and other Asian countries (Ryu et al., 1996; Wang et al., 2011). Because of its medicinal value and increasing demand, wild resources of

S. miltiorrhiza have been subjected to excessive exploitation and the species has become threatened; for this reason, cultivated Danshen is generally used for clinical purposes. However, the yield and quality of active compounds from Danshen decrease after prolonged field cultivation; in addition, viral infections and uncontrolled cross-pollination in the field may lead to varietal loss (Shan et al., 2007). In recent years, methodological advances in plant tissue culture methods have allowed its use as a viable approach for multiplication, germplasm conservation, and genetic manipulation of medicinal plants (Benson, 2008; Gonçalves et al., 2010). Hence, tissue culture may offer an efficient alternative method to improve the quality and yield of active compounds in Danshen.

The importance of Danshen in traditional medicine has stimulated the development of tissue culture methods for the production of tanshinone, such as callus and cell cultures, and the stimulation of adventitious and hairy roots (Chen et al., 2001; Hu and Alfermann, 1993; Shan et al., 2007; Zhao et al., 1999). Although tanshinone production from *S. miltiorrhiza* has improved (Shi et al., 2007; Wu and Shi, 2008; Zhang et al., 2004; Zhao et al., 2010), to the best of our knowledge, the relative contents of tanshinone in extracts of shoots and roots grown in vitro have not been

compared with that of extracts from plantlets of the same age but derived from seeds. The present study was initiated to answer this question and to examine the antioxidant activities of tissues from in vitro-grown plants and determine the free radical scavenging properties of *S. miltiorrhiza* extracts. Antioxidants can alleviate oxidative damage induced by reactive oxygen species (ROS) (Zhu et al., 2004). In general, most of the important medicinal bioactive secondary metabolites are phenols and flavonoids that inhibit life-threatening degenerative diseases, e.g., cardiovascular disease, cancer, and neurological disorders, which are induced by oxidative stress (Kinsella et al., 1993; Reddy et al., 2012). In plants, high level of oxidative stress results in an increased production of ROS, which have a harmful effect on plant cells. The most important forms of ROS are hydrogen peroxide (H₂O₂), superoxide (O₂⁻), and nitric oxide (NO) (Kumaran and Karunakaran, 2007). Increases in the levels of these ROS can cause plant cell death through oxidation stress and denaturation of macromolecules, such as proteins, DNA, and unsaturated fatty acids (Halliwell and Gutteridge, 2007). Therefore, identifying which plant tissues contain the highest contents of antioxidant compounds might lead to the identification and characterization of novel compounds for medical applications.

In this study, we explored the effects of various cytokinins and an auxin on the induction of adventitious shoots in *S. miltiorrhiza*. Proliferating shoots were then rooted and acclimatized in a greenhouse. The antioxidant characteristics, phenol and flavonoid concentrations, and tanshinone content of in vitro-regenerated (micropropagated) plants in different tissues were compared with that from plants of the same age propagated from seeds.

Materials and Methods

Plant materials and culture conditions. *Salvia miltiorrhiza* plants were obtained from Shilai town, Taian city in Shandong Province, China, and cultivated in a greenhouse belonging to the Qufu Normal University, Shandong Province, China. Healthy leaves, which were selected as explants, were disinfected using 75% (v/v) ethanol for 30 s and then rinsed three times with sterile distilled water. Thereafter, the explants were sterilized using 0.1% (w/v) aqueous HgCl₂ for 7 min followed by four rinses with sterile distilled water. The base medium used here was MS medium with 3% (w/v) sucrose and 0.65% (w/v) agar. Medium pH was adjusted to 5.9 using 0.1 N NaOH or 0.1 N HCl, and the medium was autoclaved at 121 °C for 20 min. All cultures were placed at 25 ± 1 °C under a 12-h photoperiod with 40 μmol·m⁻²·s⁻¹ photosynthetic photon flux density from cool-white fluorescent lamps (Philips 40 W tubes, NVC lighting, Huizhou, China).

