Effects of Rare Earth Elements on Growth and Determination of Secondary Metabolites under In Vitro Conditions in *Salvia miltiorrhiza*

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Abstract. *Salvia miltiorrhiza*, known as danshen, is one of most valued medicinal plants in China. Although it has been cultivated since ancient times, an optimal culture system needs to be standardized for this important species. Here, we explored the phytochemical properties of *S. miltiorrhiza* with the treatments of rare earth elements (REEs) to develop an optimal tissue culture system. Four-week-old in vitro-grown *S. miltiorrhiza* plantlets were used as explants. The experiment was conducted in a randomized block design on a Murashige and Skoog (MS) medium containing 0.2 mg·L⁻¹ naphthaleneacetic acid (NAA) to induce rooting at four different concentrations (50, 100, 200, and 300 μM) of REEs such as cerium (Ce), lanthanum (La), or praseodymium (Pr), respectively. Compared with all REEs at different concentrations, 100 μM Pr induced greater root length than Ce or La at any concentrations. Concomitantly, 0.38 μg tanshinone IIA/mg dry weight (DW) was observed, which was 54.84% higher than in the control. Similarly, chlorophyll content, antioxidant enzyme activity, and secondary metabolite were enhanced in rooting medium supplemented with 100 μM Pr. Therefore, this study showed that 100 μM Pr is an adequate concentration in the optimal culture system for promoting plant growth as well as enhancing secondary metabolite content in *S. miltiorrhiza*.

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tanshinone I, tanshinone IIA, cryptotanshinone, and dihydrotanshinone I). These compounds are relatively abundant in danshen, and they are largely responsible for its therapeutic effects (Wang and Wu, 2010).

In the Global Burden of Diseases (GBD) overview study, 17.8 million (233.1 per 100,000) people reportedly died of CVD in 2017 globally (Ram et al., 2019). Because of danshen's high medicinal value for treating CVD and increased market demand, over-exploitation of danshen now threatens its wild resource; although the danshen cropping system has been optimized, repeated cropping tends to reduce the content of the medicinal components. Concomitantly, viral infections often reduce the yield under greenhouse cultivation. Therefore, it is highly desirable to develop a standard in vitro growth system to enhance tanshinone production of *S. miltiorrhiza*.

REEs are a set of 17 elements among which 15 have been included in the periodic table as “lanthanides,” plus scandium and yttrium (Aquino et al., 2009). In recent years, REEs have been widely used for medical, industrial, agricultural, and aquaculture applications because of their properties (Hu et al., 2004). Previous studies have reported that REEs accelerated cell growth, enhanced synthesis of secondary metabolites (Wu et al., 2001; Zhang et al., 2013), and provided tolerance against fungal diseases (Liu et al., 2008). Previous studies have also found that the addition of biotic elicitors and heavy metal salts can stimulate the accumulation of tanshinone and increase its yield (Zhao et al., 2010a, 2010b). However, to the best of our knowledge, the effect of REEs on the metabolism of danshen has not been studied. Although many physiological effects of REEs on plants remain to be studied and discovered, we are interested in the effects of REEs on the secondary metabolites of in vitro-cultured plants. Recent studies have proposed that REEs are good elicitors for secondary metabolites accumulation. Further, it has been shown that REEs have no severe deleterious effects on growth at optimal levels. Indeed, cerium (Ce), lanthanum (La), and praseodymium (Pr) reportedly showed positive effects on plant growth and stimulated secondary metabolites accumulations (Chen et al., 2003; Ting et al., 2016; Wu et al., 2001).

