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Effects of $LaCl_3$ on photosynthesis and the accumulation of tanshinones and salvianolic acids in *Salvia miltiorrhiza* seedlings

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Abstract: The effects of $LaCl_3$ on the growth, photosynthetic gas-exchange characteristics, chlorophyll fluorescence, and the accumulation of tanshinones and salvianolic acids in *Salvia miltiorrhiza* seedlings were investigated. The results showed that the increase in photosynthesis induced by $LaCl_3$ might be attributed to the enhanced stomatal conductance of the leaves and the increased level of the photochemical efficiency of PS II. The accumulation of tanshinone IIA and cryptotanshinone was markedly increased with the application of $LaCl_3$ at 20 and 60 mg/L, while tanshinone I was only slightly increased. The content of salvianolic acid B was, however, decreased with the treatment of $LaCl_3$ at 200 mg/L.

Keywords: *Salvia miltiorrhiza*; $LaCl_3$; photosynthesis; chlorophyll fluorescence; tanshinone; salvianolic acid; rare earths

Traditional Chinese medicine (TCM) plays an important role in maintaining people's health[1]. The roots and rhizomes of *Salvia miltiorrhiza* (referred to in TCM as "Danshen"), a perennial herbaceous plant, have been widely used for the treatment of cardiovascular and cerebrovascular diseases in China, Japan, Australia, America and other European countries[2]. Two constituent groups of compounds, namely lipophilic tanshinones such as tanshinone I, tanshinone IIA, and cryptotanshinone, and hydrophilic phenolic acids such as salvianolic acid B and rosmarinic acid, have been recognized as the major bioactive components[3]. The ever-increasing demand for Danshen in the international market has stimulated the improvement of cultivation practices of *S. miltiorrhiza*.

Rare earth elements (REEs), which comprise elements in the lanthanide series from lanthanum (La) to lutetium (Lu), and also include scandium (Sc) and yttrium (Y), have been widely used in agriculture in China for over 30 years. The amount of agronomic land fertilized with REEs may range from 3.0×10^{11} to 2.7×10^{16} m^2 per year, which may be associated with an increase in dry mass of plants in the range of 8% to 25%. Many studies have been conducted on the physiological effects related to the application of REEs[4]. Application of REEs has been shown to increase photosynthesis and chlorophyll content and promote N metabolism[5–11]. There is, however, very little systematic research on the effect of REEs on the growth and accumulation of secondary metabolites in medicinal plants. It is therefore important to determine how REEs affect the physiology of medicinal plants, in order to ascertain whether the benefits observed in agronomic species may be transferred to the cultivation of medicinal plants.

The technology of chlorophyll fluorescence measurement has been used to research and detect the effect of environmental factors on photosynthesis, which has been considered as a new and sensitive diagnostic technique *in vivo*. In this study, the effects of La^{3+}, a representative REE, on photosynthetic gas-exchange characteristics, chlorophyll fluorescence and the contents of tanshinones and salvianolic acids in *S. miltiorrhiza* seedlings were investigated. This study was carried out in order to gain further understanding of the mechanism of REEs on the enhancement of photosynthesis efficiency, and to provide a technological guide for the application of REEs in the cultivation of medicinal plants.

1 Materials and methods

1.1 Plant culture and treatment

Seeds of *S. miltiorrhiza* Bunge (Lamiaceae) obtained from Shanxi Province, China, were sown in containers filled with a mixture of garden soil and river sand (1:1, *v/v*) and then incubated at 25±1 °C. After emergence of the third leaf, uniform seedlings were transplanted into plastic pots (10 cm

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diameter, one plant per pot) filled with the above mentioned soil mixture and grew at 28/22 °C (day/night) under a 14 h photoperiod (280 μmol/(m^2·s)).

The seedlings were used for experimentation when the sixth true leaf was developed. The $LaCl_3$ solutions, at concentrations of 20, 60 and 200 mg/L, were sprayed as a fine mist evenly on the leaves until large droplets formed. An equivalent amount of distilled water was applied as control. There were four treatments with 10 replicates for each treatment. Gas-exchange parameters and chlorophyll fluorescence of plants were measured at 9:00 am to 11:30 am daily, for 7 d following treatment. Plant growth and the accumulation of tanshinones and salvianolic acids were determined 30 d after treatment.

