J. Plant Biol. (2016) 59:247-259
DOI 10.1007/s12374-016-0526-1

ORIGINAL ARTICLE

Autotoxicity in Cucumber (*Cucumis sativus* L.) Seedlings is Alleviated by Silicon Through an Increase in the Activity of Antioxidant Enzymes and by Mitigating Lipid Peroxidation

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Received: November 7, 2015 / Accepted: February 21, 2016

Abstract Autotoxicity in plants limits their growth and that of nearby plants of the same species, which has obvious implications in crop yield and quality. Silicon (Si) has been shown to increase plant tolerance to autotoxic stress. However, the physiological mechanisms underlying the effects of Si in alleviating autotoxicity during germination in cucumber (*Cucumis sativus* L.) are unknown. Cinnamic acid derivatives, such as 3-phenylpropionic acid (PA), are a class of autotoxins present in cucumber root exudates. Our objective was to investigate Si-induced autotoxic stress tolerance in cucumber seedlings by focusing on the effects of Si on the induction of antioxidant defense pathways. We found that PA treatment significantly reduced seed germination, radicle length, lateral root number, fresh weight, AsA and GSH contents, and the activities of SOD, CAT, and APX in cucumber seedlings, while it increased membrane permeability and levels of MDA, proline, $O_2^{\bullet-}$, and H_2O_2. Application of Si enhanced growth of PA-treated plants and significantly increased germination rate, radicle length, lateral root number, fresh weight, AsA and GSH levels, and SOD, CAT, POD, and APX activities. These results suggest that exogenous Si alleviates autotoxicity caused by PA during seed germination by increasing antioxidant enzyme activities and mitigating lipid peroxidation.

Keywords: Antioxidant enzymes, Cucumber seed germination, Lipid peroxidation, Non-enzymatic antioxidant, 3-phenylpropionic acid (PA), Reactive oxygen species

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Introduction

Silicon (Si) is the second most abundant element after oxygen in the soil. Si dioxide comprises 50-70% of the soil mass. Si is taken up by plant roots in the form of silicic acid [$Si(OH)_4$], an uncharged monomeric molecule (Ma and Takahashi 2002). Plants differ widely in their capacity to absorb and accumulate Si, ranging from 0.1% to 10.0% Si (dry weight) (Epstein 1999; Ma and Takahashi 2002; Richmond and Sussman 2003). Therefore, all plants rooted in soil include some Si in their tissues. It has been demonstrated that Si can mitigate not only biotic stresses (e.g., pathogens and pests), but also abiotic stresses such as salinity, water stress, heavy metal toxicity, and nutrient imbalance (Gong et al. 2005; Liang et al. 2007; Ashraf et al. 2010; Chen et al. 2011; Mateos-Naranjo et al. 2013). However, whether exogenous Si can alleviate autotoxic stress has not been reported. Therefore, the purpose of this study was to investigate the effects of exogenous Si on autotoxicity in cucumber.

Autotoxicity is one of many diverse abiotic stresses, and it is one of the main causes of decline in crop yield and quality, which is present widely in vegetable crops, especially cucurbit crops. Previous studies have shown that cucumber plants have the capacity to express autotoxicosis by liberating autotoxins, such as 3-phenylpropionic and cinnamic acid (Yu and Matsui 1994; Yu et al. 2000). Additional research has shown that the autotoxic substances extracted from root exudates greatly increase ion leakage, and are accompanied by increased membrane permeability (Yu and Matsui 1997), and also by increased antioxidase activities (Yu et al. 2003). Therefore, it is necessary and important to investigate the mechanisms of autotoxicity in order to devise strategies to control it. It has been reported that Si

enhances resisitance to Mn-stress in cucumber through a reduction in membrane lipid peroxidation together with an increase in the activity of antioxidant enzymes (e.g. SOD, APX, dehydroascorbate reductase, and glutathione reductase) and also non-enzymatic antioxidants (e.g. AsA and GSH) (Shi et al. 2005b). The alleviation of salt stress and cadmium (Cd) toxicity by Si in plants through reduced membrane peroxidation and increased antioxidase activities and non-enzymatic antioxidants has been demonstrated in cucumber (Zhu et al. 2004), tomato (Al-Aghabary et al. 2004) and *Brassica chinensis* (Song et al. 2009). Some studies have also shown that Si can enhance the activities of SOD, CAT, and POD enzymes that decrease oxidative damage during stress (Gong et al. 2008; Shen et al. 2010). Si treatment increased the activities of SOD, POD, CAT and the content of GSH but decreased the content of malondialdehyde (MDA) in leaves and roots of barley subjected to salt stress. Si also stimulated root H^+-ATPase and H^+-PPase activities in the plasma membranes and tonoplasts, and mediated membrane uidity, suggesting that Si may affect the structure, integrity, and functions of plasma membranes (Liang et al. 1996, 2003, 2005b, 2006; Liang 1999). However, at present, there is no usable information showing that Si alleviates autotoxicity in the antioxidative system in cucumber during seed germination.

Therefore, the objective of this study was to investigate whether exogenous Si application could alleviate autotoxic stress through its effects on membrane lipid peroxidation and induction of antioxidant mechanisms during seed germination in cucumber. In order to explore the possible underlying mechanisms, we studied the effects of Si on artificially induced autotoxicity in cucumber seedlings by examining the cell ultrastructure of root tip, concentrations of PA and Si, reactive oxygen species (ROS), membrane permeability, lipid peroxidation, antioxidant enzyme activity, and non-enzymatic antioxidant concentrations in Si-treated and control plants. Trying to elucidate Si-mediated detoxication effect of autotoxin induced by PA in change of antioxidant enzymes and cell membrane electrolyte osmotic in cucumber seedlings.

Results

Symptoms of PA Toxicity and the Concentrations of PA and Si in Plant Tissues

Following treatment with 2 mM PA for 72 h, the cucumber seedling radicles showed specific morphological changes; the radicles were shorter and thicker, and the lateral roots were bulging and deformed (Fig. 1A). However, Si application successfully prevented the symptoms of PA-induced autotoxicity in roots of cucumber seedlings (Fig. 1A). Compared with the control, 2 mM PA treatment for 72 h significantly reduced germination rate, radicle elongation, the number of lateral roots, and the fresh weight (Fig. 1B, C, D, and E). When compared with the distilled water control treatment, the inhibition rate of radicle elongation reached 83.26% in the PA treatment (Fig. 1B). However, exogenous Si significantly increased the germination rate of cucumber seeds, the number of lateral roots, and the fresh weight, and also promoted radicle elongation even in the presence of PA (Fig. 1B, C, D, and E). Compared with the PA or PA + NaOH treatments, treatment with PA + Si significantly promoted cucumber seed germination (Fig. 1A).

Our study concentrated on PA toxicity and the effects of Si on ameliorating that toxicity in cucumber seedlings. To examine the influence of Si application on the PA binding potential of the seedlings, cucumber seedlings were fractionated for the extraction of PA. As shown in Fig. 2A, Si-treated seedlings showed a tendency to accumulate lower PA concentrations than did non-Si-treated seedlings under PA-induced stress. Compared with the control, the PA content in seedlings increased significantly in the PA treatment. Si treatment markedly decreased PA contents in cucumber seedlings. For example, compared with the PA treatment alone, application of Si decreased PA content in PA-treated seedlings by 42.3%. Si concentration in cucumber seedlings was 1.7 and 1.0 times as high as in the Si treatment alone and the PA + Si treatment compared to the control and PA treatment, respectively (Fig. 2B). It is important to note that the concentration of Si measured in the cucumber seedlings showed little difference between the Si-only treatment and the PA + Si treatment (Fig. 2B).

