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# Silicon-mediated alleviation of Mn toxicity in *Cucumis sativus* in relation to activities of superoxide dismutase and ascorbate peroxidase

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**Abstract**

The effects of exogenous silicon (Si) on plant growth, activities of superoxide dismutase (SOD), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), glutathione reductase (GR) and catalase, and concentrations of ascorbate and glutathione were investigated in cucumber (*Cucumis sativus* L.) plants treated with excess manganese (Mn) (600 μM). Compared with the treatment of normal Mn (10 μM), excess Mn significantly increased $H_2O_2$ concentration and lipid peroxidation indicated by accumulation of thiobarbituric acid reactive substances. The leaves showed apparent symptoms of Mn toxicity and the plant growth was significantly inhibited by excess Mn. The addition of Si significantly decreased lipid peroxidation caused by excess Mn, inhibited the appearance of Mn toxicity symptoms, and improved plant growth. This alleviation of Mn toxicity by Si was related to a significant increase in the activities of SOD, APX, DHAR and GR and the concentrations of ascorbate and glutathione.


*Keywords:* *Cucumis sativus* L; Cucurbitaceae; Excess manganese; Silicon; Lipid peroxidation; Antioxidant enzymes; Ascorbate; Glutathione

## 1. Introduction

Manganese (Mn) toxicity is a major factor limiting plant growth in both acidic and waterlogged soils. Under such conditions, large and toxic amounts of Mn can become plant-available and this can retard plant growth and development by interfering with some metabolic processes (Le-Bot et al., 1990; Lidon and Teixeira, 2000; Hauck et al., 2003). Excess Mn induces inhibition of chlorophyll biosynthesis and a decline in the photosynthetic rate (Macfie and Taylor, 1992; Hauck et al., 2002). Moreover, as a transition metal, Mn is involved in the initial formation of reactive oxygen species (ROS) and their subsequent reactions with macromolecules such as proteins, lipids, polysaccharides and nucleic acids leading to altered membrane fluidity, loss of enzyme activity and genomic damage (Lidon and Henriques, 1993; Stochs and Bagchi, 1995).

*Abbreviations:* ROS, reactive oxygen species; SOD, superoxide dismutase (EC1.15.1.1); NBT, nitroblue tetrazolium; GPX, guaiacol peroxidase (EC 1.11.1.7); APX, ascorbate peroxidase (EC 1.11.1.11); CAT, catalase (EC 1.11.1.6); DHAR, dehydroascorbate reductase (EC 1.8.5.1); GR, glutathione reductase (EC 1.6.4.2); AsA, reduced ascorbate; DHA, dehydroascorbate; GSH, reduced gultathione; GSSG, oxidized glutathione; PVP, polyvinylpyrrolidone; EDTA, ethylenediaminetetraaceticacid; TBARS, thiobarbituric acid reactive substances.

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Table 1
Effects of Si supply on shoot height, root length, shoot dry weight, root dry weight and total dry weight of 10-day treatment cucumber plants grown in nutrient solutions containing normal (10 μM) and excess (600 μM) Mn

| Treatment | Shoot height (cm) | Root length (cm) | Shoot dry weight (g/plant) | Root dry weight (g/plant) | Total dry weight (g/plant) |
|---|---|---|---|---|---|
| CK | 49.40 ± 1.70 b | 66.35 ± 2.05 b | 4.11 ± 0.16 b | 0.45 ± 0.020 b | 4.56 ± 0.18 b |
| Si | 57.23 ± 1.93 a | 71.65 ± 1.82 a | 4.75 ± 0.33 a | 0.51 ± 0.020 a | 5.26 ± 0.33 a |
| Mn | 36.45 ± 2.09 d | 43.33 ± 2.00 d | 2.87 ± 0.08 d | 0.33 ± 0.021 d | 3.20 ± 0.08 d |
| Si + Mn | 42.6 ± 3.15 c | 48.23 ± 1.45 c | 3.52 ± 0.10 c | 0.41 ± 0.020 c | 3.93 ± 0.10 c |
| $LSD_{0.05}$ | 2.04 | 3.09 | 0.55 | 0.054 | 0.21 |

Data are means ± SD of four replicates. Within a column, mean values followed by different letters (a, b, c and d) are significantly different ($p < 0.05$).