1.2 Gas-exchange measurements

The net photosynthetic rate (P_n), stomatal conductance (G_s), intercellular CO_2 concentration (C_i) and transpiration rate (T_r) were monitored with a portable photosynthesis system (Li-6400, Li-Cor, Lincoln, USA) using the third fully expanded leaf (from the apex). Measurements were conducted at a light intensity of 280 μmol/(m^2·s), leaf temperature of 25 °C, constant CO_2 of 380 μmol/mol.

1.3 Chlorophyll fluorescence

Chlorophyll fluorescence was recorded with a modulated chlorophyll fluorometer (Opti-Science, USA) on dark-adapted (for 30 min) leaves. Minimum fluorescence (F_o) was determined by illuminating the leaf with a dim red light modulated at 0.6 kHz. Maximum fluorescence of dark-adapted leaf (F_m) was obtained during a subsequent saturating light pulse (6 000 μmol/(m^2·s) for 0.7 s). The leaf was then continuously illuminated with actinic light at an intensity of 280 μmol/(m^2·s). The steady-state fluorescence (F_s) was then recorded, and a second saturating pulse of white light (6000 μmol/(m^2·s) for 0.7 s) was imposed to determine the maximum fluorescence level in the light-adapted state (F_m'). The actinic light was turned off and the minimal fluorescence level in the light-adapted state (F_o') was obtained by illuminating the leaf with far-red light for 3 s. Maximum quantum yield of PSII (F_v/F_m) and the actual efficiency of PSII (ΦPSII) were calculated as follows[12]: $F_v/F_m=(F_m-F_o)/F_m$, $\Phi\text{PSII}=(F_m'-F_s)/F_m'$. The coefficient of photochemical quenching (qP)[13] and the coefficient of non-photochemical quenching of chlorophyll (NPQ) were calculated as follows[14]: $qP=(F_m'-F_s)/(F_m'-F_o')$, $\text{NPQ}=F_m/F_m'-1$. It should be noted that the gas-exchange and fluorescence measurements were taken on the same position of the leaf.

1.4 Plant growth

Plant growth was assessed by fresh mass (FW) and dry mass (DW) of shoots and roots. At harvest (30 d after treatment), plants were washed with distilled water prior to measurement of FW. The DW was determined after drying at 75 °C for 72 h until a constant mass was reached.

1.5 Determination of tanshinones and salvianolic acids

Quantitative analysis of tanshinones and salvianolic acids in 30-day-old *S. miltiorrhiza* seedlings were performed according to the reported procedure[1]. Briefly, the dried powdered roots of *S. miltiorrhiza* (500 mg) were extracted with 75% methanol (50 ml), and sonicated (300 W, 25 kHz) for 30 min. The extract was filtered through a 0.45 μm membrane filter and 1 μl was injected for each UPLC analysis. Tanshinones (tanshinone I, tanshinone IIA and cryptotanshinone) and salvianolic acids (salvianolic acid B and rosmarinic acid) were separated on a Waters Acquity UPLC BEH C_{18} (2.1 mm×50 mm, 1.7 μm) column. The mobile phase consisted of mobile phase A (1% aqueous formic acid) and B (acetonitrile), using a gradient of 2%–4% B at 0–2.2 min, 4%–9% B at 2.2–2.3 min, 9%–18% B at 2.3–10.0 min, 42%–54% B at 10.2–15.0 min, 54%–80% B at 15.0–16.0 min, and 80% B at 16.0–17.0 min. The flow rate was 0.6 ml/min, the detection wavelength was set at 280 nm, and temperature of column component was maintained at 60 °C.

1.6 Statistical analysis

Significant differences were determined by one-way analysis of variance (ANOVA) using SPSS 13.0 software. Differences were considered significant at $p<0.05$.

2 Results and discussion

2.1 Plant growth

The effect of $LaCl_3$ treatment on the growth of *S. miltiorrhiza* seedlings is shown in Fig. 1. Treatment with 20 mg/L $LaCl_3$ significantly ($p<0.05$) improved the fresh mass of shoots by 17.4% in comparison to the control, while treatment with 60 and 200 mg/L $LaCl_3$ showed no significant difference (Fig. 1(a)). The fresh mass of the roots was not affected by the 20 mg/L or 60 mg/L treatments, whereas a non-significant decrease (16.7%) was observed with the treatment of 200 mg/L $LaCl_3$ (Fig. 1(b)). The dry mass of shoots and roots showed no significant ($p>0.05$) differences with the treatment of $LaCl_3$ (Fig. 1(c), (d)).

