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ALLEVIATION OF IRON (Fe^{2+}) TOXICITY IN GINSENG (*PANAX GINSENG* C. A. MEYER) ROOTS AND LEAVES BY CALCIUM APPLICATION IN HYDROPONICS CULTURE

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Abstract

Rusty root is a serious problem in ginseng. Iron toxicity is an important phenomenon implicated with this rust symptom. Based on earlier attempts to reduce metal toxicity by Calcium (Ca), the present study was carried out to explore if Ca can ameliorate Fe toxicity in ginseng. Excess iron led to decreased chlorophyll content in leaves followed by necrotic spots on the lamina and reddish-brown deposition at the root surface. High Ca (16 and 32 mM) significantly reduced the Fe accumulation in leaves ($p < 0.05$) and its toxicity symptoms in plants. Ca at 32 mM enhanced the activities of SOD and POD and the pigment content in the Fe stressed leaves ($p < 0.05$). Positive influence of Ca was also reflected by higher root dry biomass and major ginsenosides accumulation in the stressed roots ($p < 0.05$). Ca enables plants to withstand the deleterious effect of excess Fe and prevents rusty symptoms in roots.

Introduction

Panax ginseng is a valuable perennial herbaceous root which is used in many traditional medicines, and ginsenosides are the main active constituents (Shi *et al.* 2007). *P. ginseng* is widely cultivated in China, North Korea, South Korea, Japan, and Russia; the Changbai Mountain area is the major producer of ginseng in the world, accounting for 80% of the total production of ginseng in China and 60 - 70% of global production (Li *et al.* 2014). However, rusty root is a serious problem that is widely distributed, restricts the production and quality of ginseng (Lee *et al.* 2011a, Wang *et al.* 2016). Fe^{2+} toxicity is considered as a key factor causing ginseng rusty root due to reddish-brown deposition on root surfaces that occurred when excessive Fe^{2+} was present in the soil or nutrient solution (Wang *et al.* 1997 and Zhang *et al.* 2016). Therefore, alleviation of iron (Fe^{2+}) toxicity in *P. ginseng* is an important way to alleviate rusty root.

Calcium (Ca) is an essential nutrient element and is involved in the regulation of plant growth and development (Ahmad *et al.* 2015, Li *et al.* 2016). Ca plays an important role in metal stress. It has been reported that Ca appears to be beneficial for plants in alleviating cadmium (Cd) toxicity by reducing Cd uptake and accumulation, decreasing reactive oxygen species (ROS) production, and suppressing oxidative damage (Srivastava *et al.* 2015). Ca was also shown to alleviate root growth inhibition under Cd stress through keeping auxin homeostasis in *Arabidopsis* seedlings and improve seed quality of mustard plants by enabling them to withstand the deleterious effect of Cd (Ahmad *et al.* 2015 and Li *et al.* 2016). In addition, it was reported that Ca may alleviate aluminum (Al) toxicity by reducing Al accumulation in the Al-sensitive cultivars of highbush blueberry (Reyesdíaz *et al.* 2011). Thus, exogenous Ca can be applied to ameliorate metal toxicity in plants. Cai *et al.* (2003) suggested that Ca can ameliorate Fe^{2+} toxicity by eliminating active oxygen and maintenance of membrane stability.

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However, a few report on alleviation of Fe^{2+} toxicity in ginseng by Ca application is available. In the present study, exogenous Ca was used to alleviate Fe^{2+} toxicity and improve quality in *P. ginseng*. The objective of the present study was to investigate the alleviation mechanism of Fe^{2+} toxicity by Ca application, which is important for alleviating rusty root in ginseng cultivation.