Liming has been the most important approach used to reduce soil acidity, and to, thereby, alleviate Mn toxicity in acidic soils. However, Mn toxicity is not always economically reducible with conventional liming. Although silicon (Si) is the second mostly abundant element in soil, it is not considered to be an essential element for higher plants (Epstein, 1999). However, there is increasing evidence that Si has a number of beneficial effects on plant growth under biotic and abiotic stresses (Rogalla and Rmheld, 2002; Heckman et al., 2003; Zhu et al., 2004). During the induction of systematic acquired resistance (SAR) in cucumber, the expression of a gene encoding a novel proline-rich protein is enhanced (Kauss et al., 2003), which has C-terminal repetitive sequences containing an unusually high amount of lysine and arginine. The synthetic peptide derived from the repetitive sequences is able to polymerize orthosilicic acid to insoluble silica, which is known to be involved in cell wall reinforcement at the site of the attempted penetration of fungi into epidermal cells. The study of Kauss et al. (2003) has provided insight into a biochemical and molecular basis of Si-enhanced disease resistance. With regard to abiotic stresses, species-specific differences have been observed for interactions of Si with Mn toxicity. In rice, Si treatment changes the distribution of Mn by increasing Mn concentration in the roots but decreasing that in the tops, and therefore reduces the symptoms of Mn toxicity in leaves (Horiguchi, 1988). In cowpea, Si supply does not affect the total Mn concentration (Iwasaki et al., 2002). The alleviation of Mn toxicity by Si is due to not only the decrease of the concentration of soluble apoplastic Mn through the enhanced adsorption of Mn on the cell walls, but also the increase of the concentration of apoplastic Si in the apoplast. Rogalla and Römheld (2002) have shown the similar results that Si-mediated tolerance of Mn in *Cucumis sativus* is a consequence of stronger binding of Mn to cell walls and a lowering of Mn concentration within the symplast. The objectives of this study are to clarify the involvement of exogenous Si in lipid peroxidation, antioxidant enzymes and non-enzymatic antioxidants in cucumber under excess Mn stress and elucidate the possible mechanisms of Si-mediated alleviation of Mn toxicity in cucumber.

## 2. Results

### 2.1. Symptom and plant growth

The plants treated with excess Mn showed visible symptoms of Mn toxicity in the leaves. However, the occurrence of these symptoms was related to leaf positions. Severe chlorosis occurred in both older and younger leaves. Chlorosis was not observed in the leaves treated with excess Mn and Si. Therefore, the addition of Si inhibited the development of Mn toxicity symptoms.

Table 1 shows that the shoot height, root length, shoot and root dry weight, and total weight of cucumber plants were greatly influenced by the treatments. Si supply promoted the growth of both shoot and root ($p < 0.05$). Excess Mn greatly inhibited the plant growth ($p < 0.05$), however, this inhibition was significantly alleviated by Si addition ($p < 0.05$).

### 2.2. Mn and Si concentrations

Table 2 shows that excess Mn greatly increased the Mn concentration in the leaves ($p < 0.05$); however, Si supply significantly reduced the Mn concentration in the leaves treated with excess Mn ($p < 0.05$), but only slightly decreased the Mn concentration in the leaves treated with normal Mn. The addition of Si largely increased the Si concentration in the leaves ($p < 0.05$), however, excess Mn significantly reduced the Si concentration in the leaves treated with Si ($p < 0.05$).

