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Exogenous silicon leads to increased antioxidant capacity in freezing-stressed pistachio leaves

Ghader HABIBI[1]

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ABSTRACT

Freezing stress limits photosynthesis and growth of plants. This may be attributed to the enhancement of freezing-associated oxidative damage. In this study, we followed precisely changes in the extent of lipid peroxidation and oxidative damage in leaves of pistachio (*Pistacia vera* 'Ahmadaghaii') plants exposed to foliar-applied silicon (Si) under freezing stress. The foliar-applied Si decreased significantly damaging effects of cold on relative water content (RWC), accompanied by an increase in shoot fresh mass (SFM). In addition, pre-Si treatment caused a significant reduction of the leaf area lost by freezing. There was a remarkable increase in phenylalanine ammonia-lyase (PAL) activity during recovery. Since leaf phenolic content was not affected by supplementary Si, the possibility that exogenously applied Si directly influences the activity of PAL seems thin. In the present work, freezing stress caused great membrane damage, as assessed by lipid peroxidation, but Si application significantly reduced the membrane damage because of an efficient scavenging by superoxide dismutase (SOD) and peroxidase (POD). Under freezing, despite the increasing POD activity, Si-supplied plants accumulated the highest levels of hydrogen peroxide (H_2O_2) may act as a signal for recovery ability from freezing injury. A positive correlation was found between the concentration of malondialdehyde (MDA) and the percentage of necrotic leaf area. This study suggests that the possible mechanisms for Si enhanced freezing resistance may be attributed to the higher antioxidant defense activity and lower lipid peroxidation through leaf water retention, in addition to its role as a mere physical barrier.

Key words: antioxidant enzymes, cold stress, Evans dye, hydrogen peroxide, phenylalanine ammonia-lyase, *Pistacia vera*, malondialdehyde, relative water content

IZVLEČEK

TRETIRANJE LISTOV PISTACIJE (*Pistacia vera* 'Ahmadaghaii') S SILICIJEM POVEČA NJIHOVO ANTIOKSIDATIVNO SPOSOBNOST V MRAZNEM STRESU

Mrazni stres omejuje fotosintezo in rast rastlin, kar lahko pripišemo povečanju oksidativnih poškodb zaradi zmrzovanja. V raziskavi sta bili spremljani peroksidacija lipidov in oksidativne poškodbe listov pistacije (*Pistacia vera* 'Ahmadaghaii') izpostavljenih mraznem stresu in foliarnem tretmaju s silicijem (Si). Foliarna uporaba silicija je značilno zmanjšala učinke mraza na ravni relativne vsebnosti vode (RWC), kar je povzročilo povečanje sveže mase poganjkov (SFM). Dodatno je predtretiranje s Si povzročilo značilno zmanjšanje izgube listne površine zaradi zmrzovana. Med okrevanjem po mraznem stresu je bila opazno povečana aktivnost fenilalanin amonik-liaze (PAL). Zaradi nespremenjene vsebnosti fenolov v listih po aplikaciji Si je maloverjetno, da bi foliarno dodani Si neposredno vplival na aktivnost PAL. Mrazni stres je povzročil velike poškodbe membran, ki so bile ocenjene s peroksidacijo lipidov, a jih je uporaba Si značilno zmanjšala zaradi učinkovitega antioksidativnega delovanja superoksid dismutaze (SOD) in peroksidaze (POD). Kljub povečanju aktivnosti POD v razmerah zmrzovanja, so s Si-obravnavane rastline kopičile največje količine vodikovega peroksida (H_2O_2), ki je lahko deloval kot signal za sposobnost okrevanja po poškodbah zaradi zmrzovanja. Ugotovljena je bila pozitivna korelacija med koncentracijo malondialdehida (MDA) in odstotkom nekrotične listne površine. Raziskava kaže, da je možen mehanizen preko katerega Si povečuje odpornost na zmrzovanje večja antioksidativna obramba in manjša peroksidacija lipidov, ki se odraža v večjem zadrževanju vode poleg delovanja Si kot čisto fizikalne prepreke.