Adventitious shoot induction. Sterilized leaf explants were inoculated on MS medium containing various concentrations (0, 0.5,

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1.0, 2.0, or 3.0 mg·L⁻¹ of BA, kinetin (KT), or thidiazuron (TDZ). Thereafter, BA was chosen as the optimum cytokinin for evaluation and tested in a mixture with the auxin NAA at several concentrations (0.1, 0.2, 0.3, or 0.4 mg·L⁻¹) for its ability to induce adventitious shoot formation. While TDZ was filter-sterilized and then added to the sterilized medium, BA, KT, and NAA were added to the MS medium before pH adjustment and the sterilization step. After 6 weeks of culture, the rate of shoot induction, the number of shoots induced per plant, and the lengths of shoots were determined. Thirty explants were employed and each treatment was repeated three times.

Rooting and acclimatization. After adventitious shoot induction, elongated shoots

(2–4 cm) were inoculated on MS medium containing different concentrations of NAA (0, 0.1, 0.2, 0.3, or 0.4 mg·L⁻¹) to induce root formation. After 3 weeks, the rate of root formation, and the length and number of roots were assessed. Plantlets with roots were transferred to 8-cm-diameter pots containing a mixture of vermiculite and soil (1:1, v/v; flower nutrition soil; Deli, Fuzhou, China) and grown in a greenhouse with day/night temperatures of 25/18 °C and 75% to 80% relative humidity. The survival rate of the transplants was calculated at 4 weeks of acclimatization.

Sample preparation, phytochemical compounds extraction, and analysis of total phytochemical compounds. For phytochemical evaluation, shoot and root samples (0.5 g)

were collected from plants derived from adventitious small shoots cultured on optimal rooting media after 10 weeks and from seed-derived *S. miltiorrhiza* plants that had been grown for 10 weeks in soil. The samples were ground in liquid nitrogen and then extracted with 5 mL 80% (v/v) methanol with shaking at 500 rpm for 4 h (García-Pérez et al., 2012). Thereafter, the extracts were centrifuged at 7000 rpm (D-37520; Heraeus-Thermo, Osterode, Germany) for 10 min and the supernatant was employed in phytochemical analysis.

The total phenol content of the extracts was evaluated following the theory of Folin–Ciocalteu (Singleton et al., 1999). Partial extracts (25 µL) were diluted to 2 mL with distilled water and then added to Folin–

Table 1. Effect of different concentrations of plant growth regulators on adventitious shoot induction from the leaf explants of *Salvia miltiorrhiza*.

Treatment	6-benzyladenine (mg·L ⁻¹)	Kinetin (mg·L ⁻¹)	Thidiazuron (mg·L ⁻¹)	Shoot induction (%)	No. shoots per explant	Shoot length (mm)
1	—	—	—	20.0 ± 0.00 h ²	2.7 ± 0.33 g	15.7 ± 0.88 de
2	0.5	—	—	33.3 ± 3.33 fg	4.7 ± 0.88 d	14.3 ± 1.20 ef
3	1.0	—	—	83.3 ± 3.33 a	10.3 ± 0.33 a	24.7 ± 0.33 a
4	2.0	—	—	76.7 ± 3.33 ab	8.0 ± 0.58 b	19.0 ± 1.15 bc
5	3.0	—	—	60.0 ± 0.00 c	4.0 ± 0.00 f	17.0 ± 0.58 cd
6	—	0.5	—	40.0 ± 0.00 ef	3.3 ± 0.67 f	14.0 ± 0.00 ef
7	—	1.0	—	56.7 ± 3.33 c	3.3 ± 0.33 f	15.3 ± 0.88 def
8	—	2.0	—	73.3 ± 3.33 b	6.3 ± 0.88 c	10.7 ± 0.88 g
9	—	3.0	—	30.0 ± 0.00 g	2.3 ± 0.33 h	11.3 ± 0.88 g
10	—	—	0.5	40.0 ± 0.00 ef	4.3 ± 0.88 e	20.3 ± 0.33 b
11	—	—	1.0	73.3 ± 3.33 b	3.3 ± 0.33 f	23.0 ± 1.15 a
12	—	—	2.0	53.3 ± 3.33 cd	3.7 ± 0.33 f	16.3 ± 0.88 de
13	—	—	3.0	46.7 ± 3.33 de	4.0 ± 0.00 f	13.0 ± 0.00 fg

Values represent the mean ± SE of three replications, each with 10 explants.

²Means followed by same letter(s) within a column are not significantly different ($P < 0.05$).