Cell Ultrastructure

The cellular structure of the root tip cell appeared to be normal when treated with distilled water in the control. The cell wall and cell membrane showed a tight association with each other, and the cell membrane, cytoplasm, and organelles were plentiful and dense (Fig. 3A). However, root tip cells of cucumber seedlings were significantly deformed after treatment with PA (Fig. 3C). The cell walls appear to have become loose, the cell membranes are ruptured and bulging, and plasmolysis is evident in the root tip cell wall. The intercellular spaces have expanded, fluid has accumulated in the cells, the nuclear membranes and nucleoli have disappeared, but starch granules are present in the root tip cells.

Application of Si to PA-stressed seedlings resulted in root tip cells with cell walls, nuclear membranes, and cell organelles that resembled those in the control seedlings (Fig. 3D). In the PA+NaOH treatment, the cell walls and vacuoles

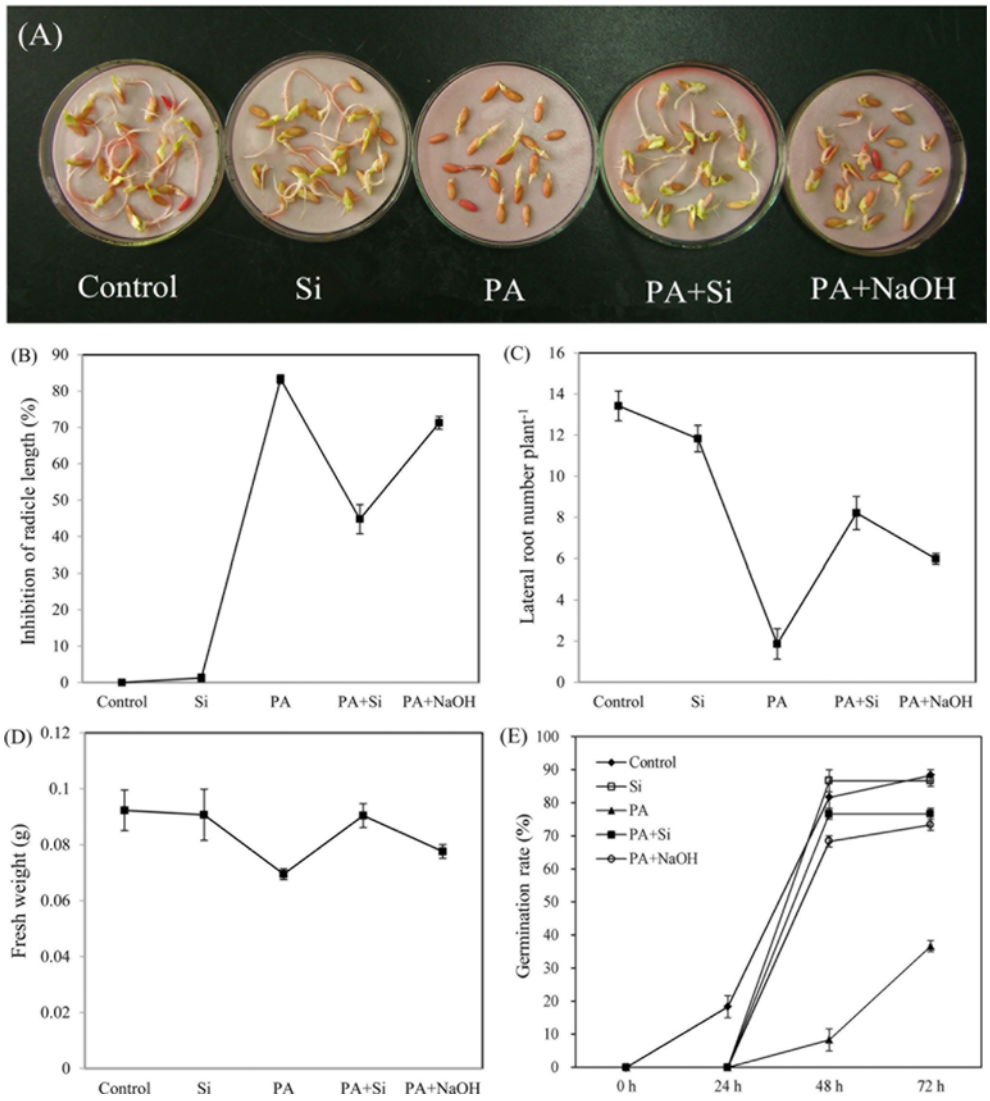

Fig. 1. Effects of exogenous Si on the germination and growth of cucumber seedlings treated with PA. (A) Effects of PA and Si on the phenotype of cucumber seedlings. The five treatments were: (1) control (distilled water), (2) 2 mM Si, (3) 2 mM PA, (4) 2 mM PA + 2 mM Si, (5) 2 mM PA + NaOH. Photographs were taken at 72 h of treatment. (B), (C) and (D) Effects of exogenous Si on inhibition of radicle length, lateral root number, and fresh weight, respectivly, in cucumber seedlings after 72 h of treatment. (E) Effects of PA and Si treatments on germination in cucumber seeds at 0, 24, 48 and 72 h of treatment. Inhibition of radicle length, lateral root number, fresh weight, and germination rate were expressed as mean ± SE (n=3, each replicate consisted of 20 cucumber seeds).

became loose and enlarged, respectively, plasmolysis is quite pronounced, and even more starch grains are present in the root tip cells (Fig. 3E).

Electrolyte Leakage Percentage (ELP), Damage Rate, and MDA Contents

ELP and damage rate are determinants of cell membrane injury. As shown in Fig. 4A, PA stress largely increased ELP in cucumber seedlings after 48 h of treatment. Si addition significantly decreased ELP, and the relative decrease in the PA + Si treatment was larger than in the PA + NaOH treatment. PA-induced stress largely increased the cell membrane damage rate in cucumber seedlings treated for 48 h (Fig. 4B). Si addition significantly decreased the cell membrane damage rate, and the relative decrease in the PA + Si treatment was also larger than in the PA + NaOH treatment.

To further determine the effects of Si and PA on membrane lipid peroxidation in cucumber seedlings, we measured the MDA contents of the cucumber seedlings at 0, 4, 8, 12, 24, 36, 48, and 60 h after treatment. Fig. 4C shows that the relative changes in MDA contents in the control and Si-treated cucumber seedlings were very similar, and reached a maximum at 48 h. MDA levels in the PA- and PA + NaOH-treated seedlings reached maximum values at 36 h and 48 h,

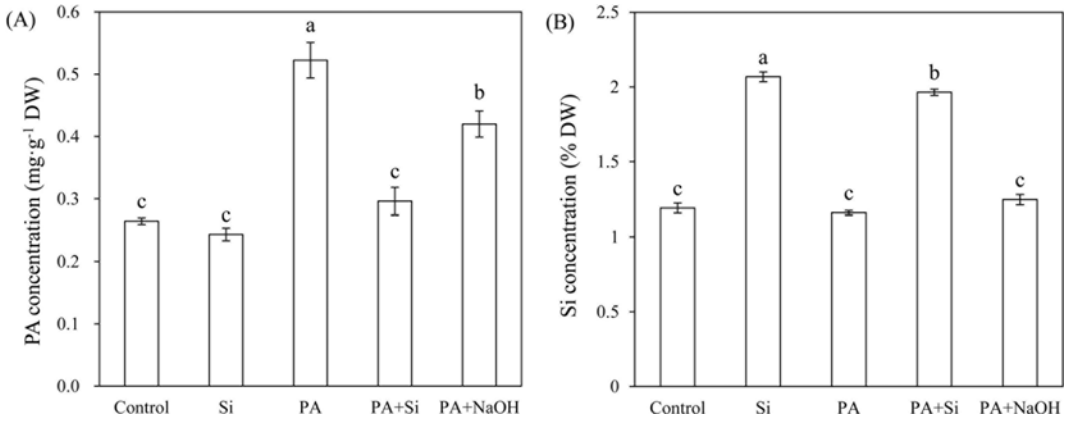

Fig. 2. PA (A) and Si (B) concentrations in cucumber seedling tissues after 72 h of treatment. Treatments consisted of (1) control plants (distilled water), (2) 2 mM Si, (3) 2 mM PA, (4) 2 mM PA + 2 mM Si, (5) 2 mM PA + NaOH. Vertical bars represent the means ± SE from three independent experiments. Different lower-case letters above the vertical bars indicate signicant differences by Duncan's multiple range tests ($P<0.05$).

