Effects of silicon on glycine-betaine, phytochelatin, and antioxidant enzymes in licorice (*Glycyrrhiza glabra* L.) under aluminum stress

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Abstract

Licorice (*Glycyrrhiza glabra* L.) is a valuable plant for the treatment of several diseases. Negative effects of aluminum stress on plants have been reported and silicon may alleviate these negative effects through promoting antioxidant system. This study was conducted to investigate the effects of silicon on glycine-betaine, phytochelatin, and antioxidant parameters in licorice plant under aluminum stress. The plants were treated with silicon (0, 0.5, and 1.50 mM) and submitted to aluminum stress (100, 250, and 400 μM). Glycine-betaine contents of roots and shoots were investigated. Also, guaiacol peroxidase (GPx), peroxidase (POX), superoxide dismutase (SOD), phenylalanine ammonia lyase (PAL), 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities, and hydrogen peroxide (H_2O_2) and root phytochelatin were assessed after treatment with aluminum. Results showed that aluminum stress increased the contents of glycine-betaine and root phytochelatin, activities of GPx, POX, SOD, PAL, DPPH radical scavenging, and H_2O_2 content ($p<0.05$). Application of silicon application also increased the contents of glycine-betaine, activities of GPx, POX, PAL, DPPH radical scavenging, and root phytochelatin ($p<0.05$) while it decreased H_2O_2 and SOD contents ($p<0.05$). Based on the findings, application of silicon is recommended for protection of licorice under aluminum stress.

Keywords: aluminum stress, antioxidant enzyme, licorice, phytochelatin**,** glycine-betaine

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Introduction

Plants are usually faced with environmental challenges during their life. Abiotic stresses such as heavy metal stress commonly occur in plants and delay their growth (Zhu, 2016). Heavy metals may have cytotoxic, genotoxic, and mutagenic effects in living organisms (Gallo-Franco et al., 2020; Gülmez et al.,2020).

Heavy metals such as aluminum result in physiological responses in plants and reduce crop yield (Fryzova et al., 2017; Gallo-Franco et al., 2020). Aluminum is commonly found as an integral component of mineral soil (Rahman et al., 2018). Formulated in some forms, e.g. $Al(OH)_2^+$, it can cause toxicity in plants. Aluminum toxicity prevents root cell division/elongation, root-hair formation, and the absorption of water and

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nutrients, increasing the development of swollen roots apices (Bojórquez-Quintal et al., 2017). Phytotoxicity of aluminum increases generation of reactive oxygen species (ROS), induces stress, and inhibits respiration in mitochondria (Rahman et al., 2018). Aluminum toxicity has also been reported to cause oxidative and DNA damage and lipid peroxidation by the increase in ROS (Amara et al., 2020; Saad-Allah and Abdelsalam, 2020). It might cause physiological and biochemical changes in plants such as the accumulation of osmolytes, soluble proteins, and sugars (Sadat Hosseini et al., 2018). Plants alleviate aluminum toxicity through intracellular mechanisms such as antioxidant pathways (Imadi et al., 2016).

Silicon is an element that improves plant tolerance to biotic and abiotic stresses, such as metal toxicity (Bhat et al., 2019; Hasanuzzaman et al., 2018; Jang et al., 2018; Khan et al., 2018; Delavar et al., 2016). On the other hand, losing silicon during aluminosilicate decomposition results in soil acidification and development of aluminum toxicity (Shetty et al., 2020). Researchers have reported that silicon supply for soil might form stable aluminum-silicon complexes and alleviate aluminum phytotoxicity (Elisa et al., 2016; Kopittke et al., 2017; Qian et al., 2016). Silicon adsorbs aluminum on its surfaces and forms a stable compound with lower phytotoxicity (Elisa et al., 2016; Hodson and Evans, 2020). A recent review article by Hodson and Evans (2020) showed that silicon application alleviates aluminum toxicity through the root apoplast and the formation of hydroxyl aluminosilicates. Other studies have reported that silicon application not only increases precipitation of toxic ion under toxicity condition, but also scavenges ROS in the plants (Chalmardi et al., 2013; Imtiaz et al., 2016). Furthermore, silicon was reported to improve osmolyte contents in rice cultivated under ionic stress (Yan et al., 2020). It maintains photosynthetic proteins (Muneer et al., 2017) and regulates stress-related genes (Nakashima et al., 2012). It was also reported to improve growth, photosynthetic and antioxidant capacities, and nutrient homeostasis in tomato (Zhang et al., 2019).