Fig. 1. Micropropagation of *Salvia miltiorrhiza*. (A) Green-colored protuberances from leaf explants; (B) Adventitious shoots induced after 3 weeks; (C) Shoot proliferation and elongation; (D–G) The plantlets rooted from the shoot base; (H) Acclimatized plants in the greenhouse (bars = 1 cm).

Ciocalteu reagent (0.5 mL). The mixture was placed in the dark for 5 min. Then, 1 mL 5% (w/v) Na₂CO₃ was added, the mixture was diluted to 5 mL with distilled water, and then incubated at 25 °C for 1 h. The reaction absorbance was recorded at 750 nm using an ultraviolet-5500 spectrophotometer (Shanghai Metash Instruments, Shanghai, China) and the content of total phenol was expressed as the content of gallic acid equivalents.

Total flavonoid content was evaluated using the aluminum chloride calorimetric method with slight modification (Kim et al., 2003). Samples (1 mL) were diluted to 4 mL with methanol (80%) and added to 0.3 mL 5% (w/v) NaNO₂ solution. The reaction mixture was kept at room temperature (25 °C) for 5 min. After this period, 0.3 mL 10% (w/v) AlCl₃ solution was added; after 6 min, 4 mL NaOH (4%, w/v) was added to the mixture. Finally, the reaction mixture was diluted to 10 mL with distilled water. The absorbance of the reaction mixture was recorded at 510 nm using an ultraviolet-5500 spectrophotometer and the total flavonoid content of each extract was calculated from a standard rutin calibration curve.

Quantification of total tanshinone using the methanol refluxing method. Plant samples (6.0 g) were lyophilized and macerated and then extracted twice using the methanol (100%, 60 mL) reflux extraction method; each extraction was performed for 2 h (Xu and Xiang, 2014). The extracted liquid was added to a 250-mL volumetric flask, brought to volume (250 mL) with methanol (100%), and shaken until fully mixed. A 5-mL aliquot of this mixture was then placed in a volumetric flask (50 mL) and brought to 50 mL with methanol (100%). After attenuation, absorbance was recorded at 269 nm using an ultraviolet-5500 spectrophotometer, and the total tanshinone content was calculated from a standard tanshinone IIA calibration curve (Lan et al., 2012; Li et al., 2012).

Antioxidant ability of plant extracts. Shoots and roots of micropropagated and seed-derived plants were ground in a solution containing phosphate buffer solution (PBS, 50 mM), 20% (v/v) glycerol, DL-dithiothreitol (1 mM), ethylenediaminetetraacetic acid (EDTA-Na₂, 1 mM), and MgSO₄ (1 mM). Briefly, the plant samples (0.5 g) were homogenized to a powder and then extracted with 5 mL of the solution described previously. The resulting suspension was centrifuged at 7000 rpm (10 min) and then at 10,000 rpm (15 min). The supernatant was used for the in vitro trials described next.

Scavenging activity of O₂⁻ radicals. The scavenging activity assay was based on the ability of extracts to inhibit formazan production by bleaching the O₂⁻ radicals generated in nitro blue tetrazolium (NBT) salt with riboflavin and light (Kumaran and Karunakaran, 2007). The extracts (0.1 mL) were added to a reaction mixture (0.1 mg NBT, 100 µM EDTA-Na₂, and 50 mM sodium phosphate buffer containing 20 µg

riboflavin; pH 7.6) and illuminated. After 20 min, the absorbance was recorded at 560 nm using an ultraviolet-5500 spectrophotometer.

Scavenging activity of H₂O₂ radicals. For the H₂O₂ scavenging experiment, extracts (0.1 mL) were added to the reaction mixture [1.2 mL distilled water and 0.2 mL H₂O₂ (100 mM) in 1.5 mL sodium phosphate buffer; pH 7.0]. After 3 min, the absorbance was recorded at 240 nm using an ultraviolet-5500 spectrophotometer against a blank solution that lacked H₂O₂ (Kumaran and Karunakaran, 2007).

Scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. For the DPPH scavenging experiment, sample extracts (40 µL) were first mixed with 1960 µL methanolic solution (0.1 mM) of DPPH; this mixture was incubated for 25 min in the dark before determining its absorbance at 517 nm using an ultraviolet-5500 spectrophotometer (Manivannan et al., 2015).