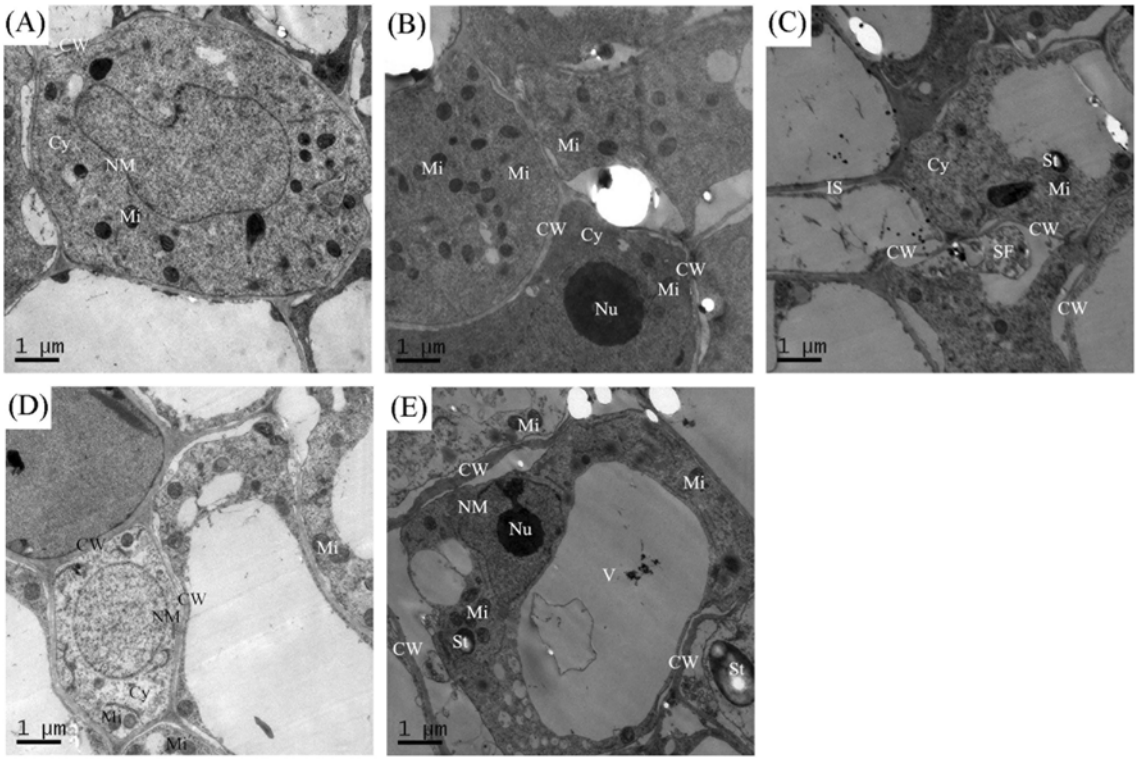

Fig. 3. Transmission electron micrographs of root tip cells in cucumber seedling after 72 h of treatment. (A) control plants (distilled water), (B) 2 mM Si, (C) 2 mM PA, (D) 2 mM PA + 2 mM Si, (E) 2 mM PA + NaOH. Scale bars indicate 1 μm. Abbreviations: CW, cell wall; Cy, cytoplasm; Nu, nucleolus; NM, nuclear membrane; Cm, cytomembrane; Mi, mitochondria; V, vacuole; IS, intercellular space; St, starch granule; SF, swelling fluid.

respectively. Compared with the control, the maximum MDA level in PA-stressed cucumber seedlings occurred earlier, and was significantly higher. However, compared with the control, the MDA level peaked in the PA + Si treatment starting at 36 h, and remained the same until 48 h, after which it declined (Fig. 4C). And compared with PA-treated seedlings, Si application caused a relative decrease in MDA content in the PA-stressed seedlings at 36 h.

$O_2^{\bullet-}$, H_2O_2, and Proline Contents

The $O_2^{\bullet-}$ levels in all treatment groups increased initially and then declined over time in cucumber seedlings. Fig. 5A shows that changes in $O_2^{\bullet-}$ contents in the control, PA, and

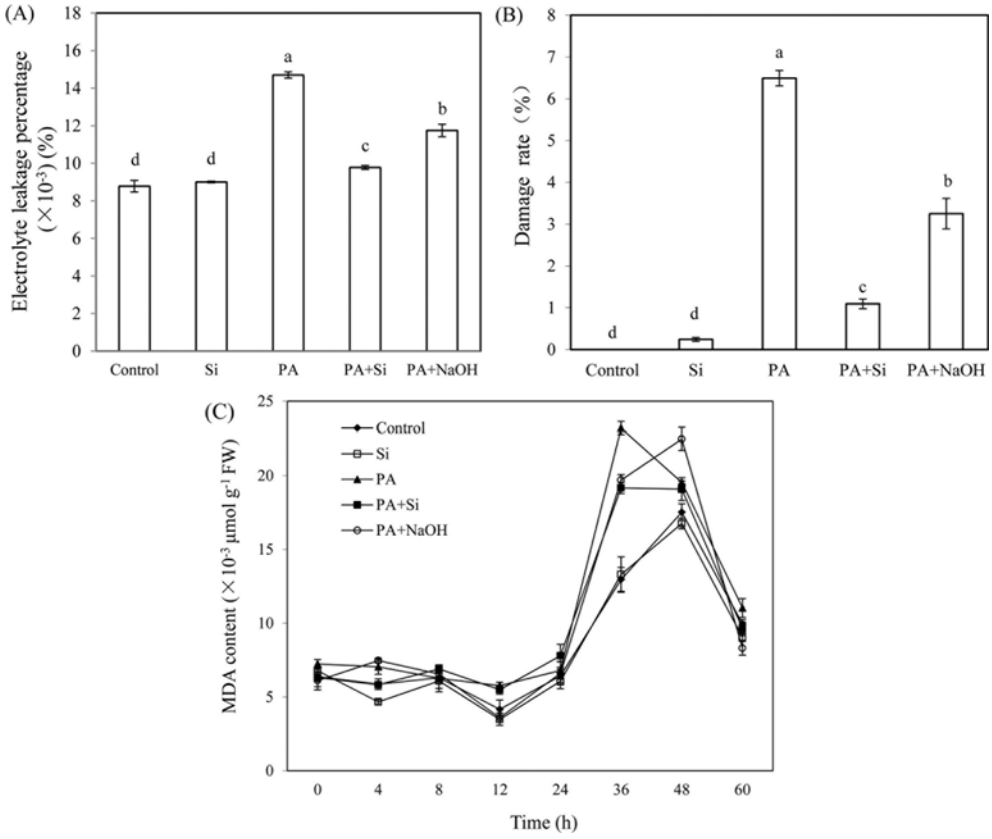

Fig. 4. Electrolyte leakage (%) (A), damage rate (B), and malondialdehyde content (C) in cucumber seedlings subjected to different treatments. Treatments consisted of (1) control plants (distilled water), (2) 2 mM Si, (3) 2 mM PA, (4) 2 mM PA + 2 mM Si, (5) 2 mM PA + NaOH. Vertical bars represent mean ± SE from three independent experiments. (A) and (B) Electrolyte leakage percentage and damage rate in cucumber seedlings after 48 h of treatment. Different lower case letters indicate signicant differences by Duncan's multiple range tests ($P<0.05$).