2.2 Photosynthetic gas exchange

The effect of $LaCl_3$ treatment on photosynthetic gas-exchange parameters of *S. miltiorrhiza* seedlings is shown in Fig. 2. P_n was increased by the treatment of $LaCl_3$ at 20 and 60 mg/L, and reached a maximum on days 5 and 4, respectively. These were estimated to be 22.9% and 24.2% higher than the control, respectively. P_n, however, was not influenced by the treatment of $LaCl_3$ at 200 mg/L (Fig. 2(a)). G_s and T_r both showed similar trends to P_n (Fig. 2(b), (c)). In contrast, treatment of 200 mg/L $LaCl_3$ significantly ($p<0.05$) increased C_i by 19.8% on day 4 (Fig. 2(d)).

An increase in photosynthesis can be attributed to many partial photosynthetic processes including enhancing photochemical activities, gas exchange and CO_2 fixation[15]. In this

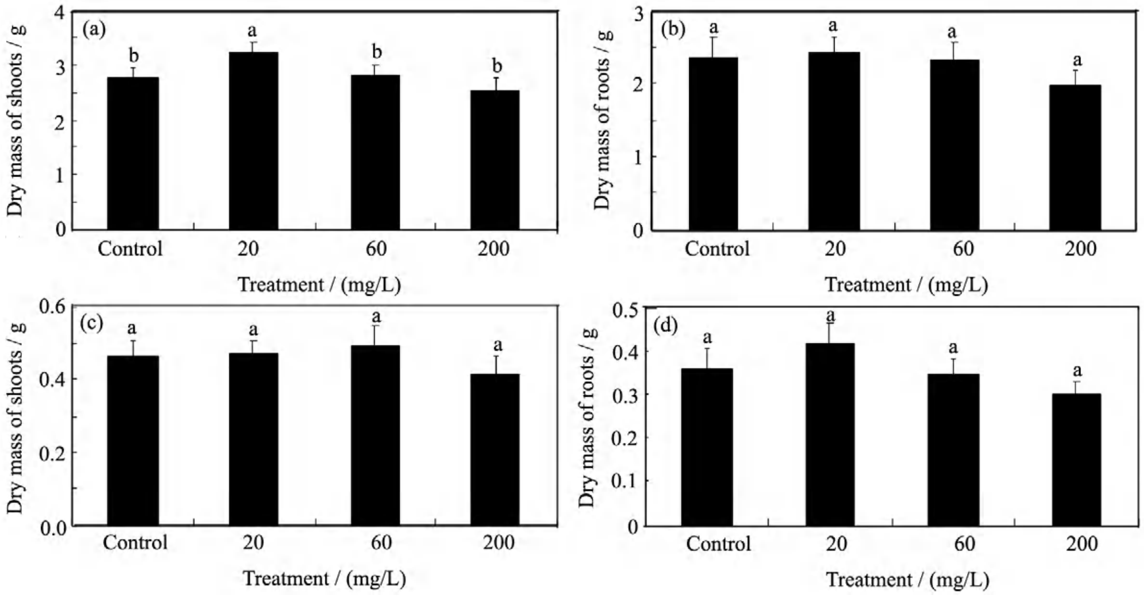

Fig. 1 Effect of $LaCl_3$ treatments on fresh mass of shoots (a), fresh mass of roots (b), dry mass of shoots (c), and dry mass of roots (d) of *S. miltiorrhiza* seedlings (Bars represent the mean ± SE (n=10), different letters indicate significant differences at p<0.05 when compared with the control according to the Duncan's multiple range test)