Ključne besede: antioksidativni encimi, mrazni stres, Evansovo modrilo-T-1824 ,vodikov peroksid, phenilalanin amoniak-liaza, *Pistacia vera*, malondialdehid, relativna vsebnost vode

[1] Department of Biology, Payame Noor University, I. R. of Iran; Email: gader.habibi@gmail.com

1 INTRODUCTION

Low temperatures severely reduce photosynthetic capacity and growth of plants that may be due to increased production of reactive oxygen species (ROS). Accumulation of ROS is capable of inducing damage to almost all cellular macromolecules including DNA, proteins and carbohydrates (Ding *et al*., 2010; Miller *et al*., 2010). The plant cells respond to elevation in ROS levels by increasing the expression and activity of ROS-scavenging enzymes in order to maintain redox homeostasis (Apel and Hirt, 2004; Miller *et al*., 2010). In addition, some plants of tropical and subtropical regions, exhibit sensitivity to cold stress and usually lack the ability for cold acclimation (Zhu *et al*., 2007; Takahashi *et al*., 2013).

Silicon (Si) has been proved to be beneficial for the healthy growth and development of many plant species, particularly from the Gramineae family (Marschner, 1995; Broadley *et al*., 2011). Si application to crops has been reported to enhance their tolerance of multiple stresses (Ma, 2004; Guntzer, 2011), including pests and pathogens (Garbuzov *et al.*, 2011; Dallagnol *et al.*, 2012), metal toxicity (Rizwan *et al.*, 2012; Habibi, 2014*a*), salt and water stress (Hattori *et al.*, 2005; Liu *et al*., 2014). The mechanisms underlying silicon's capacity to increase stress resistance are still poorly understood. It has been reported that Si causes an improvement of water use efficiency and stimulation of antioxidative defense system in winter wheat (Liang *et al*., 2008), *Paspalum vaginatum* (He *et al*., 2010) and cucumber leaves (Liu *et al.* 2009). Increase in production of antioxidants and decline of ROS generation mediated by Si causes reduction of photo-oxidative damage, maintenance of chloroplast membranes integrity and thus enhancement of plants stress tolerance (Liang *et al*., 2008; Waraich *et al*., 2011).

One of the major problems arising in some pistachio cultivation areas includes different levels of injuries caused by lower temperatures in early spring. Because of the fact that the yield of pistachio was reduced due to frost damage, the understanding of the physiological and biochemical mechanisms improving freezing tolerance of this species is very important.

We hypothesize that the possible mechanisms for Si enhanced freezing stress may be attributed to the higher antioxidant defense activity and lower membrane oxidative damage through better water retention in leaf tissues. To test this hypothesis, we examined the effect of Si on the growth parameters, leaf water retention, and the enzymatic and non-enzymatic antioxidants and the membrane lipid peroxidation of freezing-stressed pistachio plants.

2 MATERIALS AND METHODS

2.1 Plant growth and treatments:

Seeds of pistachio (*Pistacia vera* 'Ahmadaghaii') were sown in top of the cylindrical plastic pots. Pots were 14 cm in diameter and 105 cm in depth, filled with 15 kg sandy loam soil (pH 7.6, EC 1.32 dS m^{-1}, field capacity (FC) 23 %, organic carbon (OC) 1.09 %). After emergence, the seedlings were thinned to one plant per pot and irrigated with distilled water every 5 days to maintain at 90 % field capacity. plants were grown in a growth chamber located near the city of Miandoab, NW Iran (46º6' E and 36º46' N) with day/night temperature of 25 ºC/18 ºC, relative humidity of 45–55 % and daily photon flux density (PFD) of about 1100–1200 µmol m^{-2} s^{-1} throughout the experimental period. Seven weeks after sowing, half of the plants were sprayed with 10 mM Si (as K_2SiO_3, pH adjusted to 5.8 with phosphoric acid). A drop of Tween 20 (0.05 %, v/v) as surfactant was added to 500 ml of the spray solutions. Five days after the treatment, half of the control (untreated with Si) and half of the Si-treated plants were placed to a controlled environment chamber under a 12 h (1±1 °C) light (at 300 µmol m^{-2} s^{-1} photosynthetic photon flux)/12 h (–2±1 °C) dark cycle at 85 % relative humidity for 2 days. After the freezing treatment, all plants were returned to normal conditions as described above, to allow leaves to recover from freezing stress. Samples were taken 2, 48 and 96 h after recovery after cold

treatment. Each measurement was done independently and experiments were repeated at least three times.

2.2 Analysis of growth parameters:

Leaves and roots were separated and washed with distilled water, blotted dry on filter paper and after determination of fresh mass (FM) they were dried for 48 h at 70 °C for determination of dry mass (DM). Relative water content (RWC) was measured and calculated according to Lara *et al*. (2003).