PA + Si treatments showed a similar trend, reaching the maximum at 12 h. Changes in $O_2^{\bullet-}$ levels in the Si and PA + NaOH treatments showed a similar tendency, peaking at 8 h. Compared with the control, PA-induced stress significantly increased the $O_2^{\bullet-}$ content during the germination phase; however, Si application dramatically decreased $O_2^{\bullet-}$ in the cucumber seedlings treated with PA.

The H_2O_2 levels changed over time and reached the maximum at 24 h in all treatment groups. The results showed that H_2O_2 levels in cucumber seedlings in all treatment groups showed a similar trend with respect to time during the germination phase; levels peaked at 24 h, and then declined to starting levels or below by 48 h (Fig. 5B). Compared with the control, PA-induced stress significantly increased H_2O_2 content over the entire germination phase, but addition of Si dramatically decreased H_2O_2 levels in the cucumber seedlings treated with PA.

Unlike $O_2^{\bullet-}$ and H_2O_2, the proline levels in all treatment groups showed an intial decline at 8 h and then increased again before declining at the end of the experiment (Fig. 5C). The changes in proline levels in the PA, PA + NaOH, and PA + Si treatments showed similar trends, peaking at 36 h, while the control and the Si-only treatments also were nearly indistinguishable from one another over the course of the germination phase (Fig. 5C). From 12 h to 36 h, PA-induced stress significantly increased proline content in cucumber seedlings, but this increase in proline content was reversed in the PA + Si treatment.

Antioxidant Contents

The changes in AsA contents of cucumber seedlings over time are shown in Fig. 6A. AsA contents in all treatmemts showed a similar trend; they increased gradually until 24 h and then declined by 48 h. The control, Si, and PA treatments all peaked at 24 h, while the of PA + Si and PA + NaOH treatments peaked at 12 h and maintained the maximum values until 36 h (Fig. 6A). This result showed that PA-induced stress significantly lowered AsA content in all phases of germination; however, addition of Si markedly increased AsA content in seedlings treated with PA. In the non-PA-stressed (control) cucumber seedlings, Si treatment

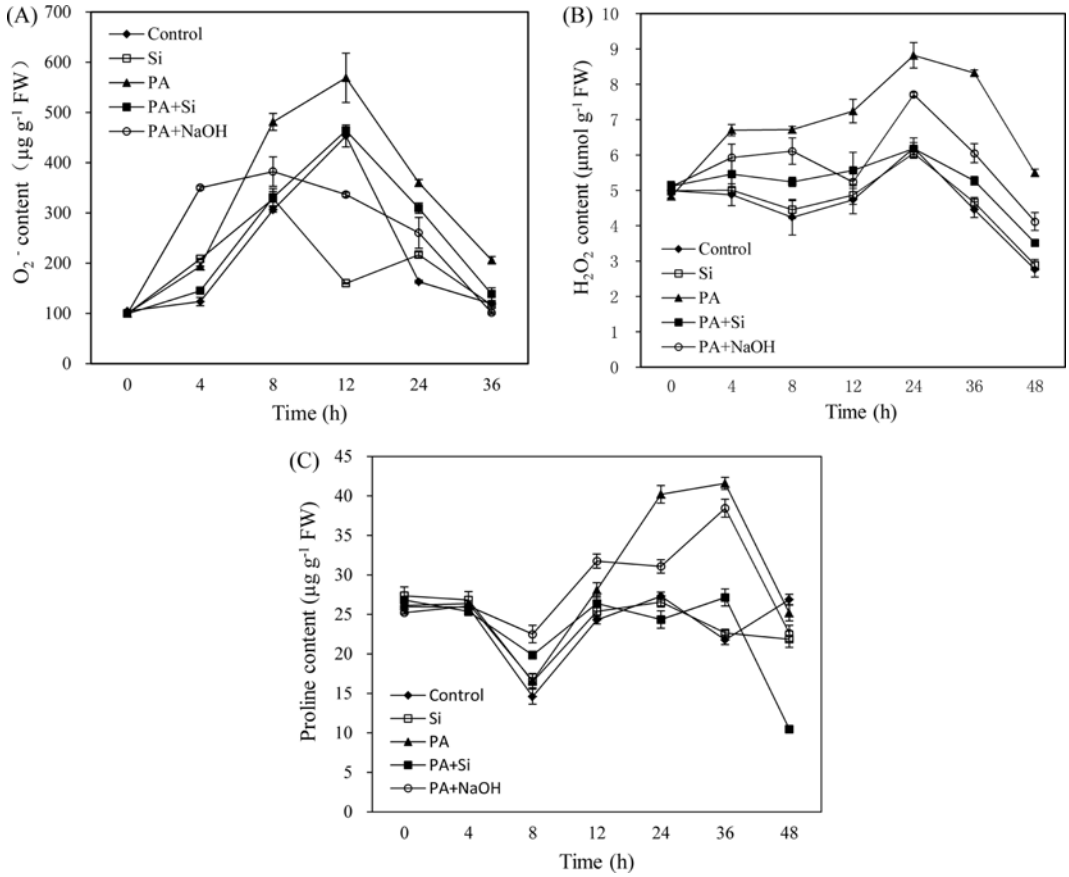

Fig. 5. Superoxide anion (A), hydrogen peroxide (B), and proline (C) contents in cucumber seedlings subjected to different treatments. Treatments consisted of (1) control plants (distilled water), (2) 2 mM Si, (3) 2 mM PA, (4) 2 mM PA + 2 mM Si, (5) 2 mM PA + NaOH. Vertical bars represent the means ± SE from three independent experiments.

significantly decreased AsA content between 12 h and 48 h of treatment.

As shown in Fig. 6B, GSH contents in cucumber seedlings increased initially in all treatment groups and then declined to starting levels at 36 h. All treatments peaked at 24 h with the exception of the PA + Si treatment, which peaked at 12 h. The changes in GSH content in the control and the Si-only treatments displayed a similar trend, as did the PA and PA + NaOH treatments. PA-induced stress significantly decreased GSH content in cucumber seedlings at 12 h and 24 h. GSH content in seedlings experiencing PA-induced stress increased dramatically after 4 h and reached a maximum at 12 h, which was 12 h earlier than in all the other treatments. In the control cucumber seedlings, GSH content increased slightly in response to Si treatment between 12 h and 36 h.

Antioxidant Enzyme Activities

To further examine the influence of Si and PA on antioxidant enzyme activities in cucumber seedlings, we measured the SOD, CAT, POD, and APX activities at 0, 4, 8, 12, 24, 36, 48, 60, and 72 h of treatment. As shown in Fig. 7A, the SOD activity in all treatment groups increased rapidly and peaked at 4 h, and there was no significant difference among treatments at 4 h. After 4 h of treatment, the results showed that PA-induced stress significantly decreased SOD activity from 8 h to 48 h compared to all other treatments. However, SOD activity increased significantly in the PA + Si treatment. There was no significant difference between Si alone and control. SOD activity decreased in the PA treatment and increased when Si was added. Therefore, PA treatment decreased SOD activity and Si application reversed the negative effects of PA on this enzyme.