Materials and Methods

Panax ginseng is a perennial herb. In the present study four-year-old ginseng seedlings and nutrient solution were prepared according to Zhang *et al.* (2016). The seedlings were placed in sand. Ten days after germination, the uniform seedlings were washed and transferred into a nutrient solution with 0.05 mM Fe and 4.0 mM Ca in 2 l plastic pots (5 seedlings per pot) for two days according to Hoagland and Arnon (1950) and Smith *et al.* (1983). Subsequently, ginseng seedlings were treated with different concentrations of Fe (Fe(II) EDTA) (0.05 mM and 0.40 mM Fe) dissolved in nutrient solution with different concentrations of Ca ($Ca(NO_3)_2$) as follows: (1) Nutrient solution (control) (C0) : Hoagland solution including 0.05 mM Fe and 4.0 mM Ca. (2) Fe^{2+} toxicity stress and Ca (C1): Hoagland solution including 0.40 mM Fe and 4.0 mM Ca. (3) Fe^{2+} toxicity stress and Ca (C2): Hoagland solution including 0.40 mM Fe and 16.0 mM Ca. (4) Fe^{2+} toxicity stress and Ca (C3): Hoagland solution including 0.40 mM Fe and 32.0 mM Ca.

Seedlings were grown in a room at 23°C, and the nutrient solution was refreshed every 3 days. Ginseng was harvested at 28 days of growth when the plant showed iron toxicity in hydroponics culture, and all physiological analyses and biochemical determinations were done in three biological replicates for each treatment.

After harvesting, ginseng roots were soaked in 20 mM Na_2-EDTA for 15 min to remove the ions adhered to the root surface. Roots and leaves were separated and oven-dried according to the method used by Shi *et al.* (2010). Dried root and leaf materials (100 mg) were finely ground, then digested and treated with H_2SO_4/HNO_3 and $HNO_3/HClO_4$ mixture according to the method of Ahmad *et al.* (2015). Fe and Ca content were determined by inductively coupled plasma optical emission spectroscopy (ICP-OES, Varian, USA).

Fresh leaf samples were weighed and homogenized under chilled conditions in potassium phosphate buffer specific for superoxide dismutase (SOD) and peroxidase (POD). SOD activity was determined by measuring the inhibition of the photoreduction of nitroblue tetrazolium (NBT) at 560 nm following the methods of Reyesdíaz *et al.* (2011) and one SOD unit was defined as the amount of enzyme leading to 50% inhibition of the NBT reduction. POD activity was determined based on the oxidation of guaiacol at 470 nm by H_2O_2 (Shi *et al.* 2010). The content of chlorophyll and carotenoids was determined according to Lichtenthaler and Wellburn (1983). Fresh leaf tissue samples were ground in 96% (v/v) ethanol. The absorbance was measured at 665, 649, and 470 nm against 96% ethanol, which was used as a blank, and the content of pigment was calculated.

Ginsenosides Rg_1, Re, Rb_1 and Rb_2 standards at least 98% purity were purchased from the National Institutes for Food and Drug control (Beijing, China). All reference standards dissolving with methanol were mixed into standard solution containing 0.21 mg/ml of Rg_1, 0.17 mg/ml of Re, 0.21 mg/ml of Rb_1, and 0.10 mg/ml of Rb_2.The dried ginseng root was weighed, and the content of four major ginsenosides, Rg_1, Re, Rb_1, and Rb_2, was quantitatively analyzed in accordance with Lee *et al.* (2011b) and Xu *et al.* (2016). The dried ginseng root was crushed and passed through a 40-mesh sieve. Each powder was suspended in 80% methanol and ultrasonically extracted for 1 hr. The extracted solution was centrifuged, and the supernatant was filtered through a 0.2 μm syringe filter. The filtrate was stored at 4°C before ultra-performance liquid chromatography (UPLC) analysis. The separation and detection of ginsenosides was performed on an ACQUITY UPLC system (Waters Corporation, Milford, MA, USA) with a PDA detector and an ACQUITY UPLC BEH C18 column (50 mm × 2.1 mm, 1.7 μm) (Fig. 1).

Statistical analysis was conducted using SAS8.0 (SAS Institute, Cary, NC, USA). Each treatment (C0, C1, C2, and C3) was repeated three times. Values reported here are the means of three replicates and are expressed as mean ± standard error (SE). Data were subjected to one-way analysis of variance, and a least significance difference (LSD) test was used to assess the significant differences between treatment groups at a significance level of $p < 0.05$.