Licorice (*Glycyrrhiza glabra* L.) belongs to Fabaceae family and has been used as a traditional medicinal plant in many countries (Sadat Hosseini et al., 2020). It grows as a wild plant in Iran (Esmaeili et al., 2019) and is known to have pharmaceutical properties including antimicrobial, anti-diuretic, anti-hepatotoxic and anti-inflammatory effects (Farag et al., 2012; Esmaeili et al., 2019; Hosseinzadeh and Nassiri-Asl, 2015). Glycyrrhizin or glycyrrhizic acid ($C_{42}H_{22}O_{16}$) is the major compound in licorice, which is much sweeter than sucrose (Behdad et al., 2020). Major compounds of licorice are stored in roots, and might be affected by the environmental factors (Gupta et al., 2016). Other phenolic compounds such as flavones, flavans, chalcones, and isoflavonoids have been identified in licorice (Farag et al., 2012). Phenolic compounds and flavonoids have antioxidant properties owing to free hydroxyl groups in their structure (Gupta et al. 2016). Studies have reported that the glycyrrhizin and phenolic compound contents of licorice might be changed in response to the soil compositions and the environmental factors (Oloumi and Hassibi, 2011; Rezaei et al., 2017; Behdad et al., 2020).

Licorice is a valuable plant owing to its pharmaceutical properties. However, aluminum toxicity may have adverse effects on its antioxidant properties, osmolyte contents, and glycyrrhizin yield. On the other hand, silicon application may reduce the negative effects of aluminum on the mentioned parameters in licorice. However, there have been no studies on the effects of silicon on antioxidant properties, osmolyte contents, and phytochelatin of licorice (*G. glabra* L.) under aluminum stress. Hence, the aim of the present study was to investigate the effects of silicon on antioxidant properties, glycine betaine, and phytochelatin under aluminum stress.

Materials and Methods

Plant material and treatment conditions

Seeds of *G. glabra* were prepared form Pakan-Bazr Company (Isfahan, Iran). The seeds were disinfected using alcohol (70%) for 60 s and then washed with distilled water. The seeds were then cultivated on a bed of perlite and irrigated with distilled water for 7 days. After emerging 2-3 leaves, the seedlings were fertigated with Long-Ashton solution (pH=5.5). The solution contained KNO_3, $Ca(NO3)_2.4H_2O$, $MgSO_4.7H_2O$, $NaH_2PO_4.2H_2O$, $FeCl_3$, $MnSO_4.4H_2O$, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$, H_3BO_3, NaCl, and $Na_2MoO_4.2H_2O$. The seedlings were then transferred to hydroponic condition with 16 h light/8 h darkness and fed with Long-Ashton solution. The temperature program was 16 ± 2 ℃ and 24 ± 2 ℃ for night and day, respectively. Hydroponic media was 1.5 L plastic pots with 2 plants per pot that aerated by an air pump that was changed every 5 days. Lighting intensity was 250 µmol $m^{-2}S^{-1}$ and in wavelengths of 400-700 nm.

The plants were treated with different concentrations of silicon in the form of $Na_2SiO_3.5H_2O$ (0, 0.5, and 1.50 mM) 30 days after planting and for 110 days. The plants were then exposed to aluminum concentrations (100, 250, and 400 µM) in the form of $AlCl_3.6H_2O$ for 18 days.

To investigate enzyme activity, the plants were submitted to aluminum stress for 4 days. Aluminum and silicon were prepared from Merk Company (Munich-Germany). The plants were harvested after aluminum stress and were weighed to record fresh weight (FW). To assess enzyme activity, the plants were fixed in liquid azote and kept in-20 ℃.

Glycine-betaine and phytochelatin assays

Glycine-betaine was assayed as reported by Grieve and Grattan (1983). Root phytochelatin contents were measured based on De Vos et al. (1992) method.

Assessment of enzymes activity and antioxidant parameters in shoot

To investigate the enzymatic activities, 0.25 g fresh tissue was ground in liquid azote, weighed, and transferred into 1.5 mL Eppendorf containing 1 mL 50 mM potassium phosphate buffer (pH=7.5) and Triton 1%. All the extraction phases were conducted in ice and the samples were stored in a refrigerator for 1 h. The extracts were centrifuged at 15000 g for 15 minutes in 4 ℃. Supernatants were used for measuring enzymatic activity. Guaiacol peroxidase (GPx) and peroxidase (POX) activities were assayed as reported by Polle et al. (1994) based on absorption of tetraguaiacol at 436 nm. Superoxide dismutase (SOD) activity was evaluated as reported by Gianopolitis and Ries (1977) by using p-nitro blue tetrazolium. In addition, phenylalanine ammonia lyase (PAL) was assayed based on the method suggested by Goldson et al. (2008). Also, the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was measured as reported by Abe et al. (1998). Finally, to determine the hydrogen peroxide (H_2O_2), method of Sagisaka (1976) was used.