PA-induced stress significantly decreased CAT activity in cucumber seedling during germination; however, Si significantly increased CAT activity in the PA + Si treatment at all timepoints except for 8 h (Fig. 7B). In the non-PA-treated cucumber seedlings, Si treatment increased CAT activity at 4 h and decreased CAT activity from 8 h to 48 h of treatment, although the effects were slight.

As shown in Fig. 7C, PA treatment did not affect POD

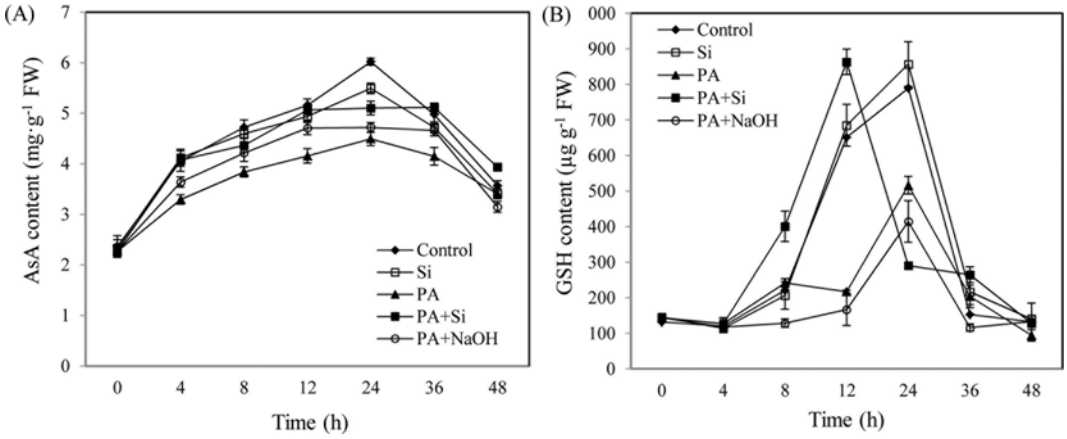

Fig. 6. Ascorbic acid (AsA) (A) and glutathione (GSH) (B) contents in cucumber seedlings subjected to different treatments. Treatments consisted of (1) control plants (distilled water), (2) 2 mM Si, (3) 2 mM PA, (4) 2 mM PA + 2 mM Si, (5) 2 mM PA + NaOH. Vertical bars represent the means ± SE from three independent experiments.

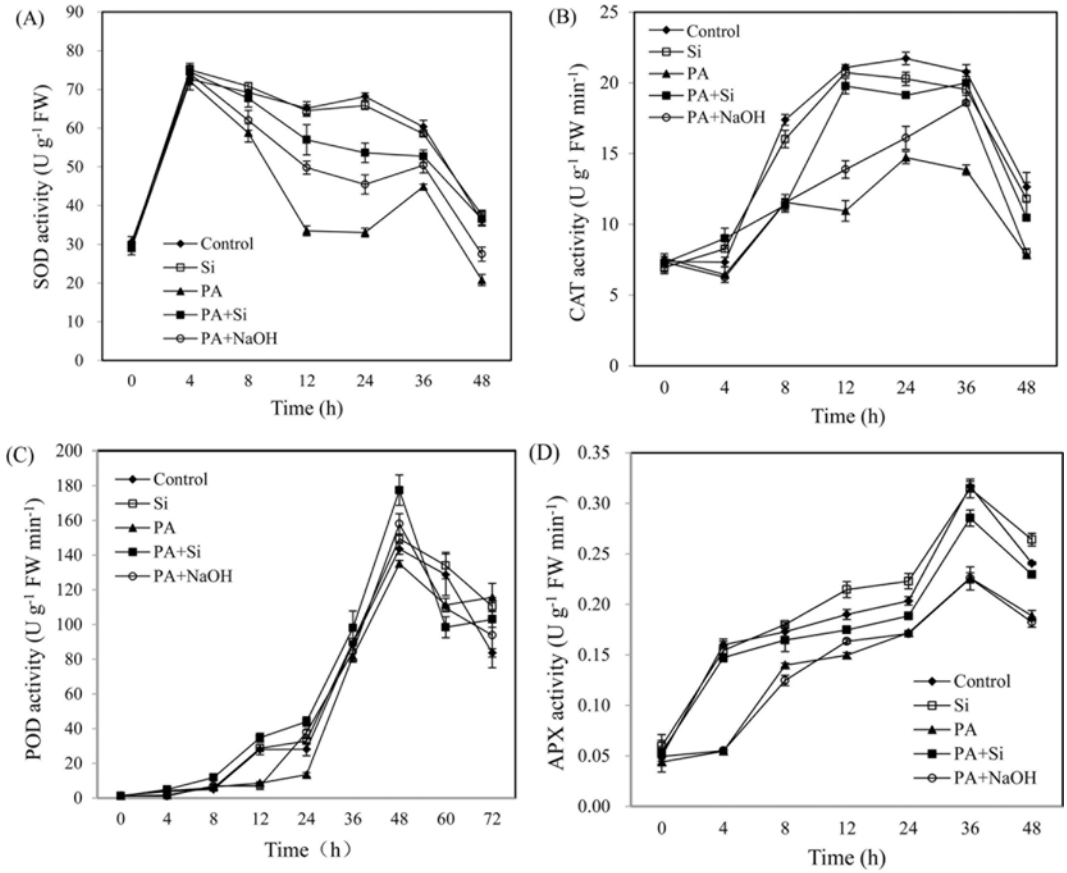

Fig. 7. Activities of superoxide dismutase (SOD) (A), catalase (CAT) (B), peroxidase (POD) (C), and ascorbate peroxidase (APX) (D) in cucumber seedlings subjected to different treatments. Treatments consisted of (1) control plants (distilled water), (2) 2 mM Si, (3) 2 mM PA, (4) 2 mM PA + 2 mM Si, (5) 2 mM PA + NaOH. Vertical bars represent means ± SE from three independent experiments.

activity from 0 h to 8 h, and generally reduced POD activity from 12 h to 60 h of treatment. Treatment with Si alone did not affect POD activity from 0 h to 8 h of treatment and significantly increased POD activity between 12 h and 48 h. POD activity peaked at 48 h in all treatments.

APX activities in all treatment groups showed a similar trend during germination in cucumber seedlings; there was an initial increase that peaked at 36 h, followed by a decrease at 48 h of treatment (Fig. 7D). Compared with the control, PA treatment significantly decreased APX activity at all

timepoints, but this was effect was alleviated in the PA + Si treatment. In the non-PA-stressed cucumber seedlings treated with Si alone, a slight increase in APX activity was observed at 12, 24, and 48 h of treatment.

Discussion

Plant Growth, Absorption of PA and Si, and Root Tip Cell Ultrastructure in Cucumber Seedlings

PA (a cinnamic acid derivative) was previously identified from cucumber root exudates (Yu and Matsui 1994; Yu et al. 2000) and was used to investigate the effect of autotoxins on cucumber seedling root growth and ROS metabolism regulation. The symptoms of PA toxicity were coupled with a reduction in plant growth (determined by radicle length), the number of lateral roots, fresh weight, and germination rate (Fig. 1B, C, D, and E), which was consistent with results of previous reports (Yu and Matsui 1994, 1997; Yu et al. 2003; Batish et al. 2008; Zhang et al. 2009). Si is known to be beneficial to plant growth and helps plants overcome abiotic and biotic stresses (Ma and Takahashi 2002; Gong et al., 2005; Guo et al., 2005; Pavlovic et al., 2013). Autotoxicity is considered to be an abiotic stress and, in this experiment, we found that application of Si significantly stimulated plant growth in PA-treated seedlings (Fig. 1). The result also shown that NaOH added (acid neutralization) also promoted plant growth in PA-treated seedlings, but the mitigating effect of NaOH was significantly weaker than Si (Fig. 1).