Scavenging activity of NO radicals. Inhibition of NO generation by plant extracts was estimated using sodium nitroprusside (SNP)-mediated NO production (Manivannan et al., 2015). Nitric oxide is produced spontaneously when SNP reacts with oxygen to produce nitrite ions, which can be estimated using Griess reagent. The reaction started when SNP (10 mM) in PBS was added to 0.1 mL extracts. The mixture was placed at 25 °C for 150 min and 0.5 mL freshly prepared Griess reagent [1% sulfanilic acid, 2% H₃PO₄, and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride] was added to the mixture after this period. Absorbance was determined at 546 nm using an ultraviolet-5500 spectrophotometer.

Free radical scavenging was assayed using ascorbic acid (AA). The rate of radical scavenging (%) was measured using the formula [(A_c-A_s)/A_c] × 100, where A_c is the optical density (OD) value of the control (which lacked the extracts), and A_s is the OD of the extracts or AA.

Statistics. All assays were performed using a stochastic experimental design with three replication treatments, and each exper-

iment was repeated three times to confirm the repeatability of results. The significance of differences between treatments was tested by analysis of variance followed by Duncan's multiple range tests at a 5% significant level, using the SPSS software package (IBM, Armonk, NY).

Results and Discussion

Effect of plant growth regulators (PGRs) on shoot induction. Plant cytokinins and auxins are important regulators in plant differentiation (Frello et al., 2002; Kordi et al., 2013; Peeters et al., 1991). In the present study, we examined the abilities of various PGRs and their concentrations to induce adventitious shoots from leaf explants of field-growing plants. Healthy leaf explants were cultured on MS medium with or without PGRs to induce shoot propagation. Explants on MS medium without PGRs produced only a few shoots. However, explants on MS medium containing a cytokinin formed several adventitious shoots (Table 1). Explants first showed differentiation at 12–14 d of culture on cytokine-supplemented medium and eventually formed green callus (Fig. 1A). After 4 weeks, the regeneration of shoots was recognized by the formation of small leaf-like tissues on the leaf edge that subsequently produced adventitious shoots (Fig. 1B).

The highest rate (83.3%) of shoot induction and largest number of shoots (10.3) were achieved on MS medium containing 1.0 mg·L⁻¹ BA (Table 1). This finding is consistent with previous reports on BA being effective for in vitro proliferation and multiplication of *S. miltiorrhiza* (Xie et al., 2004; Zhao et al., 1999), *Salvia fruticosa* (Arikat et al., 2004), and *Salvia leucantha* (Hosoki and Tahara, 1993). However, an increase in concentration to 2.0 mg·L⁻¹ BA resulted in a decrease in the rate of shoot induction and formation of brown calli on leaf edges. This result could be due to BA metabolism and induction of other endogenous hormones in the plant tissues (Sharma and Wakhlu, 2003). Most adventitious shoots on media with

Table 2. Effect of 6-benzyladenine (BA) and α-naphthalene acetic acid (NAA) combination on adventitious shoot induction of *Salvia miltiorrhiza*.

BA (mg·L ⁻¹)	NAA (mg·L ⁻¹)	Shoot induction (%)	No. shoots per explant	Shoot length (mm)
1.0	0.1	100.0 ± 0.00 a ^z	22.3 ± 0.33 a	19.5 ± 0.24 b
1.0	0.2	100.0 ± 0.00 a	11.0 ± 0.58 c	34.7 ± 0.14 a
1.0	0.3	86.7 ± 3.33 b	15.3 ± 0.33 b	13.0 ± 1.15 c
1.0	0.4	80.0 ± 5.78 b	15.0 ± 0.58 b	16.7 ± 1.67 b

Values represent the mean ± SE of three replications, each with 10 explants.

^zMeans followed by same letter(s) within a column are not significantly different (*P* < 0.05).

Table 3. Effect of α-naphthalene acetic acid (NAA) on root induction of *Salvia miltiorrhiza*.