Table 2
Effects of Si supply on the total concentrations of Mn and Si in the leaves of cucumber plants grown in nutrient solutions containing normal (10 μM) and excess (600 μM) Mn

| Treatment | Mn conc. (μg $g^{-1}$ DW) | Si conc. (μg $g^{-1}$ DW) |
|---|---|---|
| CK | 253 ± 4 c | 57.6 ± 6.6 c |
| Si | 233 ± 2 c | 600.1 ± 27.8 a |
| Mn | 11,897 ± 271 a | 53.9 ± 5.9 c |
| Si + Mn | 9120 ± 615 b | 523.6 ± 18.9 b |
| $LSD_{0.05}$ | 392 | 21.1 |

Data are means ± SD of four replicates. Within a column, mean values followed by different letters (a, b, c and d) are significantly different ($p < 0.05$).

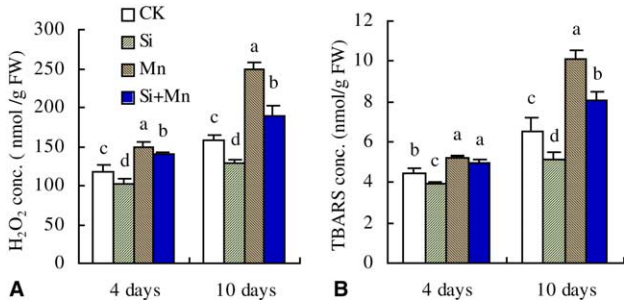


Fig. 1. Effects of Si on concentrations of $H_2O_2$ (A) and TBARS (B) in the leaves of cucumber plants grown in nutrient solution containing normal (10 μM) or excess (600 μM) Mn. Data are means ± SD of four replicates. Within the same treatment day, mean values followed by different letters (a, b, c and d) are significantly different ($p < 0.05$).

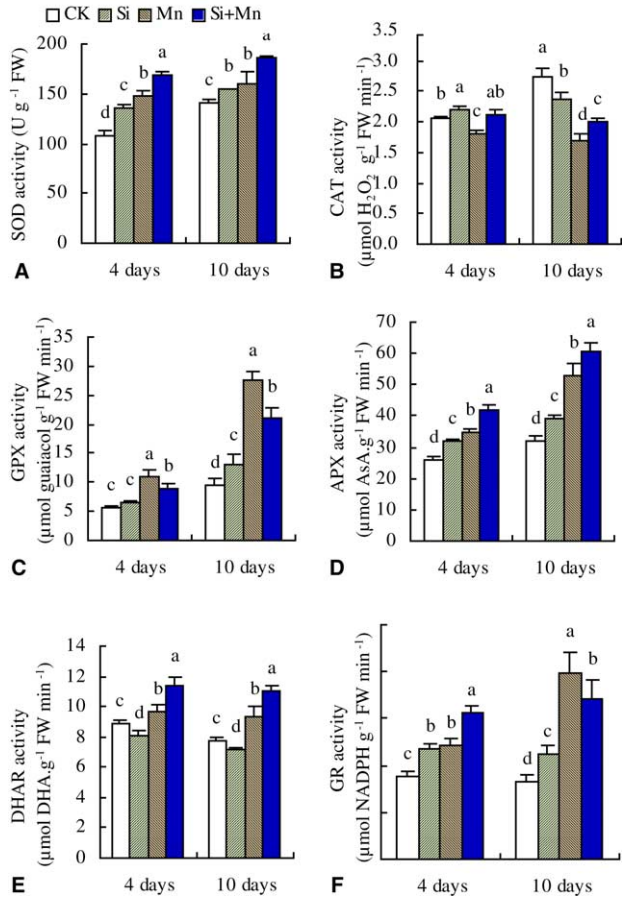


Fig. 2. Effects of Si on activities of SOD (A), CAT (B), GPX (C), APX (D), DHAR (E) and GR (F) in the leaves of cucumber plants grown in nutrient solution containing normal (10 μM) or excess (600 μM) Mn. Data are means ± SD of four replicates. Within the same treatment day, mean values followed by different letters (a, b, c and d) are significantly different ($p < 0.05$).