Secondary metabolites are generally believed to be a part of plant stress and defense responses (Bai et al., 1995; Peng et al., 2013). Thus, plant defense processes can be elicited by the enzymatic antioxidant system, including superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX) (Bowler et al., 1994). In general, secondary metabolites of most medicinal plants are flavonoids or phenols that can counter some diseases of the human body caused by oxidative stress, such as cardiovascular diseases, cancer, neurological diseases, etc. (Kinsella et al., 1993; Reddy et al., 2012). Superoxide anion (O₂⁻), followed by hydrogen peroxide (H₂O₂) and hydroxy

Salvia miltiorrhiza, the Lamiaceae family, is a perennial herb that bears large zygomorphous flowers and is known as danshen in China. Medicinal components from danshen are widely used to treat cardiovascular diseases (CVD), particularly angina pectoris and myocardial infarction (Ryu et al., 1996). Similarly, it has been applied for liver dysfunction, renal deficiency, and diabetic vascular complication (Shan et al., 2007). A previous study found that methanolic extracts obtained from danshen had a significant cytotoxicity against cultured human tumor cell lines (Ryu et al., 1996). Dried roots of *S. miltiorrhiza* have been found to contain relatively large amounts of the important secondary metabolites (tanshinones and diterpenoid pigments, such as

(·OH), are by-products of metabolic reaction, which can be scavenged by enzymatic reactions to maintain redox homeostasis (Kumaran and Karunakaran, 2007). Excess amounts of these oxidative free radicals could denature biological macromolecules such as proteins, DNA, and unsaturated fatty acids, and ultimately lead to plant cell death (Halliwell and Gutteridge, 2007). Enzymatic and nonenzymatic antioxidants can detoxify excess reactive oxygen species (ROS) in plant cells (Angkhana et al., 2019). Therefore, the antioxidant content in plants is also a standard strategy to estimate the value of medicinal plants.

Previously, we have studied the effect of plant growth regulators on seed-derived *S. miltiorrhiza* under in vitro conditions (Liu et al., 2018). However, to standardize the optimal in vitro system for *S. miltiorrhiza* culture, different plant sources (in vitro-grown adventitious shoots) were used here and treated with REEs (Ce, La, and Pr) in this study. We aimed to increase an understanding of the effects of REEs as efficient elicitors to improve growth and secondary metabolite biosynthesis in medicinal plants.

Materials and Methods

Plant materials and culture conditions. *S. miltiorrhiza* were collected in the town of Shilai, Xintai county, Shandong Province, China. Surface sterilized plants were cultured in vitro on Murashige and Skoog (MS) basal medium with 3% (w/v) sucrose and 0.75% (w/v) agar containing 1.0 mg·L⁻¹ 6-benzyl-aminopurine (6-BA) and 0.1 mg·L⁻¹ NAA. Cultures were maintained under 40 μmol·m⁻²·s⁻¹ light irradiance and a 12-h photoperiod at 25 ± 1 °C. Four-week-old well-grown shoots were inoculated for rooting on an MS medium containing 0.2 mg·L⁻¹ NAA with different concentrations (50, 100, 200, and 300 μM) of REEs, including Ce, Ce(NO₃)₃; La, La(NO₃)₃; or Pr, Pr(NO₃)₃. Growth and biochemical analysis were carried out after roots were well developed (four weeks).

Assessment of root length and chlorophyll content. The roots' lengths were measured with a Vernier caliper. Chlorophyll content was calculated with an ultraviolet-5500 spectrophotometer (Shanghai Metash Instru-

ments, Shanghai, China) (Qiu et al., 2016). Briefly, in vitro leaves (1-mm segments, 50 mg) were soaked with 2 mL of dimethyl sulfoxide (DMSO) and boiled at 65 °C until the chlorophyll extraction was completed. After cooling, total volume was brought to 10 mL with 80% acetone. Optical density (OD) was recorded at 663.6 and 646.6 nm to determine total chlorophyll concentration by this formula: $C_{chl} = 7.35A_{663.6} + 17.58A_{646.6}$.

Enzyme extraction and activity assessment. To estimate antioxidant enzyme activity, 100 mg of leaves were homogenized in a 1 mL phosphate buffer (50 mM, pH 7.0). Supernatants were centrifuged at 10,000 rpm (D-37520; Heraeus Thermo, Osterode, Germany) for 20 min at 4 °C and used for determining antioxidant enzyme activity. Finally, the antioxidant enzymes activity was calculated based on total protein (by the Bradford method).