The beneficial effects of Si are mainly associated with its high level of deposition in plant tissues, which enhances their strength and rigidity (Ma and Yamaji 2006). Si is taken up by the roots in the form of silicic acid [$Si(OH)_4$] (Ma and Takahashi 2002). Cucumber is an important culinary vegetable used worldwide, and it can actively absorb Si (Liang et al. 2005a). In the present study, we found that the concentration of Si in cucumber seedlings was increased by exogenous Si addition (Fig. 2B), and this was attributed to the higher available Si concentration in the + Si treatments (the available Si concentration in external solution the + Si treatments was increased by 73.73% in non-PA treatment and 69.40% in the PA + Si treatment compared with the control) and partially alleviated the inhibitory effect of PA on biomass accumulation (Fig. 2A). The positive linear relationship between the Si concentration in soybean seedlings and wheat flag leaves, and the available Si concentration in soil, was also discovered by Li et al. (2004) and Ma et al. (2015). Our result is also consistent with the reports that Si reduced uptake and transport of Cd in maize (Liang et al. 2005c), rice (Shi et al. 2005), and pakchoi (Song et al. 2009). Previous studies also showed that Si can alleviate the negative influence of boron toxicity in spinach shoots (Gunes et al. 2007a) and cadmium stress in *Brassica chinensis* L. (Song et al. 2009) via decreased uptake of boron and cadmium. In the present study, Si-treated cucumber seedlings exposed to PA were shown to have lower concentrations of PA than seedlings treated with PA (Fig. 2A). Thus, Si inhibited the absorption of PA in cucumber seedlings. The main reason attributed to this is that Si can increase cell wall thickness and maintain the integrity and function of the cell wall, thus inhibiting plant absorption of PA (Fig. 3D). Most of these alleviating functions are also ascribed to Si deposited in cell walls of plants (Ma and Yamaji 2006). It also has been reported that the silicification of cells proceeds from silica cells to silica bodies (Ma and Yamaji 2006). In addition to leaf blades, silicified cells are also observed in the epidermis and vascular tissues of the stem, leaf sheath, and hull in rice. The deposition of Si protects plants from multiple abiotic and biotic stresses (Ma and Yamaji 2006). The silicified cells also provide useful palaeoecological and archaeological evidence known as plant opals or phytoliths (Hodson et al. 2005).

Membrane Peroxidation and Osmotic Regulation

Liang et al. (2007) reported that Si can stimulate the antioxidant system in plants and affect the structure of the plasma membrane so as to alleviate abiotic stresses. Under drought and salt stresses, the alleviating effects of Si have been accompanied by an increase in antioxidant defense abilities (Liang et al. 2003; Zhu et al. 2004; Gong et al. 2005). Our results show that added Si greatly reduced ELP and the rate of cell membrane damage in cucumber seedlings under PA-induced stressed at 48 h of treatment, and also that the relative decrease in the PA + Si treatment was larger than in the PA + NaOH treatment (Fig. 4A, B). This result indicates that Si treatment may decrease the permeability of plasma membranes and reduce membrane lipid peroxidation to maintain membrane integrality and functions in cucumber seedlings treated with PA. Similarly, a previous study also showed that Si decreased ELP and strengthened the stability of lipids in cell membranes of cucumber under salt stress (Zhu et al. 2004). Addition of Si reduced the changes in plasma cell membrane permeability in leaf cells that was induced by salt-stressed (Liang et al. 1996; Liang 1999). It has also been reported that Si plays a role in enhancing the stability of lipids in rice cell membranes under drought and heat stresses (Agarie et al. 1998), suggesting that Si prevents the structural and functional destruction of cell membranes when rice plants are subjected to environmental stresses. In order to analyze Si-induced PA tolerance, we conducted a time-course experiment focusing on reactive oxygen metabolism, membrane peroxidation, and antioxidant activities in PA-

treated cucumber seedlings with or without Si. The results show that ROS can accumulate in seedlings experiencing PA-induced stress, resulting in lipid peroxidation as shown by the MDA increased concentrations (Fig. 4C). Si increased the contents of antioxidants, and this was coupled with reductions in the MDA and H_2O_2 concentrations in drought stressed wheat leaves (Ma et al. 2015). Similarly, in the present study, in response to PA-induced stress, the levels of MDA, $O_2^{\bullet-}$, H_2O_2, and proline decreased in cucumber seedlings treated with Si compared to the non-Si treatments (Fig. 4C; Fig. 5A, B, C), indicating that Si application can also increase the antioxidant levels in seedlings to alleviate PA-induced oxidative damage.

Antioxidant Enzymes and Non-enzymatic Antioxidants

Another important finding from our study is the Si-mediated enhancement of the antioxidant defense system in cucumber seeds and seedlings exposed to PA-induced stress. We found that the application of PA observably decreased GSH contents, but this effect was reversed by the addition of Si, and the GSH level peaked 12 h before it did in the control and the PA-treated seedlings (Fig. 6B). AsA contents also decreased in PA-treated cucumber seedlings, and this effect was also reversed somewhat by the presence of Si (Fig. 6A). AsA and GSH are strong antioxidants which function as redox buffers in the plant, protecting the plasma membranes from oxidative damage (Foyer et al. 2001). The results of this study appear to indicate that application of Si increases the activity of ROS scavengers and increased PA tolerance, consequently reducing the adverse effects of PA in suppressing germination (Fig. 1). It has been reported that the presence of Si enhanced antioxidant defense activity and increased tolerance to oxidant-induced injury in salt-stressed barley (Liang 1999; Liang et al. 2003) and cadmium-stressed pakchoi (Song et al. 2009). The functions of Si in adjusting antioxidant defense activity and inhibiting membrane peroxidation were also verified in salt-stressed cucumber (Zhu et al. 2004), drought stressed wheat (Gong et al. 2005), excess manganese-stressed cucumber (Shi et al. 2005), excess boron-stressed spinach and wheat (Gunes et al. 2007a, b), and cold-stressed wheat (Liang et al. 2008). The results of these previous studies seem to show that the Si enhanced antioxidant defense system is a common response to diverse types of abiotic stresses in both Si-accumulating and non-Si-accumulating plants. Our study also offers supporting evidence that exogenous application of Si plays an important role in increasing ROS biosynthesis to alleviate oxidative stress.

In the AsA-GSH cycle, APX plays a major role in eliminating H_2O_2. In the present study, the activity of APX was significantly decreased (Fig. 7D), and the content of H_2O_2 was dramaticlly increased (Fig. 5B) in cucumber seedlings treated with PA, implying that reduced APX function resulted in higher H_2O_2 levels in PA-stressed cucumber seedlings. Nevertheless, the application of Si restored much of the APX activity (Fig. 7D) and decreased the content of H_2O_2 (Fig. 5B) in PA-stressed cucumber seedlings, indicating that Si can improve plant tolerance to PA-induced oxidative damage by enhancing the speed of the AsA-GSH cycle. The production of reactive oxygen derivatives increases when plants are exposed to various environmental stresses. Plants have an effective system of reactive oxygen species scavenging that protects them from damaging oxidation reactions (Foyer et al. 1994). In this system, the defense mechanisms are mainly due to the presence of antioxidative enzymes. Numerous changes in the activities of antioxidant enzymes were detected in cucumber seedlings under autotoxic stress (Fig. 6A, B, C, D), notably decreases in SOD, CAT, and APX activity (Fig. 7A, B, D). Therefore, ROS might accumulate in PA-stressed cucumber seedlings, causing lipid peroxidation, as indicated by the elevated levels of MDA (Fig. 4), and also increased levels of $O_2^{\cdot-}$, H_2O_2, and proline (Fig. 5A, B, C) in the cucumber seedlings after treatment with PA. The results show that PA treatment reduced the activity of antioxidant enzymes and weakened the ability of the cells to remove ROS in cucumber seedlings after a certain time. These results were consistent with the results of Zhang et al. (2014), but inconsistent with those of Ye et al. (2006) and Ding et al. (2007). Also, we found no effect on POD activity after PA treatment in this experiment (Fig. 7C). This was not consistent with the results of Yu et al. (2003), who detected an increase in POD activity in cucumber root triggered by root extracts and root exudates of cucumber. However, the effect of autotoxicity on the changes in antioxidant enzymes is extremely complicated and depends on the treatment time, plant species, and genotypes. In this study, the differences in results of autotoxicity on the activities of antioxidant enzymes were also detected with treatment time.