2.3 Assay of enzymes activity and related metabolites:

Activities of antioxidant enzymes were determined according to the methods described elsewhere (Habibi 2014*b*). For the determination of superoxide dismutase (SOD, EC 1.15.1.1) activity, enzyme was extracted in 25 mM HEPES pH 7.8 with 0.1 mM EDTA and the supernatant was added to the reaction mixture contained 0.1 mM EDTA, 50 mM Na_2CO_3 pH 10.2, 13 mM methionine, 63 µM nitroblue tetrazolium chloride (NBT), 13 µM riboflavin. One unit of SOD was defined as the amount of enzyme which produced a 50 % inhibition of NBT reduction under assay conditions. For the determination of catalase (CAT, EC 1.11.1.6) activity, samples were homogenized with 50 mM phosphate buffer pH 7.0 and assayed spectrophotometrically by following the degradation of H_2O_2 at 240 nm. Reaction medium contained 50 mM phosphate buffer pH 7 and 10 mM H_2O_2. Peroxidase (POD, EC 1.11.1.7) activity was determined using the guaiacol test at 470 nm. The enzyme was extracted by 10 mM phosphate buffer pH 7.0 and assayed in a solution contained 10 mM phosphate buffer, 5 mM H_2O_2 and 4 mM guaiacol. Ascorbate peroxidase (APX, EC 1.11.1.11) activity was assayed by following reduction in absorbance at 290 nm as ascorbate was oxidized according to the method of Boominathan and Doran (2002). The reaction mixture contained 50 mM phosphate buffer pH 7, 0.2 mM EDTA, 0.5 mM ascorbic acid and 50 µg bovine serum albumin (BSA). Lipid peroxidation was estimated from the amount of malondialdehyde (MDA) formed in a reaction mixture containing thiobarbituric acid (Sigma) at 532 nm. MDA levels were calculated from a 1,1,3,3-tetraethoxypropane (Sigma) standard curve. The hydrogen peroxide (H_2O_2) contents in the leaves were assayed according to the method of Velikova et al. (2000). Leaves were homogenized in ice bath with 0.1% (w/v) TCA. The extract was centrifuged at 12,000 × g for 15 min, after which to 0.5 ml of the supernatant was added 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI, the reaction was improved for 1 h in the dark and measured spectrophotometrically at 390 nm. The content of H_2O_2 was given on a standard curve.

The total soluble proteins were measured according to the Bradford protein assay (Bradford, 1976).

To assay for PAL activity, leaf samples were ground in 50 mM sodium phosphate buffer (pH 7.0) containing 2 % (w/v) polyvinylpolypyrrolidon (PVPP), 2 mM EDTA, 18 mM β-mercaptoetanol and 0.1 % (v/v) Triton X-100. After centrifugation (15000 g for 15 min at 4 °C), PAL was assayed in the supernatant by measuring the formation of cinnamic acid at 290 nm according to modified method of Zucker (1965). Enzyme extracts were incubated at 30 °C for 60 min with 5 mM L-phenylalanine in 60 mM sodium borate buffer (pH 8.8). One unit (U) of PAL activity was defined as the amount of the enzyme that produced 1 nmol cinnamic acid per h. Total phenolic content was determined using the Folin-Ciocalteau method as modified by Velioglu *et al*. (1998). Gallic acid was used for constructing the standard curve. Results were expressed as mg gallic acid (GA) per gram of the fresh weight.

2.4 Measurement of cell death:

Cell death was measured according to the method described by Schützendübel *et al*. (2001). After cold and Si treatments, leaf tips were inserted in Evans blue solution (0.025 % (w/v) Evans blue in water) for 30 min, followed by washing with water for 15 min. The trapped Evans blue was released from the leaves by homogenizing leaf tips in 1.6 mL of 50 % (v/v) MeOH and 1 % (w/v) SDS, and then centrifuged for 15 min. The optical density of the supernatant was determined at 600 nm and expressed on the basis of fresh mass. The percentage of necrotic area was calculated by measuring separately green and necrotic leaf area according to Irigoyen *et al*. (1996).

Leaves were prepared for determination of Si (Jaiswal 2004) using Inductively-Coupled Plasma-Atomic Emission Spectrometry (*ICP-AES, INTEGRA XL2, GBC,* Australia).