NAA (mg·L ⁻¹)	Root induction (%)	Root length (mm)	No. roots per explant	Nodular rooting (%)
0.0	23.3 ± 3.33 c ^z	16.3 ± 0.33 d	1.7 ± 0.33 c	10.0 ± 0.00 a
0.1	88.3 ± 3.33 a	38.0 ± 1.15 a	2.2 ± 0.07 b	6.7 ± 3.33 a
0.2	96.7 ± 3.33 a	34.7 ± 0.33 b	5.3 ± 0.05 a	0.0 ± 0.00 b
0.3	43.3 ± 8.82 b	22.3 ± 1.33 c	1.2 ± 0.07 c	0.0 ± 0.00 b
0.4	30.0 ± 5.77 bc	11.7 ± 0.67 e	1.3 ± 0.06 c	0.0 ± 0.00 b

Values represent the mean ± SE of three replications, each with 10 explants.

^zMeans followed by same letter(s) within a column are not significantly different (*P* < 0.05).

different concentrations of TDZ showed hyperhydricity, resulting in their malformation and translucent or glassy appearance, with low lignification and poor regeneration. Hyperhydricity typically occurs under stress conditions during tissue culture but has been reported after application of high concentrations of PGRs such as TDZ (Caboni et al., 1999; Ghimire et al., 2012). Overall, the combination of cytokinin analogs and auxin improved the rate of adventitious shoot induction (Burdyn et al., 2006; Ghimire et al., 2012; Zheng et al., 2009). In the present study, MS media containing BA ($1.0 \text{ mg}\cdot\text{L}^{-1}$) and NAA (0.1 , 0.2 , 0.3 , or $0.4 \text{ mg}\cdot\text{L}^{-1}$) showed a concentration-related increase in the rate of shoot regeneration compared with BA alone (Table 2). The largest number of shoots per explant (22.3) was found on MS medium supplemented with $1.0 \text{ mg}\cdot\text{L}^{-1}$ BA and $0.1 \text{ mg}\cdot\text{L}^{-1}$ NAA (Fig. 1C), whereas the highest rate of shoot induction (100%) was observed on MS medium supplemented with $1.0 \text{ mg}\cdot\text{L}^{-1}$ BA and $0.2 \text{ mg}\cdot\text{L}^{-1}$ NAA [although fewer shoots per explants (11.0) were seen on this medium].

Effect of NAA on rooting and acclimatization. Plant auxins such as indole-3-butyric acid, indole acetic acid, or NAA have been applied to induce root formation in tissue explant cultures (Caboni et al., 1999). In our previous study, the addition of $0.05 \text{ mg}\cdot\text{L}^{-1}$ NAA to MS medium resulted in efficient rooting in *Haworthia turrida* explants (Liu et al., 2017). Here, healthy shoots (2–4 cm in length) were cut from elongated shoots and cultured on MS media with NAA (0 , 0.1 , 0.2 , 0.3 , or $0.4 \text{ mg}\cdot\text{L}^{-1}$) to induce rooting. Roots emerged from the shoot base within 10 d of culture on rooting medium and efficient induction of the root system was observed at three weeks of culture (Fig. 1D and E). The highest rate of rooting was achieved with $0.2 \text{ mg}\cdot\text{L}^{-1}$ NAA, as 96.7% of the explanted shoots showed induced root formation, with an average root length of 34.7 mm and an average root number of 5.3 (Table 3). Well-rooted plantlets (Fig. 1F and G) were moved to pots containing nutritive soil, placed within the greenhouse, and generated true leaves after ≈ 10 d. In general, a 95% survival rate was achieved at 4 weeks within the greenhouse (Fig. 1H).

Assessment of phytochemicals

Determination of total phenol and flavonoid content. Natural phenolic and flavonoid compounds are known to have positive effects on inflammation and cardiovascular disease and to show antioxidant activities (Alothman et al., 2009; Jagtap et al., 2011; Xanthopoulou et al., 2010). The properties of the different phytochemical components of different tissues of *S. miltiorrhiza* are essential to its medicinal properties. Here, we determined the total phenol and flavonoid contents of extracts from shoots and roots of regenerated plants, and shoots and roots of seed-derived plants (Fig. 2). Overall, shoot extracts had higher total flavonoid (Fig. 2A) and