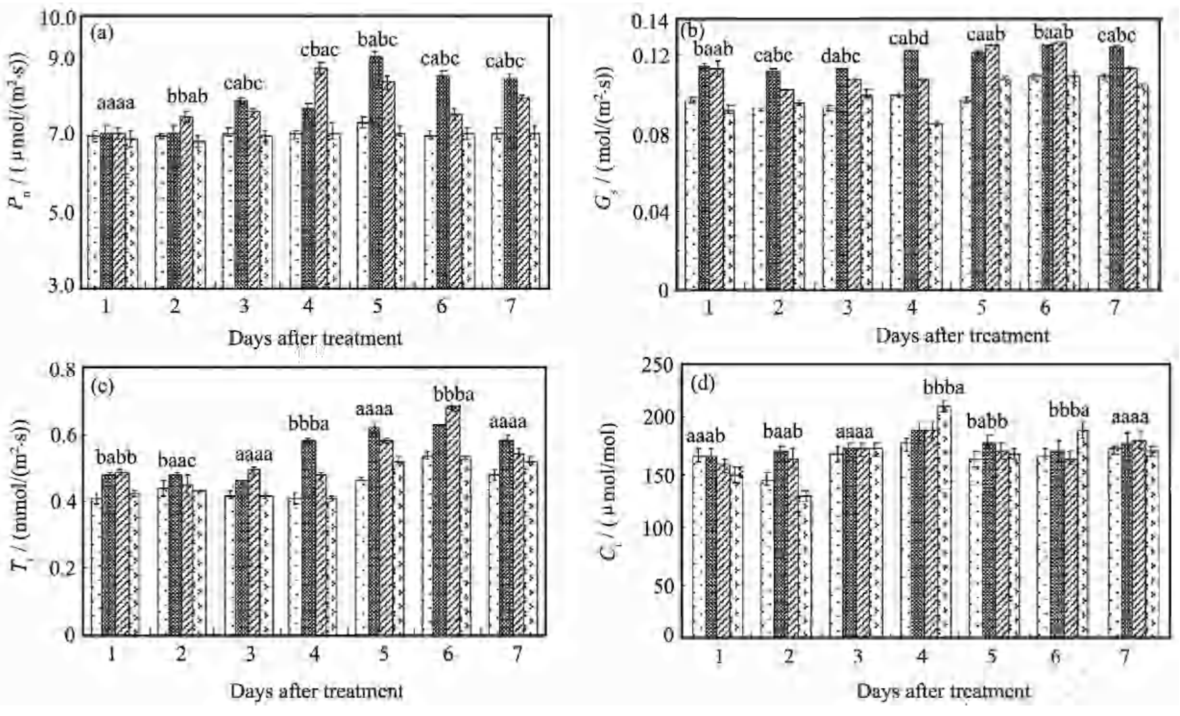

Fig. 2 Effect of $LaCl_3$ on P_n (a), G_s (b), T_r (c), and C_i (d) of *S. miltiorrhiza* seedlings for 7 d after treatment (Bars represent the mean ± SE (n=10); different letters indicate significant differences at p<0.05 when compared with the control according to the Duncan's multiple range test)

study, treatment with 20 and 60 mg/L $LaCl_3$ induced an increase of P_n, accompanied by an increase of G_s and T_r, while 200 mg/L $LaCl_3$ had little effect on G_s, T_r and P_n. This suggests that the effect of $LaCl_3$ on photosynthesis involved the enhancement of the stomatal opening, which corresponds with results obtained by Liu and Hao[16]. A significant positive correlation exists between G_s and P_n[17], and the induction of stomatal opening by $LaCl_3$ may be one of the contributing mechanisms to the increase in photosynthesis observed with $LaCl_3$ at 20 and 60 mg/L.

2.3 Chlorophyll fluorescence

As shown in Fig. 3(a), the F_v/F_m ranged from 0.80 to 0.86 and did not show any significant differences (p<0.05) with the treatment of $LaCl_3$. The *qP* was markedly enhanced with the treatment of $LaCl_3$ at 20 and 60 mg/L, but reduced with 200 mg/L $LaCl_3$ (Fig. 3(b)). The *Φ*PSII showed a similar trend to that observed with *qP*, as shown in Fig. 3(c). The NPQ, however, showed a different trend to the other parameters measured (Fig. 3(d)). It was decreased markedly by the treatment of $LaCl_3$ at 20 and 60 mg/L by 32.6% and 41.5% lower than the control respectively on day 4. Treatment with $LaCl_3$ at 200 mg/L, however, increased the NPQ by 27.4% and 19.9% compared to the control on days 1 and 4, respectively.