### 2.3. $H_2O_2$ concentration and lipid peroxidation

Compared to the control, excess Mn significantly increased $H_2O_2$ concentration in the leaves ($p < 0.05$), especially 10 days after the treatment. Si supply decreased $H_2O_2$ concentration at normal Mn and excess Mn, but more considerably at excess Mn for 10 days after the treatment ($p < 0.05$) (Fig. 1A).

Lipid peroxidation was estimated by TBARS concentration. Changes in TBARS concentration were similar to those of $H_2O_2$ concentration. Excess Mn greatly increased TBARS concentration ($p < 0.05$), whereas Si supply significantly reduced the accumulation of TBARS ($p < 0.05$) (Fig. 1B).

### 2.4. Antioxidative enzymes

Si supply increased superoxide dismutase (SOD) activity in the leaves at normal Mn and excess Mn. Excess Mn significantly increased SOD activity ($p < 0.05$) and the highest SOD activity was observed in the Si + Mn treatment (Fig. 2A).

A slight increase at 4 days and a slight decrease at 10 days were observed in catalase (CAT) activity in the leaves with Si supply. CAT activity was significantly inhibited by excess Mn, especially at 10 days of treatment ($p < 0.05$); however, Si supply significantly alleviated the inhibition of CAT activity caused by excess Mn (Fig. 2B).

Excess Mn significantly increased guaiacol peroxidase (GPX) activity in the leaves ($p < 0.05$). Si supply slightly increased GPX activity at normal Mn, but significantly decreased its activity at excess Mn ($p < 0.05$) (Fig. 2C).

Excess Mn considerably promoted ascorbate peroxidase (APX) activity in the leaves ($p < 0.05$), and the effect was more obvious with the increasing duration of treatment. Si supply significantly increased APX activity. The highest APX activity was observed at 4 and 10 days of the Si + Mn treatment (Fig. 2D).

Excess Mn significantly increased dehydroascorbate reductase (DHAR) activity in the leaves ($p < 0.05$). The addition of Si reduced DHAR activity at normal Mn, but significantly increased DHAR activity at excess Mn ($p < 0.05$) (Fig. 2E).

Excess Mn significantly increased glutathione reductase (GR) activity in the leaves. Si increased GR activity at normal Mn for 4 and 10 days of the treatment and at excess Mn for 4 days of the treatment, but decreased GR activity at excess Mn for 10 days of the treatment (Fig. 2F).

### 2.5. Ascorbate concentration

Fig. 3 shows that excess Mn significantly decreased the concentrations of reduced ascorbate (AsA), dehydroascorbate (DHA) and total ascorbate in the leaves ($p < 0.05$). Si supply increased the concentrations of AsA and total ascorbate at normal Mn for 4 days of the treatment and at excess Mn for 10 days of the