Superoxide dismutase (SOD) activity was measured by the change in absorbance at 560 nm during incubation of the extracts (100 μL) at 25 °C with 75 μM nitroblue tetrazolium (NBT), 10 μM ethylene diamine tetraacetic acid (EDTA), 2.0 μM riboflavin in 50 mM phosphate buffer (pH 7.8) in a total volume of 3 mL. SOD activity was determined by the formula $(A_{ck} - A_e)/(A_{ck} \times 50\% \times W)$, where A_{ck} is the OD value of the control, A_e is the OD of extracts, and W is the sample protein content (Siddiqui et al., 2012). CAT activity was assessed by the change in absorbance at 240 nm during incubation of the extracts (100 μL) at 25 °C with 10 mM H₂O₂ in a 50 mM phosphate buffer (pH 7.0) in a total volume of 3 mL (Wang et al., 2004b). POD activity was detected at 460 nm with 18 mM guaiacol and 5 mM H₂O₂ in a 50 mM sodium acetate buffer (pH 5.4) in a total volume of 3 mL of enzyme extract (Manivannan et al., 2015a). APX activity was measured in a reaction mixture containing a 50 mM potassium phosphate buffer (pH 7.0), 5 mM ascorbic acid, 0.1 mM H₂O₂, and 100 μL enzyme extract. The reaction was induced by addition of H₂O₂, and the absorbance was recorded at 290 nm for 3 min at 30 s intervals. One enzyme unit was defined as a 0.1 change in absorbance in 30 s (Nakano and Asada, 1981).

Estimation of ROS scavenging activity. In vitro roots samples (100 mg) were lyophilized and extracted overnight with 5 mL of

80% (v/v) methanol at 150 rpm in a rotating shaker. The resulting homogenates were centrifuged at 10,000 rpm for 10 min, and the supernatants were used for the in vitro assays (Manivannan et al., 2015b).

Superoxide (O₂⁻) scavenging activity of the extract was determined by bleaching the superoxide radical produced by nitro-blue tetrazolium salt with riboflavin and light. Extraction (0.1 mL) was added to the 2 mL reaction mixture [0.1 mg NBT, 12 mM EDTA, and 20 μg of riboflavin sodium phosphate in a 50 mM buffer (pH 7.6)], and the light was radiated. Absorbance was measured at 590 nm after 90 s (Kumaran and Karunakaran, 2007).

For the H₂O₂ scavenging experiment, 0.6 mL H₂O₂ (2 mM) was mixed with the extract (0.1 mL) and 0.8 mL distilled water, then incubated for 10 min. Absorbance was measured at 240 nm according to the method described by Kumaran and Karunakaran (2007).

The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging ability was analyzed by mixing sample extracts (40 μL) with a 0.1-mM methanolic solution of DPPH (total 2 mL) and allowed to stand for 25 min under dark conditions. Absorbance of the sample was measured at 517 nm (Manivannan et al., 2015b).

Radical scavenging percentages for O₂⁻, H₂O₂, and DPPH were calculated using the formula $[(Ac - As)/Ac] \times 100$, where Ac is the absorbance value of the control (i.e., reaction mixture without extract) and As is the OD value of the extract.

For the ·OH scavenging experiment, the reaction mixture (4 mL) contained 1 mL of FeSO₄ (4 mM), 1 mL of H₂O₂ (4 mM), 1 mL of sodium salicylic acid (4 mM), and 1 mL of sample or water, as reference. Absorbance at 510 nm was measured after incubation for 1 h at 37 °C. The percentage of ·OH scavenging was calculated as: $[1 - (A_1 - A_2)/A_0] \times 100$, where A_1 is the absorbance of the sample; A_0 is the absorbance of the reaction without any sample; and A_2 is the absorbance of the reagent blank without sodium salicylic acid addition (Smironoff and Cumbes, 1989).