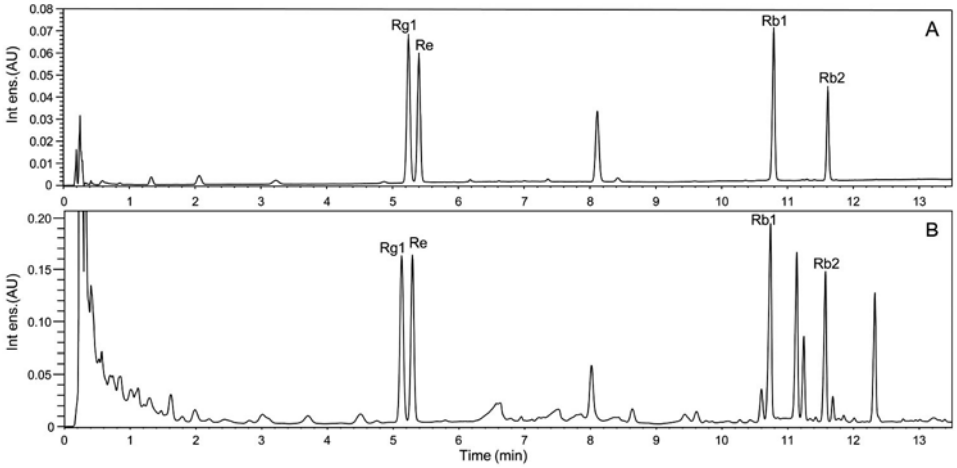

Fig. 1. Panaxoside content determined by ultra-performance liquid chromatography (UPLC). (A) UPLC chromatogram of ginsenoside Rg1, Re, Rb1, Rb2 standard and (B) UPLC chromatogram of sample.

Results and Discussion

Reddish-brown deposits at the root surface and necrotic spots on the leaf appeared under Fe^{2+} toxicity stress (C1) (Fig. 2A and 2C), which are recognized as typical Fe^{2+}-induced toxicity symptoms. In cultivated rice, the exposure of plants to Fe^{2+}toxicity also showed typical symptoms of Fe^{2+} toxicity, such as leaf bronzing on the leaves and formation of a red coat on the roots (Dufey *et al.* 2014). Also, a Fe plaque formed by oxidizing Fe^{2+} at the root surface has been suggested as a tolerance mechanism for excluding high amounts of Fe^{2+} in the soil or nutrient solution from the plant (Lee *et al.* 2013, Zhang *et al.* 2016). Excessive Fe accumulated in leaves (Fig. 3A), and free Fe catalyzes an oxidative stress through the formation of reactive oxygen species (ROS), which causes serious damage to the plant (Quinet *et al.* 2012).

No visible symptoms on the leaves and roots in the presence of Fe^{2+} toxicity and Ca (C2 or C3) were observed. Higher doses of Ca (16 and 32 mM against 4.0 mM in control Hoagland nutrient solution) significantly reduced toxicity symptoms in plants fed with higher concentration of Fe (0.40 mM instead of a value of 0.05 mM) in the control nutrient solution (Fig. 2B and 2D). In roots, no significant change in the uptake of Fe elements was observed (Fig. 3A). The addition of Ca (C2 and C3) significantly enhanced the accumulation of Ca under Fe^{2+} toxicity treatment in roots ($p < 0.05$) (Fig. 3B). In leaves, the Fe accumulation under iron stress was brought down from 1.31 mg/g level to < 0.3 mg/g level with 16 and 32 mM Ca ($p < 0.05$), while Ca concentration was increased from 3.20 mg/g to 6.19 mg/g when Ca was co-fed at 32 mM levels ($p < 0.05$) (Fig. 3A, B). Owing to high resemblance in chemical character with Fe, Ca and Fe share transporters for their uptake and transport inside the cell. Therefore, increased Ca in the nutrient solution has an antagonistic effect against Fe bioaccumulation, and might compete with Fe for its uptake. This competition and uptake were also reported in plants exposed to silver (Ag) and Cd (Oukarroum *et al.* 2013, Srivastava *et al.* 2015).