Data analysis

The present study was conducted as a factorial arrangement, consisting of silicon (0, 0.5, and 1.50 mM) and aluminum (100, 250, and 400 µM) with 3 replications based on a completely randomized design. The data were normally distributed according to the Kolmogorov-Smirnov test. Analysis of variance was used, followed by the Duncan post hoc test.

Results

Glycine-betaine

Concentration of shoot glycine-betaine (Fig.I. A) progressively increased with increasing the concentration of aluminum (100, 250, and 400 µM) compared with plants in the control group (0 µM) ($p<0.05$). Application of silicon (1.50 mM) increased shoot glycine-betaine compared to other concentrations (0 and 0.50 mM).

Concentration of root glycine-betaine (Fig. I. B) was significantly higher in the plants treated with aluminum (100, 250, and 400 µM) compared to the plants grown under non-stress condition ($p<0.05$). Application of silicon (1.50 mM) only increased concentration of root glycine-betaine under 0 and 100 µM aluminum, but it did not have significant effects at higher concentrations of aluminum ($p>0.05$). However, shoot/root ratio of glycine-betaine (Fig. I. C) was significantly higher in plants grown under stress condition compared to

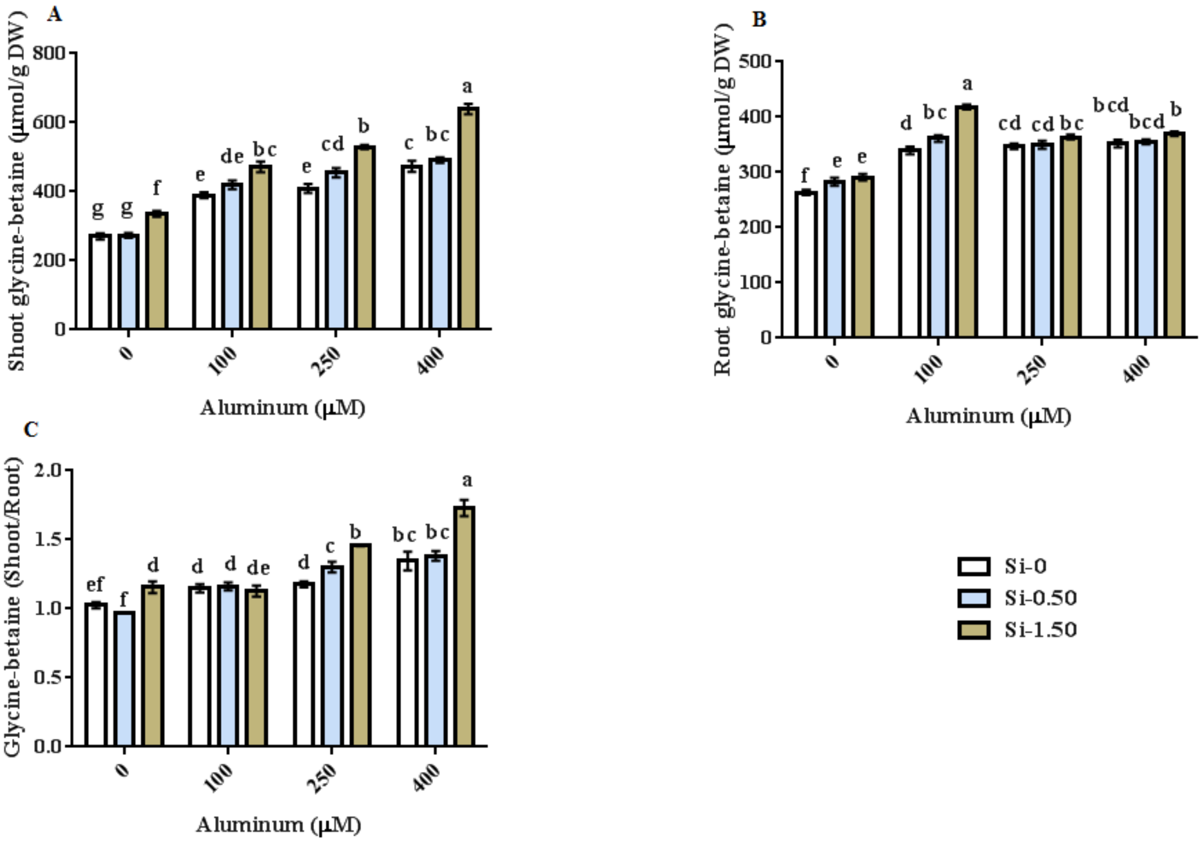

Fig. I. Effects of silicon on glycine-betaine concentration of roots and shoots in licorice grown under aluminum stress; the data are shown as means ± S.E. Superscripts (a-g) show significant differences among groups ($p<0.05$).

those grown under non-stress condition ($p<0.05$). Silicon treatment increased the ratio of shoot/root glycine-betaine under the concentrations of 250 and 400 µM aluminum compared to no application of silicon ($p<0.05$).

Enzyme activity and oxidative parameters

The results for GPx (Fig. II. A) and POX (Fig. II. B) activities showed significant effects for silicon ($p<0.05$), aluminum ($p<0.05$), and interaction between silicon and aluminum ($p<0.05$). Aluminum stress progressively increased GPx and POX activities in a concentration-dependent manner compared to non-stressed plants. The treatment of the plants with silicon increased GPx and POX activities compared to the plants not treated with silicon. SOD activity (Fig. II. C) was higher in plants treated with 250 and 400 µM aluminum compared with non-stressed plants and those treated with 100 µM aluminum. Moreover, results showed that silicon application increased SOD activity compared to control group while it decreased SOD activity under stress condition. The stress progressively increased PAL activity (Fig. II. D) compared to non-stressed plants. Application of silicon at the highest concentration (1.50 mM) increased PAL activity compared to other concentrations. The results for DPPH radical scavenging (Fig. II. E) showed that stress significantly increased DPPH radical scavenging compared to non-stressed plants ($p<0.05$). However, silicon application increased scavenging under stress condition. Hydrogen peroxide content (Fig. II. F) significantly increased with the induction of stress while silicon application significantly decreased hydrogen peroxide content ($p<0.05$).

Root phytochelatin

Root phytochelatin content (Fig. III) in the plants grown under aluminum stress was significantly higher compared to non-stressed plants ($p<0.05$).