Extraction and quantitation of total phytochemical compounds. Dried samples were ground in a 1 mL methanol solution, followed by extraction by ultrasonication for 30 min and centrifugation at 5000 rpm

Table 1. Effect of Ce(NO₃)₃, La(NO₃)₃, and Pr(NO₃)₃ on root growth and chlorophyll content of *S. miltiorrhiza*.

Treatment	Ce ³⁺ (μM·L ⁻¹)	La ³⁺ (μM·L ⁻¹)	Pr ³⁺ (μM·L ⁻¹)	Root length (cm)	Chlorophyll content (mg·g ⁻¹ fresh wt)
1	—	—	—	4.40 ± 0.16 h ^z	1.61 ± 0.02 e ^z
2	50	—	—	6.38 ± 0.07 f	1.85 ± 0.02 d
3	100	—	—	7.77 ± 0.01 c	3.08 ± 0.04 b
4	200	—	—	5.33 ± 0.12 g	2.40 ± 0.04 c
5	300	—	—	2.45 ± 0.08 j	1.63 ± 0.02 e
6	—	50	—	7.39 ± 0.15 d	2.37 ± 0.06 c
7	—	100	—	6.20 ± 0.10 f	2.39 ± 0.09 c
8	—	200	—	6.08 ± 0.09 f	1.73 ± 0.05 de
9	—	300	—	3.58 ± 0.12 i	1.64 ± 0.09 e
10	—	—	50	8.26 ± 0.12 b	2.27 ± 0.04 c
11	—	—	100	8.85 ± 0.15 a	3.80 ± 0.02 a
12	—	—	200	6.73 ± 0.09 e	3.11 ± 0.08 b
13	—	—	300	1.77 ± 0.15 k	1.75 ± 0.07 de

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

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

Fig. 1. In vitro effect of REEs on root growth of *S. miltiorrhiza*. (A) Inhibition of root growth. (B) Root in blank treatment. (C) Promotion of root growth. (D) Comparison of root growth of *S. miltiorrhiza* (bar = 1 cm). Roots (a-e) were cultured for 4 weeks from adventitious buds: a, 300 μM $\text{Pr}(\text{NO}_3)_3$; b, 300 μM $\text{Ce}(\text{NO}_3)_3$; c, control; d, 200 μM $\text{Ce}(\text{NO}_3)_3$; e, 100 μM $\text{Pr}(\text{NO}_3)_3$.

(D-37520; Heraeus Thermo) for 10 min; the supernatants were employed in phytochemical analysis.

The Folin-Ciocalteu theory was used to evaluate the total phenolic content of extracts (Singleton et al., 1999). Some extracts (100 μL) were diluted with 2 mL of distilled water and then added to Folin-ciocalteu reagent (1 mL). The reaction mixture was placed in the dark for 5 min and then mixed with 3 mL of 2% (w/v) Na_2CO_3 and diluted to 10 mL using distilled water. After incubation at 25 $^\circ\text{C}$ for 1 h, the reaction mixture absorbance was recorded at 760 nm using an ultraviolet-5500 spectrophotometer. Finally, the equivalent content of gallic acid was used as the content of total phenol.

Total flavonoid content was determined by aluminum chloride calorimetric analysis (Kim et al., 2003). Samples (100 μL) were mixed with methanol (80%) to 4 mL, and a 0.3 mL 5% (w/v) NaNO_2 solution. The reaction mixture was placed at room temperature (25 $^\circ\text{C}$) for 5 min and added with 0.3 mL of

10% (w/v) AlCl_3 . After 6 min, 4 mL of 4% (w/v) NaOH was added. Finally, the reaction mixture was brought to a final volume of 10 mL with distilled water. The absorbance of the reaction mixture was recorded at 510 nm with a ultraviolet-5500 spectrophotometer, and total flavonoid content of each extract was calculated by a standard calibration curve.

Total tanshinone content of the extracts was evaluated following quantitative analysis of multicomponents by single-marker (Li et al., 2012). We selected standards tanshinone IIA to represent total tanshinone content. Samples (100 μL) were added to 3 mL ethanol solution, and absorbance was recorded at 269 nm using an ultraviolet-5500 spectrophotometer; total tanshinone content was calculated using a standard tanshinone IIA calibration curve (Lan et al., 2012; Li et al., 2012).