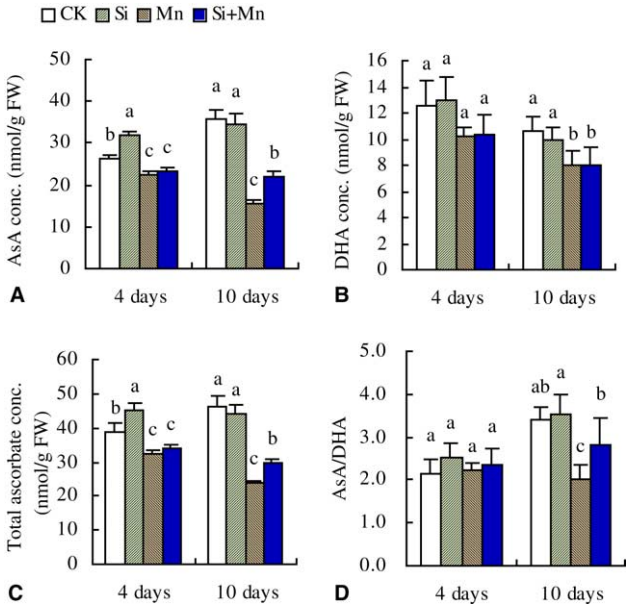


Fig. 3. Effects of Si on AsA concentrations (A), DHA concentrations (B), total ascorbate concentrations (C) and AsA/DHA ratio (D) in the leaves of cucumber plants grown in nutrient solution containing normal (10 μM) or excess (600 μM) Mn. Data are means ± SD of four replicates. Within the same treatment day, mean values followed by different letters (a, b and c) are significantly different ($p < 0.05$).

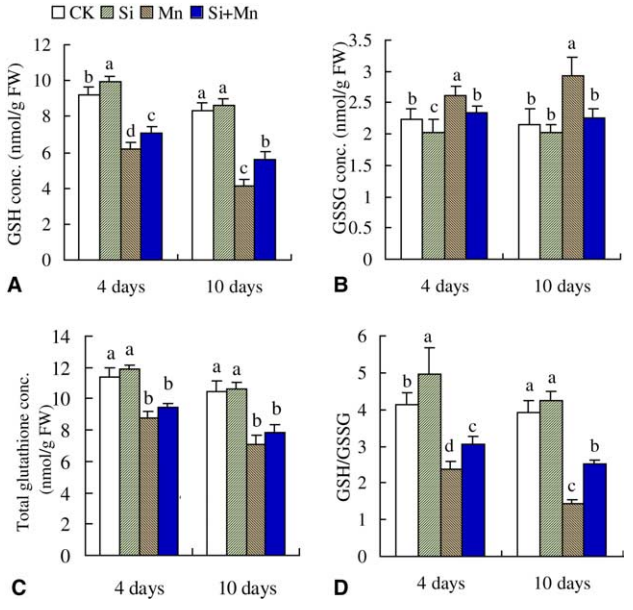


Fig. 4. Effects of Si on GSH concentrations (A), GSSG concentrations (B), total glutathione concentrations (C) and GSH/GSSG ratio (D) in the leaves of cucumber plants grown in nutrient solution containing normal (10 μM) or excess (600 μM) Mn. Data are means SD of four replicates. Within the same treatment day, mean values followed by different letters (a, b, c and d) are significantly different ($p < 0.05$).

treatment. The ratio of AsA/DHA was significantly reduced by excess Mn for 10 days of the treatment, however, this reduction was alleviated by Si supply.

### 2.6. Glutathione concentration

Excess Mn considerably decreased the concentrations of GSH and total glutathione ($p < 0.05$) (Fig. 4). Si supply significantly increased GSH concentration at excess Mn ($p < 0.05$). Excess Mn increased GSSG concentration when Si was not added. The ratio of GSH/GSSG was significantly reduced by excess Mn, however, this reduction was alleviated by Si supply.

## 3. Discussion

Being an essential micronutrient, Mn improves plant growth at low concentrations, but if present at a high level, Mn retards plant growth by interfering with normal cellular metabolic events (Houtz et al., 1988; Subrahamanyam and Rathore, 2000; Fecht-Christoffers et al., 2003). Si improves plant growth under biotic and abiotic stresses (Epstein, 1999; Ma, 2004). In the present study, Si supply mitigated excess Mn toxicity to cucumber plants (Table 1), which was consistent with the previous findings (Horiguchi, 1988; Iwasaki and Matsumra, 1999; Rogalla and Römheld, 2002). Rogalla and Römheld (2002) have reported that Si-mediated tolerance in cucumber is a consequence of stronger binding of Mn to cell walls and a lowering of Mn concentration within the symplasts. In the present study, Si supply significantly decreased the Mn concentration in cucumber leaves (Table 2), but the apoplastic Mn was not investigated.