Chlorophyll fluorescence analysis has commonly been used as one of the most powerful and widespread techniques regarding photosynthesis[18]. The light energy absorbed by chlorophyll molecules in leaves is consumed in three ways that are competitive: photochemical reactions, heat dissipation, and fluorescence (non-photochemical). In general, F_v/F_m, ΦPSII, and qP have been described as photochemical-quenching parameters, and NPQ as a non-photochemical-quenching parameter[19]. In this study, the parameter, F_v/F_m, showing the maximum quantum yield of PSII, was not affected obviously with the treatment of $LaCl_3$, indicating that F_o and F_m were affected proportionally by $LaCl_3$. ΦPSII, qP and NPQ fluctuated simultaneously at all dynamic frequencies suggesting that the absorbed energy was redistributed quickly in the different pathways. However, a greater increase in qP in *S. miltiorrhiza* seedlings treated with 20 and 60 mg/L $LaCl_3$ indicated that much of the excitation energy captured by antenna pigment can be used to propel the photosynthetic electron transfer. On the other hand, a decrease in the non-photochemical quenching (NPQ) reflects the decreased thermal dissipation in order to avoid photodamage at the pigment level. It implies that the appropriate concentration of $LaCl_3$ enhances P_n by exploiting the harvested light energy more efficiently in photosynthesis.

2.4 Contents of tanshinones and salvianolic acids

The effect of $LaCl_3$ on the content of tanshinones and salvianolic acids in *S. miltiorrhiza* seedlings is shown in Fig. 4. The content of tanshinone I was significantly improved ($p<0.05$) by 35.8% with the treatment of 60 mg/L $LaCl_3$, and treatment with 20 and 200 mg/L $LaCl_3$ showed a slight, though not significant, increase (Fig. 4(a)). The content of tanshinone IIA was increased significantly ($p<0.05$) by 23.9% and 21.51% higher than the control by the treatment of 20 and 60 mg/L $LaCl_3$, respectively. The content of tanshinone IIA, however, was not significantly affected by 200 mg/L $LaCl_3$ (Fig. 4(b)). The effect of $LaCl_3$ on the cryptotanshinone content showed a similar trend to that of tanshinone IIA (Fig. 4(c)). The content of salvianolic acid B in the seedlings was decreased by the treatment of 200 mg/L $LaCl_3$, although it was not obviously affected by the treatment of 20 and 60 mg/L $LaCl_3$ (Fig. 4(d)). The content of rosmarinic acid in the seedlings was not affected by the treatment of $LaCl_3$ (Fig. 4(e)).

In this study it was shown that 20 and 60 mg/L $LaCl_3$ treatments could promote the synthesis of tanshinones, although little influence or inhibition (200 mg/L) on the content of salvianolic acids was observed. Tanshinones (tanshinone I, tanshinone IIA, and cryptotanshinone) have similar chemical structures and biosynthetic pathways. Further studies on molecular basis of La-induced biosynthesis of secondary metabolite may help to elucidate the mechanisms of the effects of $LaCl_3$ on *S. miltiorrhiza* seedlings.

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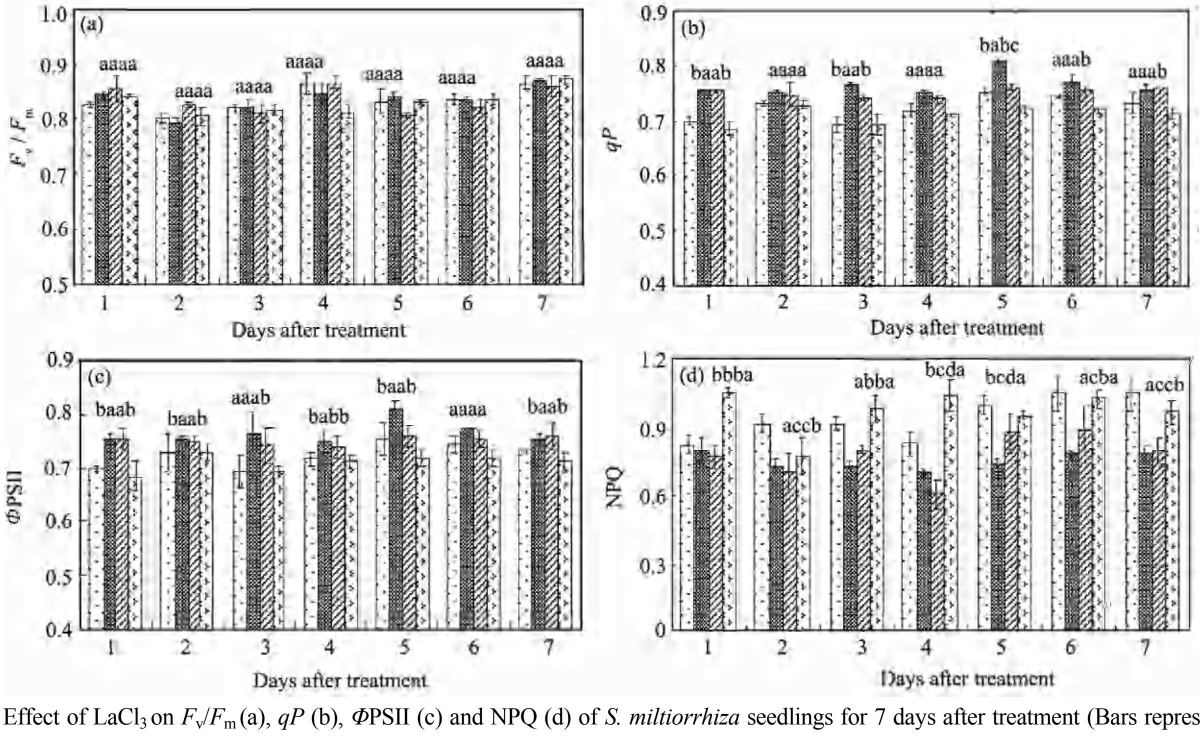