The activity of antioxidant enzymes changed variably under different treatments (Fig. 3C,D). Ca supplementation enhanced the activities of major antioxidant enzymes SOD and POD in the Fe stressed leaves, respectively ($p < 0.05$) (Fig. 3C, D), proving that Ca may further initiate the enzyme protective system to eliminate ROS and relieve the damage caused by toxicity. This was also suggested in *Brassica juncea* and rice exposed to Cd toxicity (Ahmad *et al.* 2015, Srivastava *et al.* 2015). Fe^{2+} toxicity treatment (C1) decreased the content of chlorophyll a (chla), leading to a significant decrease of total chlorophyll content in leaves when compared with the control plants (C0) (Table 1). The addition of Ca (C2 and C3) alleviated the negative influence of excess Fe on the total chlorophyll content (Table 1). Similarly, the content of carotenoids was increased in the nutrient solution with 32 mM Ca concentration (C3) compared with Fe^{2+} toxicity stress (C1) (Table 1). The addition of Ca reduced Fe content in leaves, and alleviated the negative influence of excess Fe on the pigment content. Previously it was also clearly demonstrated that application of Ca enhanced Chl and carotenoid biosynthesis in Cd-stressed plants (Ahmad *et al.* 2015).

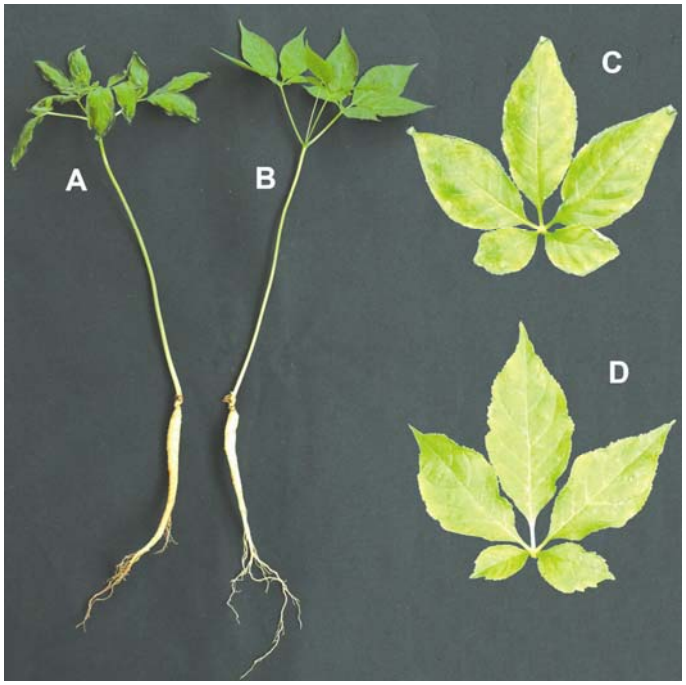

Fig. 2. Effect of Ca on characteristics of roots and leaves under Fe^{2+} toxicity stress.(A) 0.4 mM Fe + 4.0 mM Ca, (B) 0.4 mM Fe + 16.0/32.0 mM Ca, (C) 0.4 mM Fe + 4.0 mM Ca and (D) 0.4 mM Fe + 16.0/32.0 mM Ca.

Table 1. Chlorophyll and carotenoid content in leaves of *P. ginseng*.

Treatment	Chl a (mg/g)	Chl b (mg/g)	Chl a+b (mg/g)	Car (mg/g)
C0	2.67 ± 0.09b	1.18 ± 0.02ab	3.85 ± 0.11b	0.94 ± 0.06ab
C1	2.04 ± 0.09a	0.94 ± 0.04a	2.98 ± 0.12a	0.79 ± 0.02a
C2	2.46 ± 0.27ab	1.37 ± 0.19b	3.84 ± 0.42b	0.91 ± 0.09ab
C3	2.70 ± 0.16b	1.22 ± 0.09ab	3.92 ± 0.25b	1.01 ± 0.08b

Values are means ± SE from three replicates, and significant differences among the treatments ($p < 0.05$) are indicated by different letters.