Statistical analysis. All experiments were in a completely randomized design with three replications. Additionally, each experiment

Fig. 2. Effect of $\text{Ce}(\text{NO}_3)_3$, $\text{La}(\text{NO}_3)_3$, and $\text{Pr}(\text{NO}_3)_3$ on antioxidant enzyme activities of plant tissue cultures. The activity of superoxide dismutase (A); catalase (B); peroxidase (C); and ascorbate peroxidase (D) estimated in the plant tissue culture. Data are means $\pm$ SE from three replicates. Different letters on the bars corresponding to the different treatments indicate statistically significant difference at $P < 0.05$ by Duncan's multiple range test.

Fig. 3. Effect of $Ce(NO_3)_3$, $La(NO_3)_3$, and $Pr(NO_3)_3$ treatments on free radical scavenging activities potentials of danshen plant tissue cultures. Superoxide radical scavenging potential (A); Hydrogen peroxide scavenging percentage (B); Hydroxyl radical scavenging capacity (C); DPPH radical scavenging potential (D). Data are the means $\pm$ SE from three replicates. Different letters on bars of the different treatments indicate statistically significant difference at $P < 0.05$ by Duncan's multiple range test.

was repeated three times to verify the reproducibility of the results. The significance test of the differences among treatments was conducted by analysis of variance, and Duncan's multiple range test was performed at the 5% significance level by the SPSS.19 software package to separate significantly different means (IBM, Armonk, NY).

Results

Effects of REEs on root length and chlorophyll content. The highest root length (8.85 cm) of *S. miltiorrhiza* was observed at 100 μ M Pr (Table 1). All REEs induced more roots than those in the control at 100 μ M. In other words, both 200 μ M and 300 μ M reduced root elongation relative to 100 μ M. Especially, root growth was inhibited at 300 μ M in all treatments (Fig. 1, Table 1). Promotion of root growth by low concentration and inhibition at high concentration was consistently observed in all experiments. Further, we found that increased chlorophyll content

consistently correlated with greater root length (Table 1).

Effect of REEs on SOD, CAT, POD, and APX activities. As shown in Fig. 2, the highest SOD activity was observed at 100 μ M $Pr(NO_3)_3$ (35.80 U/mg protein), which was $\approx$ 34.51% higher than SOD activity in controls. However, SOD activity was significantly inhibited by 200 μ M $La(NO_3)_3$; and when the concentration was up to 300 μ M, all REE treatments inhibited SOD activity (Fig. 2A). In turn, Ce promoted CAT activity at 50 μ M, but further increase in Ce concentration decreased it. On the other hand, CAT was stimulated more at 100 μ M Pr, which was followed by La. Especially, about an 80.48% increase in CAT activity was observed under 100 μ M Pr over the control level (Fig. 2B). The POD activity was not much variation in the range between 50 to 200 μ M concentration for Ce, Pr, or La. Although POD activity was reduced at 300 μ M, no significant difference was detected between REEs at this high concentra-

tion. And 50 μ M La increased the APX activity to a similar extent relative to other REEs, but an unexpected decrease in APX activity was observed at 100 μ M (Fig. 2D).

Effect of chemical elicitors on the free radical scavenging potential. Treatment with REEs treatments significantly enhanced the free radical scavenging potential of the danshen root extracts in comparison with the control (Fig. 3). The best O_2^- scavenging potential of an extract (85.58%) was recorded in the 100 μ M Pr treatment, followed by La (Fig. 3A). However, H_2O_2 was higher at 100 μ M Pr than at any other concentration, and no significant difference was observed between REEs treatments (Fig. 3B). Free radical scavenging activity of danshen extracts elicited by treatment with 100 μ M Pr resulted in the best $\cdot OH$ scavenging potential of 92.99%, followed by the 50 μ M $La(NO_3)_3$ treatment (Fig. 3C). When the concentration of La went to 50 μ M, the $\cdot OH$ scavenging activity was inhibited. DPPH activity was increased at 50 μ M in all REEs treatments.