Excess heavy metals increase the oxidative damage mediated by ROS and therefore promote TBARS accumulation in plants due to increased lipid peroxidation (Groppa et al., 2001; Verma and Dubey, 2003). In the present study, a similar response was also observed in cucumber plants treated with excess Mn. Si supply decreased $H_2O_2$ and TBARS concentration at excess Mn (Fig. 1), indicating that Si alleviated oxidative stress caused by Mn toxicity.

Aerobic organisms face constant risk from toxic ROS, especially under stress conditions. Plants possess antioxidant defense systems, comprised of enzymatic and non-enzymatic components, which normally maintain ROS balance within the cell. SOD constitutes the first line of defense against ROS, which is crucial for the removal of $O_2^{\bullet -}$ in the compartments where $O_2^{\bullet -}$ radicals formed (Takahashi and Asada, 1983). In the present study, a significant enhancement in SOD activity was observed in cucumber plants when exposed to excess Mn (Fig. 2A), suggesting an important role in removing $O_2^{\bullet -}$ induced by excess Mn. Si supply increased SOD activity under both normal and excess Mn level (Fig. 2A), and therefore increasing the $O_2^{\bullet -}$ scavenging. Similar results have been observed in barley and

cucumber under salt stress (Liang, 1999; Zhu et al., 2004). However, the reason was unclear.

The decomposition of $O_2^{\cdot-}$ is always accompanied by production of $H_2O_2$, which rapidly diffuses across the membrane and is toxic as it acts both as an oxidant as well as a reductant (Foyer et al., 1997). $H_2O_2$ can be scavenged in the cell either by CAT, GPX or APX. Catalase is a universally present oxidoreductase that decomposes $H_2O_2$ to water and molecular oxygen and it is one of the key enzymes involved in the removal of toxic peroxides. A decline in CAT activity under Mn toxicity was observed in the present study (Fig. 2B). Similar decrease in CAT activity was also reported in Fe, Cu and Cd toxicity (Gallego et al., 1996). Addition of Si significantly alleviated the decrease in CAT activity of the leaves treated with excess Mn (Fig. 2B).

In plants, GPX plays an important role in scavenging $H_2O_2$; however under excess Mn stress, its function becomes more complex. As early as in 1950, Kenten and Mann (1950) found a close relationship between the oxidation of Mn in the presence of peroxidase (GPX) and phenols. The GPX-catalyzed formation of polyphenols, which are responsible for the browning of the leaves, is accompanied by the formation of phenoxy radicals (Takahama and Oniki, 1992; Takahama, 1993). In the present study, excess Mn caused a significant elevation in GPX activity (Fig. 2C), which could be responsible for the brown speckle formation under excess Mn. Si supply significantly decreased GPX activity at excess Mn (Fig. 2C). This reduction of GPX activity may be related to the inhibition of brown speckle formation and therefore the toxicity symptoms were inhibited.

Ascorbate–glutathione cycle plays an important role in detoxification of ROS through successive oxidation and reduction reactions involving ascorbate and glutathione (Rennenberg, 1982; Kuźniak and Sklodowska, 1999). In the process of this cycle, APX plays a most important role in removing $H_2O_2$, and DHAR and GR can provide substrate for APX by catalyzing reaction. In the present study, activities of APX, DHAR and GR were increased in cucumber plants when exposed to excess Mn (Fig. 2D–F), suggesting the important role of APX in the detoxification of $H_2O_2$. DHAR and GR could keep higher substrate for APX by increasing activity, as has been reported in Mn-stressed common bean and in Zn-stressed *Brassicaceae* (González et al., 1998; Prasad et al., 1999). Addition of Si increased activities of APX, DHAR and GR under excess Mn stress (Fig. 2D–F), indicating that Si supply could increase cucumber plants tolerance to oxidative stress under Mn toxicity by increasing the efficiency of ascorbate–glutathione cycle. The influence of Si on APX activity at excess Mn was different to that of GPX, suggesting that APX was more important in $H_2O_2$ scavenging at excess Mn.