The results of the present experiment indicated that Si addition increased the activities of SOD, CAT, POD, and APX in the PA-treated cucumber seedlings (Fig 7A, B, C, D), and also relieved the lipid peroxidation as shown by the reduction in H_2O_2, $O_2^{\cdot-}$, MDA, and proline contents (Fig. 4C; Fig. 5A, B, C), suggesting that oxidative injury due to PA toxicity can be alleviated by the application of Si. This defense mechanism to oxidative stress may play a vital role in preventing plants from sustaining autotoxin damage. The results of the present study are in line with the findings of Zhang et al. (2014) and Liang (1999) in cucumber and barley, respectively, which showed that addition of Si reduced the permeability of the plasma membrane of leaf cells and reduced the level of lipid peroxidation. The useful

functions of Si are most evident in plants exposed to various abiotic and biotic stresses (Ma and Yamaji 2006), and this was supported by the results of our study.

We studied the roles of Si in increasing tolerance to autotoxic stress in germinating cucumber seedlings and provided compelling evidence that Si acts to alleviate the effects of PA treatment. It is notable that, while sodium silicate ($Na_2SiO_3 \cdot 9H_2O$) is alkaline and PA is acidic, the pH of the 2 mM PA + NaOH treatment was the same as in the of 2 mM PA + 2 mM Si treatment (see Materials and Methods). Our results show that exogenous application of NaOH had a small positive effect on plant growth and other parameters in the PA-stressed seedlings, and the main alleviating mechanism of NaOH was acid-base neutralization. However, the exogenous NaOH did not have comparable effects to Si, suggesting that simple acid neutralization is not the only mechanism of protection against autotoxic stress in this study. Several studies in monocots (rice and wheat) and dicots (cucumber) have shown that plants supplied with Si can produce phenolics and phytoalexins in response to abiotic and biotic stresses (Ma and Yamaji 2006). Ma and Takahashi (2002) reported that Si might play an active role in enhancing host resistance to plant diseases by stimulating defense reaction mechanisms. Si is also able to activate some defense mechanisms. For example, in roots of cucumber plants infected and colonized by *Pythium*, Si was found to enhance the activity of chitinases, peroxidases, and polyphenoloxidases (Chérif et al. 1994). In conclusion, all the results presented here provide unequivocal evidence to indicate that Si protects the radical per se under PA-induced stress and also promotes plant growth; the beneficial effects were more visible in seedlings subjected to PA-induced stress than in the controls.

Conclusion

The addition of Si significantly reduced the levels of ELP, and MDA, and lowered the endogenous concentrations of PA, $O_2^{\bullet -}$, proline, and H_2O_2, while increasing the relative activities of SOD, CAT, POD, and APX and the contents of AsA and GSH in PA-stressed cucumber seedlings. The beneficial effects of Si were more obvious under conditions of PA-induced stress. These results suggest that Si may act to alleviate autotoxic stress in cucumber seedlings by reducing the permeability of plasma membranes and reducing membrane lipid peroxidation, and also by protecting the cell wall and membrane integrity and function. A marked increase in the activities of antioxidant enzymes and an increase in antioxidant levels in PA-stressed seedlings following Si application suggests that Si may be involved in the metabolic or physiological activities in cucumber seedlings that are affected by PA-induced autotoxic stress.

Materials and Methods

Plant Materials, Growth Conditions, and Treatments

Cucumber (*Cucumis sativus* L. cv. 'Xinchun No. 4') seeds were germinated at 25ºC in darkness. Twenty seeds were put in petri dishes (9 × 9 cm) on two layers of filter paper saturated with 10 mL of either distilled water or the treatment solutions for 72 h. Solutions were renewed and the number of germinating seeds was counted every day, and the germination rate was then calculated.

Autotoxicity was initiated by treating seedlings with 2 mM 3-phenylpropionic acid (PA; Sigma, St Louis, MO, USA). Si treatment was applied as sodium silicate ($Na_2SiO_3 \cdot 9H_2O$, AR). The experiment was conducted in a growth chamber at the Vegetable Cultivation and Physiology Lab of Gansu Agricultural University in Lanzhou, Gansu (northwest China).

Treatments, with three independent replications, consisted of (1) control (distilled water), (2) 2 mM Si, (3) 2 mM PA, (4) 2 mM PA + 2 mM Si, (5) 2 mM PA + NaOH (the pH was adjusted to be the same as in the 2 mM PA + 2 mM Si treatment). The concentrations of PA and Si used here were selected based on the results of preliminary exprements (data not shown).

Determination of Radicle Length, Number of Lateral Roots, and Fresh Weight

The standard of seed germination was defined as the germ length reached to be the seed full-length of cucumber. The fresh weights of 20 seedlings from each treatment, with three independent replications, were measured and used to calculate averages. For each treatment, three replicates were performed. The radicle length was defined as being from the root tip to the junction between the radicle and hypocotyl (with embossmen), and was measured with a ruler for each seedling. The lateral roots were considered to be those attached to the main root that were visible to the naked eye. For each treatment, 20 seedlings were selected for evaluation and the average radicle length and number of lateral roots were determined.

Cell Ultrastructure Observations

Tissue squares (each 1 × 3 mm) were excised with scissors from the root tip of cucumber seedlings 72 h after treatment according to the method of Kim et al. (2002). The specimens were fixed in a solution consisting of 3% (v/v) glutaraldehyde and 8% (w/v) saccharose in 0.2 M phosphate buffered saline (pH 7.4) at 4ºC overnight and then washed with the same buffer three times for 15 min each. The specimens were postfixed with 1% (w/v) osmium tetroxide in the same buffer at 4ºC for 5 h and washed with 0.2 M phosphate buffered saline (pH 7.4) three times for 15 min each. The postfixed specimens were dehydrated in a graded ethanol series (50, 70, 80, 90, and 100 %) three times for 15 min each and two times in 100 % acetone for 15 min each. The specimens were further treated with acetone:resin (1:1) at room temperature for 5 h and 100% resin at room temperature overnight and embedded in Spurr's medium. Ultrathin sections (approximately 50 nm thick) were made with a diamond knife using an ultramicrotome (MT-X; RMC Inc., Tucson, AZ). The sections were mounted on copper grids and stained for 7 min each with 2% uranyl acetate and Reynolds' lead citrate (20). The sections were examined by transmission electron microscopy (TEM) using a model JEM-1230 transmission electron microscope (JEOL Ltd., Tokyo) at an accelerating voltage of 80 kV. For each specimen, more than three

ultrathin sections were examined with the microscope.