Fig. 4. Effect of $\text{Ce}(\text{NO}_3)_3$, $\text{La}(\text{NO}_3)_3$, and $\text{Pr}(\text{NO}_3)_3$ treatments on the secondary metabolites of *S. miltiorrhiza*. Total flavonoid content (A); total phenol content (B); and total tanshinone content (C) of plant tissue cultures. Data are the mean $\pm$ SE from three replicates. Different letters (a, b, c) on bars of the different treatments indicate statistically significant difference at $P < 0.05$ by Duncan's multiple range test.

Optimal DPPH scavenging percent (83.71%) was observed in the 50 μM Ce treatment, followed by the 100 μM $\text{Pr}(\text{NO}_3)_3$ treatment (Fig. 3D). Overall, low concentrations (50 and 100 μM) improved the scavenging ability of oxidized free radicals, whereas high concentrations inhibited the ability of the scavenging enzyme activities.

Effect of REEs on the accumulation of bioactive compounds. Rare earth elements had significant effects on the synthesis of bioactive compounds, such as total flavonoids, total phenols, and total tanshinone (Fig. 4). Like the antioxidant enzyme activities, the highest concentration of REEs (300 μM) significantly reduced the production of the total flavonoids. The greatest content of total flavonoids was registered in the danshen extracts treated with 100 μM Ce and Pr (Fig. 4A). Similarly, the synthesis of total phenols was significantly enhanced by the treatment with 100 μM REEs. The most content of total phenol was recorded in the danshen extracts treated with the 100 μM Pr, namely 62.69 μg gallic acid·mg⁻¹, i.e., 73.48% better than total phenol content mea-

sured in the controls (Fig. 4B). Similarly, accumulation of total tanshinone was triggered by the increasing concentrations of REEs. A decrease in tanshinone content at higher concentrations (200 and 300 μM) was correlated with a decrease in total flavonoids and total phenol contents. Most importantly, the improvement of tanshinone content was achieved at 100 μM Pr, which caused a 54.84% increase more than control content (Fig. 4C). Similarly, 100 μM Ce and La resulted in a 41.21% and 51.89% increase in tanshinone content relative to control levels, respectively.

Discussion

Plant-derived secondary metabolites are important medicinal resources. Danshen extracts reportedly show important secondary metabolites with pharmaceutical activities (Ryu et al., 1996). However, increasing market demand and difficulties in cultivation of danshen pose a challenge that increases content of tanshinones, which are important active medicinal compounds (Shan et al.,

2007). Plant tissue culture is one of the techniques that could be used for year-round production of danshen. Furthermore, micropropagation might improve the medical properties and tanshinone content of in vitro cultures (Liu et al., 2018). Therefore, the present study was conducted to promote the phytochemical properties of in vitro-grown danshen. Additionally, the effects of REEs, such as $\text{Ce}(\text{NO}_3)_3$, $\text{La}(\text{NO}_3)_3$, and $\text{Pr}(\text{NO}_3)_3$ as supplements to induce medicinal compound accumulation were also explored. The induction of adventitious buds and roots was achieved using leaf explants cultured with a combination of 1.0 mg·L⁻¹ BA, 0.1 mg·L⁻¹ NAA, and 0.2 mg·L⁻¹ NAA in the growth medium (Liu et al., 2018). At low concentrations, especially at 100 μM Pr, three REEs could improve root elongation and chlorophyll content (Figs. 1 and 2). Similar results were observed in studies that reported that the appropriate concentration of REEs improved the root elongation of radish, tomato, and peach (Philippe et al., 2014; Song et al., 2003). The enhancement of photosynthetic rate was observed in spinach with

treatments of REEs (Brown et al., 1990; Hong et al., 2002). And the inhibition of root elongation at high REE concentrations in danshen correlated with the decreased root growth in Chinese cabbage at high Pr concentration (Tang and Tong, 1988).