Fe^{2+} toxicity treatment (C1) decreased the root dry weight markedly, and application of Ca to Fe^{2+} toxicity-stressed plants reduced the negative effect of excess Fe on root dry weight ($p < 0.05$) (Table 2). Ginsenoside, exhibiting various pharmacological and physiological effects, is the main active ingredient in *P. ginseng* (Oh *et al.* 2014), and Rg_1, Re, Rb_1, and Rb_2 are the four major ginsenosides in extracts of *P. ginseng* root (Shi *et al.* 2007).The quantity of Rg_1 decreased, whereas the content of Re and Rb_1 increased significantly in the roots under Fe^{2+} toxicity treatment (C1) (Table 2). The contents of four industrially important major ginsenosides, Rg_1, Re, Rb_1, and Rb_2, further increased under the addition of Ca (C2 and C3) treatment (Table 2). Especially, when 32 mM

Table 2. Dry weight and ginsenoside content in *P. ginseng* roots.

Treatment	Dry weight (g)	Rg_1 (mg/g)	Re (mg/g)	Rb_1 (mg/g)	Rb_2 (mg/g)
C0	1.32 ± 0.04c	5.44 ± 0.24b	4.85 ± 0.27a	6.75 ± 0.16a	3.88 ± 0.26a
C1	0.83 ± 0.02a	4.44 ± 0.32a	6.53 ± 0.28b	8.33 ± 0.33b	4.14 ± 0.18a
C2	1.12 ± 0.05b	5.97 ± 0.15b	6.93 ± 0.26b	8.80 ± 0.35b	5.14 ± 0.38b
C3	1.14 ± 0.05b	6.77 ± 0.21c	6.82 ± 0.31b	10.74 ± 0.13c	6.06 ± 0.14c

Values are means ± SE from three replicates, and significant differences among the treatments ($p < 0.05$) are indicated by different letters.

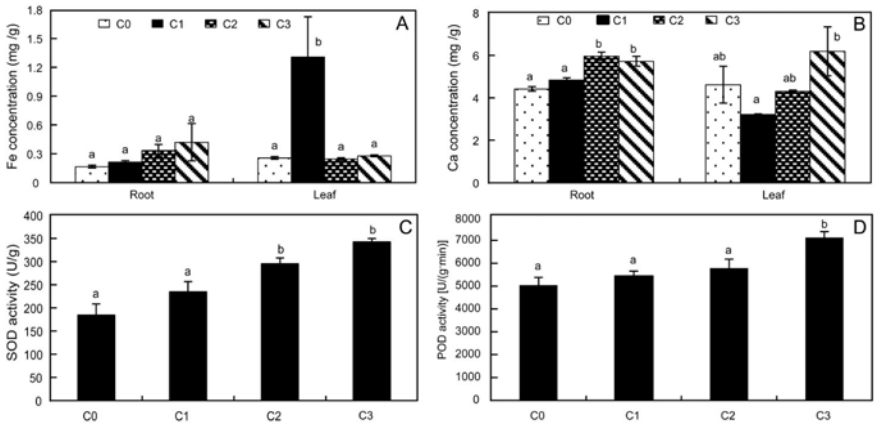

Fig. 3. Effect of Ca on element content and antioxidase activityunder different treatments. Significant differences among the treatments ($p < 0.05$) are indicated by different letters. (A) Fe content, (B) Ca content; (C) SOD, (D) POD.

Ca was also incorporated in the nutrient solution with 0.4 mM level of Fe stress (4.44, 6.53, 8.33 and 4.14 mg/g dry tissue, respectively), the contents of four ginsenosides Rg_1, Re, Rb_1 and Rb_2 in the roots also improved (6.77, 6.82, 10.74 and 6.06 mg/g dry wt., respecetively). Thus, Ca could increase ginsenoside contents and improve the root quality of ginseng plants under iron stress based on the measurement of root dry weight and ginsenoside contents.

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