Ascorbate and glutathione are two important antioxidants. They are redox buffering in the apoplasts and protect the plasmalemma from oxidation (Foyer et al., 2001; Pignocchi and Foyer, 2003). AsA and GSH are readily oxidized by catalytic transition metals with an appreciable rate at acid or neutral pH (Buttner, 1988; Gallego et al., 1996). In the present study, AsA and GSH concentrations decreased greatly in cucumber leaves treated with excess Mn (Figs. 3A and 4A), and similar results have been obtained from Cu-stressed *Phaseolus vulgaris* (Gupta et al., 1999). The decline of AsA concentration reduced the availability of substrate for APX, resulting in elevation in $H_2O_2$ accumulation. Monodehydroascorbate, as a result of APX catalysis and certain non-enzymatic reactions, is unstable and highly reactive, and it disproportionates spontaneously to DHA that is reduced by DHAR using GSH as a reductant. The elevation of DHAR activity in cucumber leaves at excess Mn (Fig. 2E) could increase the conversion of DHA to AsA. However, AsA concentration decreased in excess Mn treatment, which may be due to increasing oxidation of AsA and prevention of AsA formation at excess Mn. On the other hand, the decrease in DHA concentration led to a decrease in the substrate of DHA to AsA catalyzed by DHAR, resulting in a lower AsA/DHA ratio. Addition of Si increased AsA level at excess Mn (Fig. 3A), which probably attributed to increased DHAR activity or lower oxidative stress caused by Si. GSH is oxidized to GSSG during the conversion of DHA to AsA, which depends on GR to reform GSH by oxidizing NAD(P)H to $NAD(P)^+$ (Foyer et al., 1994). Although GR activity significantly increased under Mn stress treatment (Fig. 2F), the GSH concentration was reduced and the GSSG concentration was increased (Fig. 4A and B), probably due to shortage of NAD(P)H as substrate during the reaction.

In the review of Epstein (1999), Si is a major constituent of many plants, but is not generally classified as 'essential'. Although Si has been regarded as an essential element in a number of species of the Poaceae and Cyperaceae it has not been possible to demonstrate that Si is essential to all higher plants because direct evidence is still lacking that Si is part of the molecule of an essential plant constituent or metabolite (Epstein, 1999). From the present experiment, it was difficult to conclude that the lowering of Mn concentration within the symplast observed by Rogalla and Römheld (2002) was a prerequisite for the increases of SOD and APX activities. Liang et al. (2003) and Zhu et al. (2004) have also observed increases of SOD and APX activities under salt stress by Si addition in barley and cucumber. They strongly suggest that Si may be involved in the metabolic or physiological and/or structural activity in higher plants exposed to abiotic and biotic stresses. The results of the present study also support this conclusion.

In summary, the results in the present study indicated that at excess Mn Si decreased the Mn concentration, increased the activities of SOD, APX and the ascorbate–glutathione cycle, reduced concentrations of $H_2O_2$ and TBARS in the leaves, as a result, the Mn toxicity to cucumber plants were alleviated.