Determination of PA and Si Contents in Cucumber Seedlings

Cucumber seedling samples at 72 h after treatment were dried at 105ºC for 15 min and 80ºC for 12 h, then ground to a powder for determination of PA and Si. Samples of leaf powder (0.25 g) were then soaked in 10 mL of methanol for 0.5 h and were ultrasonically extracted for 45 min. After vacuum ltration through a 0.22 μm membrane, the filtrate was used for determination of the PA concentration in the leaf using a Waters 2487 High Performance Liquid Chromatography system (Waters Technologies, US). The chromatographic column used was a Waters C18 (4.6 × 250 mm, 5 μm). The mobile phase was acetonitrile-1% acetic acid (95:5), and the injection volume was 20 μL. Chromatography was performed at 254 nm and 25ºC with a flow-rate of 1.0 mL·min^{1} for 4 min. The protocol used was based on the improved method of Dai et al. (2012).

Si in seedling tissue was measured using the blue silicon-molybdous method according to Van der Vorm (1987). Dried and ground samples (0.3 g) were put into a nickel crucible with 10 mL 4 M NaOH solution and digested by heating for 3 h at 550ºC. The ash was then transferred to 250 mL plastic test tubes. Color development was completed by adding 10 mL of extracting solution and 1 mL of a reagent mixture consisting of 1.8 M H_2SO_4 and ammonium molybdate (0.38 M). After 10 min, 4 mL of 0.67 M tartaric acid ($C_4H_6O_6$) and 1 mL of a reducing reagent mixture consisting of 0.056 M Na_2SO_3, 6.3 M 1-Amino-2-naphthol-4-sulfonic acid ($C_{10}H_9NO_4S$) and 0.87 M $NaHSO_3$ were added, and the mixture was diluted with distilled water to 50 mL and mixed. After 30 min, the absorbance was measured at 640 nm.

Determination of Electrolyte Leakage Percentage (ELP), Damage Rate, and Membrane Lipid Peroxidation

ELP was used to evaluate plasma membrane permeability and was measured using an electrical conductivity meter (DDS-307A). The method used was that of Lutts et al. (1996). Cucumber seedling samples (0.5 g) at 48 h after treatment were placed in individual stoppered triangular flasks containing 10 mL of deionized water. The samples were incubated at 25ºC on a shaker at 100 rpm for 24 h. The electrical conductivity of the solution (EC1) was measured after incubation. The samples were then placed in a boiling water bath for 10 min and the second measurement (EC2) was determined after cooling the solutions to room temperature. ELP was calculated as EC1/EC2 and converted into percent. The damage rate was calculated using the following formula:

$$\text{Damage rate (\%)} = [1-(1-\text{ELP})/(1-\text{ELP}_{CK})] \times 100.$$

Membrane lipid peroxidation in cucumber seedlings was determined by measuring the MDA content using the method of Dhindsa et al. (1981). The samples (0.5 g) of cucumber seedlings were extracted with 4 mL of 10% trichloroacetic acid, followed by centrifugation at 10,000 × *g* for 10 min. The resulting supernatant (2 mL) was mixed with 2 mL of 0.6% thiobarbituric acid and was then heated at 100ºC for 20 min. The absorbance was determined at 450, 532, and 600 nm.

Measurement of $O_2^{\bullet-}$, H_2O_2, and proline contents

The content of $O_2^{\bullet-}$ was assayed as described by Elstner and Heupel (1976). Samples (0.5 g) of the cucumber seedlings were ground in the presence of liquid nitrogen and were homogenized in 10 mL of 65 mM phosphate buffer (pH 7.8). After centrifugation at 12,000 ×*g* at 4ºC for 10 min, 2 mL of the supernatant was mixed with 1.5 mL of 65 mM phosphate buffer (pH 7.8) and 0.5 mL of 10 mM hydroxylammonium chloride and then incubated at 25ºC for 20 min. The color reaction was performed by adding 2 mL of 17 mM sulfanilamide and 2 mL of 7 mM α-naphthylamine an incubating for 30 min. The mixture was centrifuged at 12,000 × *g* for 10 min, and the absorbance at 530 nm was immediately measured. The concentration of $O_2^{\bullet-}$ was computed according to a standard curve of $NaNO_2$ reagent.

The H_2O_2 concentration of the cucumber seedlings was determined colorimetrically based on the method of Mukherjee and Choudhuri (1983). To determine H_2O_2 contents, seedling samples (0.5 g) were extracted with cold acetone (4ºC). The supernatant (2 mL) was mixed with 0.1 mL of 5% (w/v) titanium sulfate and 0.2 mL concentrated ammonia liquor. The mixture was then centrifuged at 3,000 ×*g* for 10 min. After centrifugation, the supernatant was discarded, and 5 mL of 2 M H_2SO_4 was added to the precipitate. The solution absorbance was measured at 415 nm.

The content of proline in the cucumber seedlings was determined at 520 nm by the method of Bates et al. (1973).

Measurement of Non-enzymatic Antioxidant Contents

Cucumber seedlings (0.5 g) were ground in liquid nitrogen and were homogenized in 2 mL of 6% trichloroacetic acid (TCA). After centrifugation at 12,000 ×*g* at 4ºC for 20 min, the supernatant was used to assay for AsA content based on the method of Kampfenkel et al. (1995).

For GSH determination, 0.5 g samples of cucumber seedlings were ground with liquid nitrogen, homogenized in 10 mL of 0.5 mM $EDTANa_2$-3% trichloroacetic acid and centrifuged at 10,000 ×*g* at 4ºC for 10 min. The supernatant was subjected to GSH measurement as described by Guri (1983).

Antioxidant Enzyme Activity Assays

Cucumber seedlings (0.5 g samples) were ground in liquid nitrogen, and suspended in 5 mL of phosphate buffer (50 mM, pH 7.8) containing 5 mM EDTA, 2 mM AsA and 2% polyvinylpyrrolidone (PVP). After centrifugation at 12,000 ×*g* for 20 min at 4ºC, the supernatant was used for determination of the activities of SOD (EC 1.15.1.1), CAT (EC 1.11.1.6), APX (EC 1.11.1.11), and POD (EC 1.11.1.7) (Ramiro et al. 2006).

The activity of SOD was determined by inhibiting the photochemical reduction of nitroblue tetrazolium (NBT) as described by Hwang et al. (1999). One unit of SOD was defined as the amount of enzyme that inhibits NBT reduction by 50%. The activity of CAT was measured at 240 nm through the degradation of H_2O_2 ($\varepsilon = 40\ M^{-1}\ cm^{-1}$) (Pereira et al. 2002). The activity of APX was determined by following the decrease in AsA oxidized by H_2O_2 ($\varepsilon = 2.8\ M^{-1}\ cm^{-1}$) and measuring the change in absorbance at 290 nm for 1 min (Nakano and Asada 1981). POD (EC 1.11.1.7) activity was assayed according to the method of Liang et al. (2003) with minor modifications; 0.1 mL of the enzyme extract was mixed with 2.6 mL guaiacol (0.3% in 50 mM phosphate buffer, pH 6.5) and 0.3 mL 0.6% H_2O_2 and the change in absorbance was measured at 470 nm for 2 min.

Statistical Analysis

Seeds were arranged randomly in a manually-controlled climate chamber with three replicates per treatment. The data presented was the mean±SE of three replicates for each group. Statistical differences between treatments were analyzed using Duncan's multiple range test. Differences were considered significant at a probability level of $P < 0.05$. All statistical analyzes were performed using the Statistical Package for Social Sciences for Windows (version 17.0; SPSS, Inc., Chicago, IL, USA).

Acknowledgements

This research was financially supported by the National Natural

Science Foundation of China (31260493), and the Special Fund for Agro-scientific Research in the Public Interest (201203002).

Author's Contributions

JX and RB conceived and designed the experiments. RB, CW, and JY performed the experiments. RB and JX analyzed the data. JX, JY, and JL contributed reagents/materials/analysis tools. RB and JX contributed to the writing of the manuscript. JX, WL, XX, and ACU contributed to the modification of the manuscript.

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