phenol contents (Fig. 2B) than root extracts, regardless of whether they were from plants derived from in vitro culture or from seeds. Similar results have been reported for *Aloe arborescens* and *Psoralea drupacea* (Amoo et al., 2012; Lystvan et al., 2010). The above-ground parts of plants are often adapted to a higher rate of synthesis of secondary metabolites than underground tissues (Bourgaud et al., 2001). The roots of micropropagated plants had higher phenol contents than those of seed-derived plants. The total flavonoid contents were upregulated in the shoots of plant micropropagated in a medium with PGRs, but did not differ significantly in the roots of the two

plant groups (Fig. 2A). The variations in flavonoid and phenolic contents in different tissues could be due to disparities in tissue formation and accumulation of phytochemicals, plant physiology, or inherent hormone levels (Surveswaran et al., 2010). Previous studies reported that different types and contents of PGRs have modified the contents of secondary metabolites in plants (Baskaran et al., 2012; Palacio et al., 2008). Baskaran et al. (2014) reported that PGRs had a significant effect on the synthesis and accumulation of phenolic compounds and flavonoids in in vitro-derived plants of *Coleonema pulchellum*. Nevertheless, our results demonstrate that the contents of

Fig. 2. Phytochemical contents of extracts from micropropagated and seed-derived *Salvia miltiorrhiza* plants. Total flavonoid (A) and total phenol (B) contents present in shoot (IS) and root (IR) extracts of micropropagated plants, and shoot (SS) and root (SR) extracts of seed-derived plants. Different capital letters among bars indicate statistically significant differences at $P \leq 0.05$, according to Duncan's multiple range test.

Fig. 3. Tanshinone content of extracts from micropropagated and seed-derived *Salvia miltiorrhiza* plants. The tanshinone content of shoot (IS) and root (IR) extracts of micropropagated plants, and shoot (SS) and root (SR) extracts of seed-derived plants. Different capital letters among bars indicate statistically significant differences at $P \leq 0.05$, according to Duncan's multiple range test.

phytochemicals in micropropagated and seed-derived plants were similar, and thus that micropropagated plants might be used as a substitute of seed-derived plants when exploited for their medicinal benefits.

Assessment of total tanshinone content in tissue extracts. The level of tanshinone was identified in tissue extracts (Fig. 3). The highest level of tanshinone was $8.77 \mu\text{g}\cdot\text{mg}^{-1}$ fresh weight (FW) in roots of micropropagated plants and $8.52 \mu\text{g}\cdot\text{mg}^{-1}$ FW in roots of seed-derived plants. The lowest level of tanshinone was $3.18 \mu\text{g}\cdot\text{mg}^{-1}$ FW in shoot extracts of seed-derived plants and $3.46 \mu\text{g}\cdot\text{mg}^{-1}$ FW in shoots extracts of

micropropagated plants. The slight increase in tanshinone in micropropagated plants might be due to the plant tissue culture environment or to the effect of endogenous hormones. Previous studies reported that the in vitro environment might increase the production of secondary metabolites by modifying primary metabolism (Close and McArthur, 2002; Gould et al., 2000). In addition, levels of secondary metabolites may be greatly affected by the nutrients and plant hormones used during tissue culture (Baskaran et al., 2012). Higher production of secondary metabolites in micropropagated plants than in natural populations

has also been reported in *Swertia japonica* (Ishimaru et al., 1990) and *Gentiana lutea* (Menković et al., 2000).

Free radical scavenging ability

Scavenging ability of O_2^- radical. Extracts from all tissues inhibited the production of blue formazan by scavenging O_2^- radicals produced by the NBT-riboflavin-light complex. The relative scavenging ability of each tissue extract was compared with that of a control and AA (Fig. 4A). Although all extracts efficiently inhibited O_2^- , roots of micropropagated plants showed the highest scavenging activity (77.48%). Furthermore,

Fig. 4. Free radical scavenging ability of extracts from micropropagated and seed-derived *Salvia miltiorrhiza* plants. Superoxide (A), hydrogen peroxide (B), 2,2-diphenyl-1-picrylhydrazyl radical (C), and nitric oxide (D) scavenging activities of shoot (IS) and root (IR) extracts of micropropagated plants, and shoot (SS) and root (SR) extracts of seed-derived plants and of ascorbic acid (AA). Different capital letters among bars indicate statistically significant differences at $P \leq 0.05$, according to Duncan's multiple range test.

significant differences were found for the relative O_2^- scavenging abilities of shoots between micropropagated plants (71.47%) and seed-derived plants (65.22%). The rate of AA scavenging was 77.23%. Superoxide radicals are greatly detrimental to plant cell organelles and can act as precursors for ROS production (Dewir et al., 2006; Manivannan et al., 2015). In addition, excess production of O_2^- in cells can enhance dismutation, which results in the production of H_2O_2 that further increases oxidative stress (Halliwell and Gutteridge, 2007). Therefore, plant cells require an efficient antioxidant system to scavenge O_2^- .