To further explore the effects of REE on the growth of danshen, we measured the antioxidant enzymes activities including SOD, CAT, POD, and GPX. As Fig. 2 shows, the different treatments distinctly affected the activities of antioxidant enzymes. In particular, SOD, CAT, and APX activities were significantly increased under Pr at 100 μ M. Our results were similar to those in a study reporting that low concentrations of REEs had a positive effects on plant growth and simultaneously increase yield, metabolite production, and quality; whereas excessive concentrations of REEs inhibited plant growth by negatively altering photosynthesis and antioxidant enzyme activity (Chen et al., 2015). According to Feng et al. and Wu et al. (Feng et al., 1999; Wu et al., 2001), the induction of ROS could increase plasma membrane permeability to a proper concentration in long-term REEs-treated cells than in control cells, which may lead to the enhancement of nutrient uptaking and promotion of plant growth (Diatloff et al., 2008; Hong et al., 1999, 2000; Huang et al., 2012; Wu et al., 2001). Oxidative stress induced by the elicitors could stimulate antioxidant enzymes (such as SOD, CAT, POD, and GPX) to protect the plant cell from the harm of free radicals. During the process of redox homeostasis, the primary reaction is catalyzed by SOD, which provides the first line of defense against the toxic effects of ROS by catalyzing the dismutation of O_2^- into H_2O_2 and O_2 ; subsequently H_2O_2 is catalyzed into H_2O and O_2 by CAT or peroxidases such as POD or APX (Ali et al., 2006).

According to previous reports, exogenous application of chemical elicitors, such REEs, might stimulate plant secondary metabolism to improve the medicinal value of plants by interfering with their signaling process (Chen et al., 2003; Ting et al., 2016; Wu et al., 2001). We speculated that the accumulation of endogenous ROS such as O_2^- , H_2O_2 , $\cdot OH$, and DPPH under REEs treatment might induce secondary metabolites to resist oxidative stress (Kumaran and Karunakaran, 2007). The induction of ROS production upon exogenous application of REEs has been evidenced in cell suspension cultures of *Vicia faba* (Wang et al., 2012). In addition, REEs might promote the synthesis of flavonoids through enhanced PAL activities (Liu et al., 2008). Although the effect of the elicitor molecules varied among plant species (Wyttenbach et al., 1998), $Pr(NO_3)_3$ significantly enhanced the production of total flavonoids and total phenols in danshen. Like our study, lower concentration of REEs reportedly increased the production of secondary metabolites, whereas high concentrations had negative effects in *Tetrastigma hemsleyanum*, *Arnebia euchroma*, and *Saussurea medusa* (Ge et al., 2006; Wu et al., 2001; Yuan et al., 2002). Flavonoids and phenols are nonenzymatic antioxidants and aromatic sub-

stitutions involved in various processes such as pigmentation, root nodule formation, and cell signaling pathways. Higher concentration of metabolite contents, which was observed in the plants with the treatments of REEs, may associate with increasing expression of biosynthetic genes (Wang et al., 2004a). Therefore, further study is needed explore biosynthetic pathway(s) involving REEs in the production of tanshinone in danshen.

Overall, our results showed that the highest phytochemical content, antioxidant enzyme activity (SOD, CAT, and APX) and oxidation radical scavenging activity (O_2^- , H_2O_2 , and $\cdot OH$) in danshen were caused by 100 μ M $Pr(NO_3)_3$.

Conclusion

Our study clearly demonstrated the effects of $Ce(NO_3)_3$, $La(NO_3)_3$, and $Pr(NO_3)_3$ on danshen root elongation and photosynthetic efficiency. Highest enhancement effects on in vitro danshen cultures were achieved at 100 μ M $Pr(NO_3)_3$; this concentration promoted growth of in vitro danshen plants, and increased antioxidant activities and synthesis of bioactive compounds. Further studies are needed to elucidate the regulatory mechanism of $Ce(NO_3)_3$, $La(NO_3)_3$, and $Pr(NO_3)_3$ underlying the promotion of plant growth and the accumulation of secondary metabolites of pharmaceutical interest.

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