Fig. 3 Effect of $LaCl_3$ on F_v/F_m (a), qP (b), ΦPSII (c) and NPQ (d) of *S. miltiorrhiza* seedlings for 7 days after treatment (Bars represent the mean ±SE (n=10); different letters indicate significant differences at $p<0.05$ when compared with the control according to the Duncan's multiple range test)

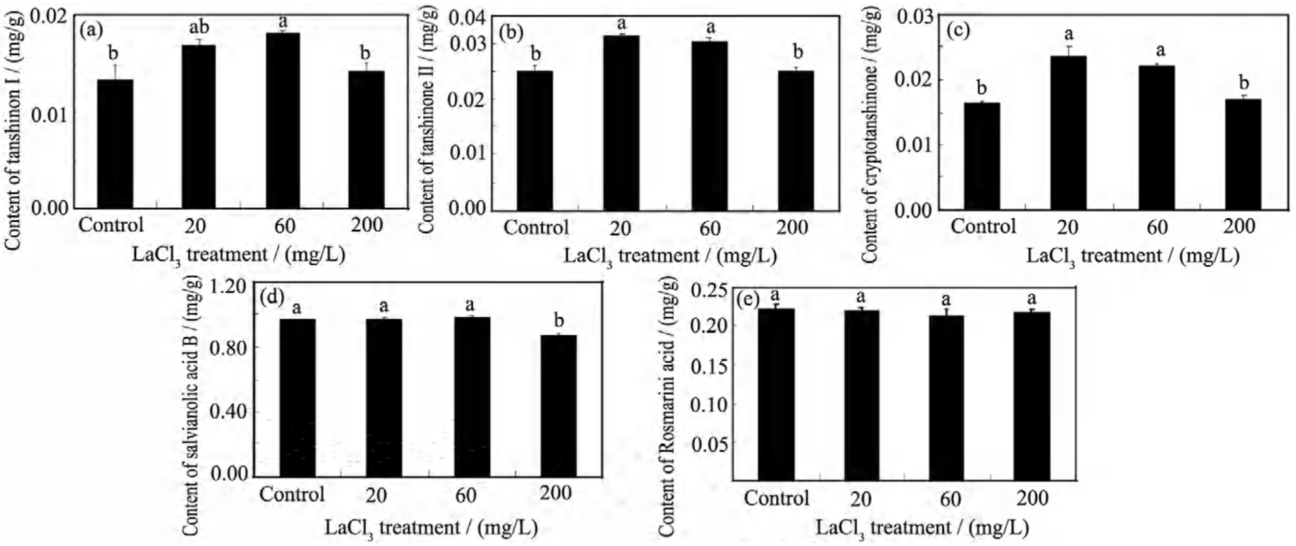

Fig. 4 Effect of LaCl$_3$ on the content of tanshinone I (a), tanshinone IIA (b), cryptotanshinone (c), salvianolic acid B (d), and rosmarinic acid (e) in *S. miltiorrhiza* seedlings for 7 d after treatment (Bars represent the mean ±SE (n=10); different letters indicate significant differences at $p<0.05$ when compared with the control according to the Duncan's multiple range test)

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