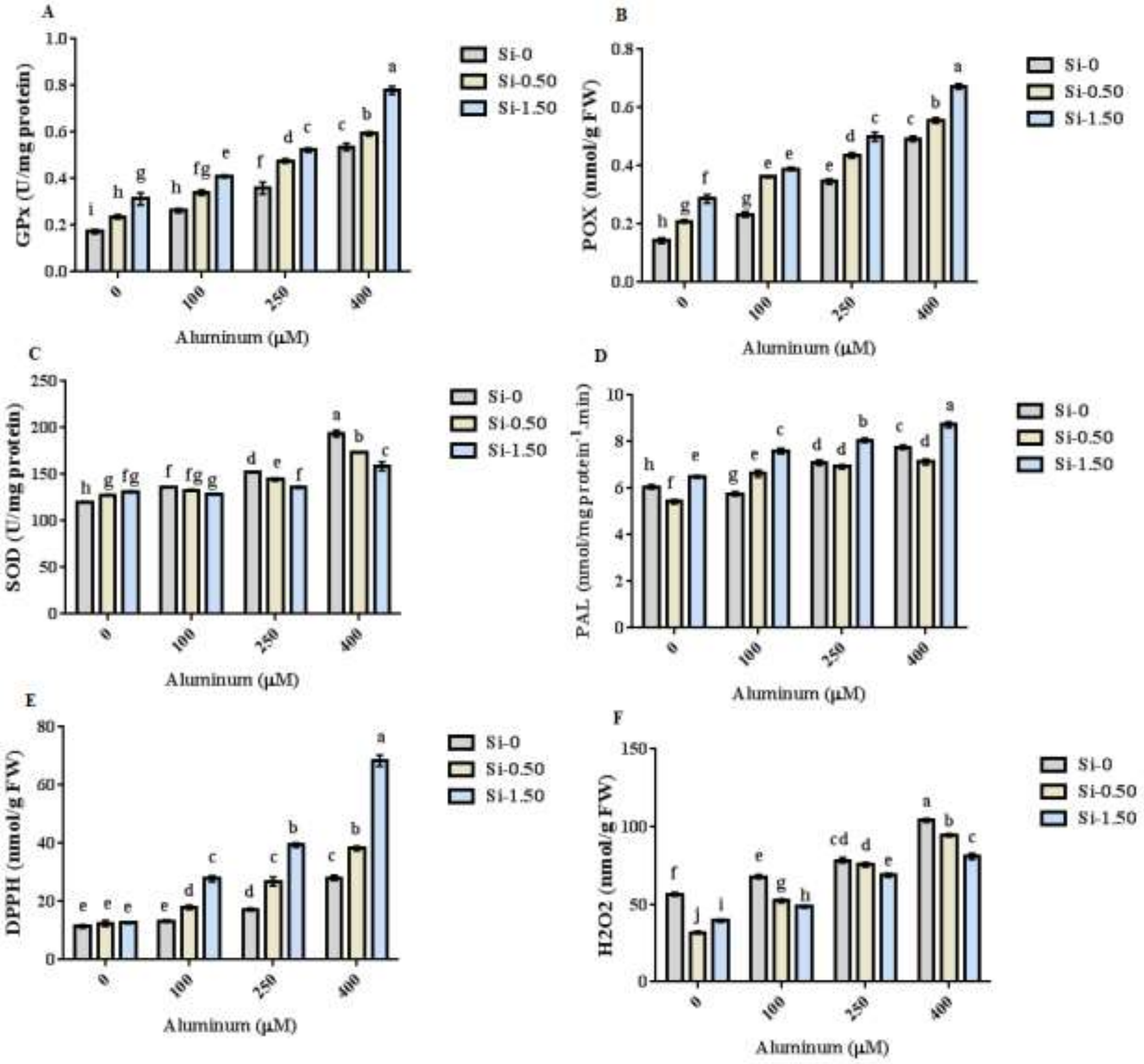

Fig. II. Effects of silicon on enzymatic activities of guaiacol peroxidase (A), peroxidase (B), superoxide dismutase (C), phenylalanine ammonia lyase (D), DPPH radical scavenging (E), and hydrogen peroxide (F) in licorice grown under aluminum stress; superscripts (a-i) show significant differences among groups ($p<0.05$).

The highest content was observed in the plants treated with 400 µM aluminum. Application of silicon increased root phytochelatin compared with control group ($p<0.05$).

Discussion

Licorice is a valuable medicinal crop, which may be affected by metal stresses with a result of reduced yield. Metal stresses can influence its production and compounds. Strategies must be considered to alleviate the effects of metal stresses on plants. In the current study, we investigated the effects of

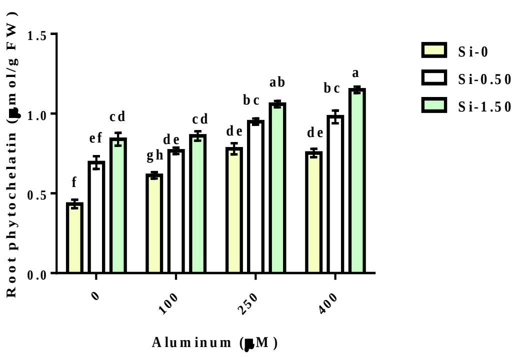

Fig. III. Effects of silicon on the root phytochelatin in licorice grown under aluminum stress; the data are shown as means ± S.E. Superscripts (a-h) show significant differences among groups ($p<0.05$).

silicon on some parameters of licorice under stress condition.

Stress increased the glycine-betaine contents of shoots and roots. In sum, glycine-betaine contents were significantly higher in shoots compared with roots and this could be attributed to their accumulation in chloroplast. Previous studies have reported that glycine-betaine is mainly accumulated in chloroplast and has a role in safeguarding the effectiveness of photosystem II in abnormal condition, e.g. metal stress (Jitender, 2011; Yildirima et al., 2015). Accumulation of glycine-betaine provides enough nitrogen for better root growth and germination and improves photosynthesis (Ali et al., 2020). It also activates antioxidant system for protection of plants via activating antioxidant enzymes (Rasheed et al., 2017). Our findings confirmed the effects of glycine-betaine on antioxidant enzymes because antioxidant enzymes activity was significantly higher under stress condition compared with non-stress condition. Antioxidant enzymes activity might be increased in response to glycine-betaine accumulation. Silicon application was reported to increase root water uptake under drought stress via active accumulation of soluble sugars and amino acids (Sonobe et al., 2011). The increase in osmolyte accumulation by addition of silicon might be attributed to improved defensive response of the plant to stress conditions.