2.5 Statistical analysis:

Experiment was undertaken in complete randomized block design with 4 pots as 4 independent replications. Statistical analyses were carried out using Sigma stat (3.5) with Tukey test ($p < 0.05$). Correlation analysis using Spearman Rank Order Correlation in Sigma Stat (3.5) were conducted to determine the relationship between the measurement metabolites and the percentage of necrotic area.

3 RESULTS

As shown in Table 1, freezing alone significantly reduced the shoot fresh weight of pistachio plants. Addition of 10 mM Si under cold conditions significantly increased the shoot fresh weight of plants as compared with freezing alone. No significant changes of SDM, RFM and RDM were found by foliar application of Si under both freezing and normal temperatures. Cold alone decreased relative water content (RWC) by 13 % after treatment for 96 h recovery, but foliar-applied Si decreased significantly damaging effects of cold on RWC. Freezing alone increased necrotic leaf area by 7.6 % after treatment for 96 h recovery, but the increase was only 3.4 % when Si was applied. Si content was elevated by foliar application of Si, but it was not affected by freezing stress during all treatment periods.

Table 1: Effect of Si supplementation on the shoot fresh mass (SFM), shoot dry mass (SDM), root fresh mass (RFM), root dry mass (RDM), relative water content (RWC), necrotic leaf area, and the content of Si in pistachio plants after 96 h recovery after freezing treatment. Data of each row indicated by the same letters are not significantly different ($p < 0.05$). Data are the mean ± SD ($n = 4$)

Parameters	Control		96 h Rec	
	−Si	+Si	−Si	+Si
SFM (g plant^{-1})	10.4±2.21 ^{a}	10.5±1.86 ^{a}	7.00±1.11^{b}	8.91±1.03 ^{a}
SDM (g plant^{-1})	0.91±0.16 ^{a}	0.84±0.08 ^{a}	0.77±0.10 ^{a}	0.86±0.06 ^{a}
RFM (g plant^{-1})	3.73±0.40 ^{a}	4.16±0.69 ^{a}	3.56±0.22 ^{a}	3.81±0.43 ^{a}
RDM (g plant^{-1})	1.07±0.36 ^{a}	0.98±0.21 ^{a}	0.87±0.27 ^{a}	0.92±0.13 ^{a}
RWC (%)	70±3.2 ^{a}	73±1.8 ^{a}	57±3.0 ^{b}	69±2.4 ^{a}
Necrotic leaf area (%)	00.0±00.0 ^{c}	00.0±00.0 ^{c}	7.60±1.10 ^{a}	3.40±0.52 ^{b}
Leaf Si (mg g^{-1} DM)	0.79±0.22 ^{b}	2.16±0.85 ^{a}	0.86±0.33 ^{b}	2.48±0.52 ^{a}

Freezing treatment dramatically increased the PAL activity. Compared with freezing treatment alone, the PAL activity was not affected after 2, 46 and 96 h recovery, by supplementary Si following the freezing treatment (Fig. 1). Similarly, the leaf phenolic content was not influenced by supplementary Si. The percentage of Evans dye uptake increased under freezing stress in the non-Si-treated plants. In Si-supplemented plants, however, uptake of Evans dye did not rise upon cold exposure during the experimental period.

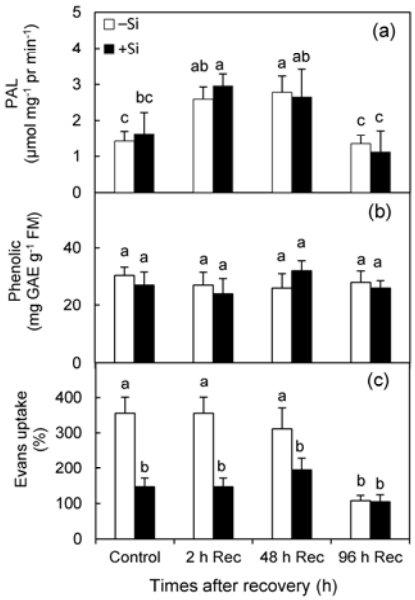

Figure 1: Changes in specific activity of phenylalanine ammonia lyase (PAL) (a), the concentration of total phenolics (b) and the percentage of uptake of Evans dye (c) in pistachio plants grown with (+Si) or without Si (-Si) supplementation after 2, 46 and 96 h recovery after freezing treatment. Bars indicated with the same letter are not significantly different ($p < 0.05$). Data are the mean ± SD ($n = 4$).