Scavenging ability of H_2O_2 radicals. The capacity of tissue extracts to suppress H_2O_2 radicals was compared with that of AA (Fig. 4B). The rates of H_2O_2 scavenging ranged from 48.56% to 85.45%. As expected, AA was the most efficient scavenger (85.46%), followed by shoots of micropropagated plants (85.45%) and shoots of seed-derived plants (80.61%). Root extracts showed moderate scavenging activity: 55.59% in micropropagated plants and 48.56% in seed-derived plants. In general, H_2O_2 is nonreactive, but it can cause the generation of other harmful free radicals, such as hydroxyl radicals, and result in plant cell death. Therefore, it is essential for cells to possess an efficient antioxidant system to scavenge H_2O_2 radicals and optimize the cellular environment. Previous reports indicated that phenolic compounds can act as scavengers of H_2O_2 (Halliwell and Gutteridge, 2007). Therefore, the existence of a high level of phenolic compounds might have contributed to the improved H_2O_2 scavenging capacity of micropropagated plants.

Scavenging ability of DPPH radicals. The DPPH assay has been generally used to determine the antioxidant capacity of extracts from in vitro-regenerated plant tissues (Allothman et al., 2009). Here, all extracts exhibited DPPH radical scavenging ability (Fig. 4C). The highest rate of DPPH scavenging was found in shoots of micropropagated plants (84.68%), with a lower rate in shoots of seed-derived plants (75.38%). In root extracts, scavenging rates of 63.78% and 62.50% were found for micropropagated and seed-derived plants, respectively. Because DPPH is a purple-colored molecule that turns yellow after receiving an electron from an antioxidant and can be spectrophotometrically recorded, the level of antioxidants in a mixture containing DPPH can be assessed by its level of discoloration (Surveswaran et al., 2010). Ahmad et al. (2010) reported increased radical scavenging activity in micropropagated shoots of *Piper nigrum*.

Scavenging ability of NO radicals. All extracts tested here showed ability to scavenge NO radicals (Fig. 4D). Extracts from shoots of micropropagated and seed-derived plants showed a similar rate of NO radicals scavenging: 83.48% and 83.54%, respectively. Moreover, NO inhibition in roots from micropropagated and seed-derived plants were 65.54% and 65.30%,

respectively (Fig. 4D). In biological systems, excess production of NO radicals is associated with a range of diseases, such as cancer, chronic inflammatory disorders, and atherosclerosis (Moncada et al., 1991). Cells, therefore, require the ability to reduce excess production of NO radicals to prevent disease.

Conclusions

The present study developed an efficient propagation system for *S. miltiorrhiza*. The highest efficiency of shoot induction was obtained on MS medium with $1.0\text{ mg}\cdot\text{L}^{-1}$ BA and $0.1\text{ mg}\cdot\text{L}^{-1}$ NAA, and MS medium supplemented with $0.2\text{ mg}\cdot\text{L}^{-1}$ NAA was the best for *S. miltiorrhiza* rooting. This study also demonstrated that the tissues of micropropagated *S. miltiorrhiza* contain abundant flavonoid and phenolic compounds and that extracts showed scavenging activities to commonly formed cellular free radicals. The information provided here will be of importance to large-scale micropropagation of *S. miltiorrhiza* for commercial purposes and for germplasm conservation. Moreover, the tanshinone contents and scavenging potential of free radicals in *S. miltiorrhiza* tissue extracts reported here will be of value for therapeutic applications. The difficulty of maintaining high-quality plants from seeds could be circumvented by using plant tissue culture, without affecting the medicinal value of *S. miltiorrhiza*.

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