Antioxidant enzyme activity increased under stress condition and the maximum activity was observed under the highest concentration of aluminum. Results also showed increased hydrogen peroxide and DPPH radical scavenging. Similar to our findings, other studies have reported increase in activity of antioxidant enzymes under stress condition (Nasrollahi et al., 2016; Sadat Hosseini et al., 2018; Zhang et al., 2017). Induction of stress in plants increases the production of reactive oxygen and radicals (Sadat Hosseini et al., 2018; Zhang et al., 2017; Enteshari et al., 2011). Efficient destruction of radicals and hydrogen peroxide needs antioxidants. Thus, licorice increases antioxidant enzyme activity in response to the induced stress. Results showed that maximum DPPH scavenging and antioxidant activity was observed at 400 µM aluminum concentration. In this study, the plants showed maximum activity in response to the highest oxidant level. Hydrogen peroxide is converted into H_2O by peroxidase enzymes in plants. In the present study, GPx and POX were measured as peroxidase enzymes. Guaiacol is utilized as a non-specific artificial substrate for the assessment of POD activity in plants. Results showed that aluminum stress changes the activity of antioxidant enzymes, and the change rate depends on the levels of stress. Enzymatic compounds might be directly involved in scavenging radicals and/or act through non-enzymatic compounds (Zhang et al., 2017). In sum, licorice responds to stress by increasing antioxidant enzymes activity and decreasing hydrogen peroxide. PAL activity increased in response to the stress. It is a key enzyme for the production of phenolic compounds in plants. Plant phenolics are biosynthesized biogenetically via the shikimate/phenylpropanoid pathway and play an important role as chemopreventive agents. These compounds act as effective free radical scavengers in cells (Ahmadi et al., 2020).

PAL activity might be increased in response to stresses (Caretto et al., 2015; Smirnov et al., 2015) and this suggests that it has a role for adaptation of the plants to stresses. Thus, PAL activity also increased in response to aluminum stress. Under stress condition, silicon application increased the activity of POX, GPx, DPPH scavenging, and PAL while it decreased SOD activity and hydrogen peroxide content. Participation of silicon in antioxidant activity in plants has previously been reported (Chalmardi et al., 2013; Imtiaz et al., 2016). Silicon accumulation in plants preserves them from adverse effects of various stresses by scavenging free radicals through ROS (Emamverdian et al., 2018). Silicon promotes antioxidant enzyme activity in plants submitted to metal stress (Dubery, 2014; Gagoonani et al., 2011; Ma et al., 2011).

In this study, SOD activity was lower in silicon treatments and this might be attributed to the regulation of antioxidant activity of silicon by other enzymes and/or by phytochelatin. Silicon also increased PAL activity even though the mechanism of action is unknown. It may increase PAL activity by involvement in gene pathways. Aluminum stress increased root phytochelatin and the treatment with silicon increased its contents. Similarly, other studies have reported the increase in phytochelatin content under stress condition (Ghori et al., 2019; Pirzadah et al., 2019; Morkunas et al., 2018). There are many reports that indicate a positive correlation between phytochelatin synthesis and accumulation of metal ions (Singh, and Chauhan, 2011; Yang et al., 2013; Delhaize et al., 2012).

The increase in accumulation of phytochelatin could be attributed to its effects on metal detoxification. Ghori et al. (2019) showed that phytochelatin transports metal complexes and starts a complex cascade of metal detoxification in the vacuole. Parallel to our findings, other studies have reported the increasing effects of silicon on phytochelatin in various plants (Bari et al., 2020; Bhat et al., 2019; Emamverdian et al., 2018). The effects of silicon on increasing root phytochelatin contents could be attributed to its effects on the expression of genes involved in the synthesis of metallothioneins. The treatment with silicon stimulated the expression of genes of the synthesis of metallothioneins and decreased the expression of genes encoding heavy metal transporters (Ahanger et al., 2017).

Conclusion

Overall, application of silicon alleviated the negative effects of aluminum stress on antioxidant status, regulated osmolyte contents, and increased phytochelatin in licorice. Results suggest that silicon application mainly act through antioxidant pathway and can help increase the production of phytochelatin in licorice. This is a preliminary study and opens a window for future studies. Silicon application is suggested for improvement of antioxidant status in licorice.

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