Freezing caused a significant increase in the activities of all analyzed antioxidant enzymes with the exception of APX. As shown in Fig. 2, the activities of antioxidant enzymes increased under freezing stress while Si application caused further increase being significant for SOD and POD activities. Freezing stress induced membrane damage as was indicated by higher MDA concentration (Fig. 3). However, the addition of Si to the freezing treatment significantly decreased MDA content compared with the corresponding freezing-treatment with no Si added. Cold stress caused a significant accumulation of hydrogen peroxide, and the continuation of the recovery time with or without Si application caused a further accumulation of H_2O_2. A positive correlation was found between the concentration of MDA and the percentage of necrotic leaf area ($r = 0.96$, $p < 0.01$ in cold treatment; $r = 0.76$, $p < 0.01$ in cold+Si treatment, Fig. 4). There was not a significant correlation between the concentration of H_2O_2 and the percentage of necrotic leaf area.

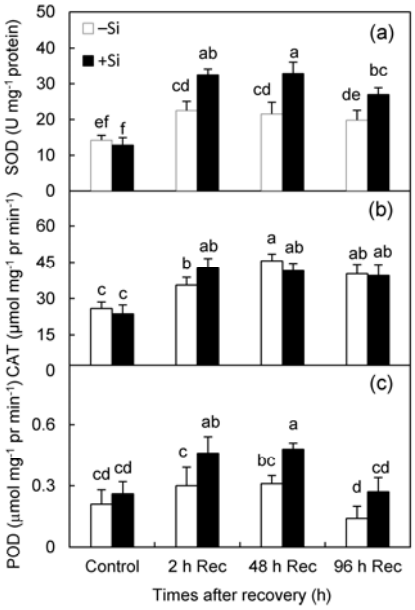

Figure 2: Effect of foliar-applied Si on (a) the specific activity of superoxide dismutase (SOD), (b) catalase (CAT) and (c) peroxidase (POD) in pistachio after 2, 46 and 96 h recovery after freezing treatment. Bars indicated with the same letter are not significantly different ($p < 0.05$). Data are the mean ± SD ($n = 4$).

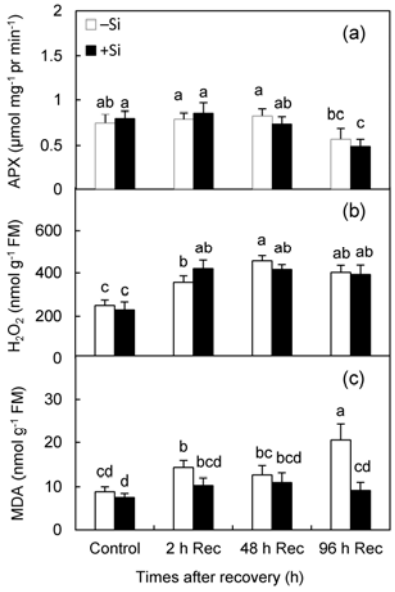

Figure 3: Effect of foliar-applied Si on (a) the specific activity of ascorbate peroxidase (APX), (b) the concentration of hydrogen peroxide (H_2O_2) and (c) malondialdehyde (MDA) in pistachio after 2, 46 and 96 h recovery after freezing treatment. Bars indicated with the same letter are not significantly different ($p < 0.05$). Data are the mean ± SD ($n = 4$).

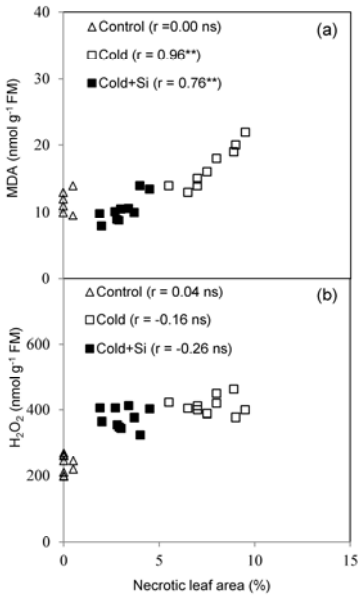

Figure 4: Correlations between the concentration of (a) malondialdehyde (MDA) and the percentage of necrotic leaf area and (b) between the concentration of hydrogen peroxide (H_2O_2) and the percentage of necrotic leaf area in pistachio plants grown with or without Si supplementation after 2, 46 and 96 h recovery after freezing treatment: ns, *, and **: non-significant, significant at the 5 % and 1 % levels of probability, respectively.

4 CONCLUSION

Freezing-sensitive plants exposed to low temperature often show signs of water stress due to decreased root hydraulic conductance, leading to associated decreases in leaf water and turgor potential, followed by a reduction of growth (Waśkiewicz *et al*., 2014). In this study, we have shown that the foliar-applied Si decreased significantly damaging effects of cold on RWC, accompanied by an increase in SFM. The main mechanism for such roles of Si in maintaining higher water content in leaf tissues is hypothesized to be the reduced transpirational water loss via reduction of both cuticular and stomatal transpiration, and through improvement of water uptake via increased volume and weight of roots (Cooke and Leishman, 2011; Sonobe *et al*., 2011). Leaf necrosis is considered as a typical external sign of freezing injury in freezing-sensitive plants. In this work, pre-Si treatment caused a significant reduction of the leaf area lost by freezing.

It has been demonstrated that cold stress induces the activity of PAL, which is the prime intermediary in the biosynthesis of phenolics, and is considered by most authors to be one of the main lines of cell acclimation against stress in plants (Levine *et al*., 1994). In the present study, there was a remarkable increase in PAL activity during recovery. These results are consistent with other authors who consider PAL to be one of the prime elements of cell acclimation against thermal stress in plants (Levine *et al*., 1994; Bharti and Khurana, 1997). The results obtained by Hossain *et al*. (2007) with oat leaves have demonstrated that Si reduces the activity of PAL. In the present experiment, leaf phenolic content was not affected by supplementary Si. Therefore, the possibility that exogenously applied Si directly influences the activity of PAL seems thin.

Earlier experiments support the positive correlation between higher activities of antioxidant enzymes and freezing tolerance (Zhang *et al*., 2011; Kishimoto *et al*., 2014). The magnitude of oxidative damage is usually measured by MDA (an end product of membrane lipid peroxidation), and Evans dye absorption, two markers for the ROS-mediated cell membrane damage (Liu *et al*., 2009). In the present work, freezing stress caused great membrane damage, as assessed by lipid

peroxidation, but Si application significantly reduced the membrane damage because of an efficient scavenging by SOD and POD (Fig. 2). There is data supporting that Si increases the activity of antioxidant enzymes such as POD and SOD (Liu *et al*., 2009), which in turn protect plants against ROS generation and lipid peroxidation. Recently, we have reported that the stability of plasma membranes in leaves of drought-stressed pistachio were mediated by addition of Si (Habibi and Hajiboland, 2013), which is associated with Si-enhanced antioxidant defense capacity in drought-stressed plants (Waraich *et al*., 2011). Thus, it is clear that Si can enhance antioxidant defense activity in plants under drought and freezing stress, resulting in decreased membrane oxidative damage, improved stability of cell membranes and enhanced stress tolerance.

In this research, despite the increasing POD activity, Si-supplied plants accumulated the higher levels of H_2O_2 after freezing treatment. The increase in the concentration of H_2O_2 may act as a signal for recovery ability from freezing injury. The significant correlation between the concentration of MDA and the percentage of necrotic leaf area confirmed the idea that, even if active oxygen formation was increased, the defense mechanisms had sufficient capacity or could be induced, with the result that damage was not apparent. The results indicated that the application of Si could prevent lipid peroxidation of stressed pistachio plants obviously because of higher POD and SOD activities.

In conclusion, Si-supplemented cold-stressed plants exhibited better protection from oxidative damage, and this ability was associated with the higher CAT and SOD activities and the lower level of lipid peroxidation. These data indicate that an application of Si can be used to promote the induction of the antioxidant system in plants, thereby improving stress resistance. One of the major problems arising in some pistachio cultivation areas includes different levels of injuries caused by lower temperatures in early spring. Our results suggest that improvement of plant tolerance to cold stress by Si supplementation is achieved by activation of antioxidant defense capacity. In addition, results demonstrated that the possibility that exogenously applied Si directly influences the activity of PAL seems thin. However further research is needed to solve the relation between the phenolic content and cold tolerance in pistachio plants upon Si supplementation.

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