## 4. Experimental

### 4.1. Plant material

Cucumber (*C. sativus* L. cv. Jinchun 5) seeds were germinated on moisture filter paper in an incubator at 28 °C for 2 days. The germinated seeds were sown in vermiculite in the greenhouse of Zhejiang University. After 12 days, the seedlings were transferred to 10 l plastic containers containing aerated full nutrient solution: 5 mM $Ca(NO_3)_2$, 5 mM $KNO_3$, 2.5 mM $KH_2PO_4$, 2 mM $MgSO_4$, 29.6 μM $H_3BO_3$, 10 μM $MnSO_4$, 50 μM Fe–EDTA, 1.0 μM $ZnSO_4$, 0.05 μM $H_2MoO_4$, 0.95 μM $CuSO_4$, three seedlings per container. The pH was adjusted close to 6.5 by adding $H_2SO_4$ or KOH. After 9 days of pre-culture, the treatments of excess manganese and silicon addition were started by adding additional $MnSO_4$ and potassium silicate ($K_2SiO_3$)) to the nutrient solution. Additional K introduced by $K_2SiO_3$ was subtracted from $KNO_3$ and the resultant nitrate loss was supplemented with dilute nitric acid. The experimental design consisted of a control (CK: 10 μM $MnSO_4$ and no added Si) and three treatments (Si: 10 μM $MnSO_4$ and 1.0 mM Si; Mn: 600 μM $MnSO_4$ and no added Si; Si + Mn: 600 μM $MnSO_4$ and 1.0 mM Si) were arranged in a randomized, complete block design with four replicates, giving a total of 16 containers. The light period was 12 h, and the air temperature was 23–32 °C and 18–24 °C day and night, respectively. The nutrient solutions were changed every 3 days. The youngest fully developed leaves were taken for assays.

### 4.2. Plant growth measurement

After 10 days of treatment, shoot height and root length were measured. The plants were harvested, divided into shoots and roots, dried at 70 °C to constant weight and weighted.

### 4.3. Determination of Mn and Si concentrations

Mn concentration in cucumber leaves was determined by atomic absorption spectrometry after digested with $H_2SO_4$ and $H_2O_2$.

Si concentration in cucumber leaves was determined according to the modified method of Van der Vorm (1987). About 0.3 g samples of cucumber leaves were ashed in porcelain crucibles for 5 h at 550 °C. The ash was washed from the crucibles into 100-ml polyethylene bottles, which were weighed before and after filling with approximately 50 ml of 0.08 M $H_2SO_4$ solution. To the suspension 2 ml of 40% HF solution was added. This suspension was shaken for 2 h and left overnight. Two ml aliquot of the suspension in transferred to a clean polyethylene bottle. Fifty ml of 0.32% $H_3BO_3$ solution were added and shaken for 2 h. From the resulting solution 10 ml aliquot was taken and added color reagent, consisting of a 1:1 mixture of 0.08 M $H_2SO_4$ and an ammoniumheptamolybdate (20 g $(NH_4)_6MoO_7 \cdot 7H_2O$ per liter). After shaking, the solution was left to stand for 5 min. Then 5 ml of 3.3% tartaric acid and 5 ml of 0.4% AsA solution were added. Upon shaking, the absorbance was measured after 25 min at 811 nm.

### 4.4. Determination of $H_2O_2$ concentration

Tissue was homogenized in cold acetone in the ratio 1 g tissue to 2 ml acetone. Titanium reagent (2% $TiCl_2$ in conc. HCl) was added to a known volume of extract supernatant to give a Ti (IV) concentration of 2%. The Ti–$H_2O_2$ complex, together with unreacted Ti, was then precipitated by adding 0.2 ml of 17 M ammonia solution for each 1 ml of extract. The precipitate was washed five times with ice acetone by resuspension, drained, and dissolved in 1 M $H_2SO_4$ (3 ml). The absorbance of the solution was measured at 410 nm against blanks, which had been prepared similarly but without plant tissue (Brain et al., 1984).

### 4.5. Determination of lipid peroxidation

Lipid peroxidation was measured in terms of TBARS (Heath and Packer, 1968). About 0.3 g of tissue homogenized in 3 ml of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000*g* for 10 min and 3 ml of 20% TCA containing 0.5% (w/v) thiobarbituric acid (TBA) was added to 1 ml of supernatant. The mixture was heated at 95 °C for 30 min and the reaction was stopped by quickly placing in an ice-bath. The cooled mixture was centrifuged at 10,000*g* for 10 min, and the absorbance of the supernatant at 532 and 600 nm was read. After substrating the non-specific absorbance at 600 nm, the TBARS concentration was determined by its extinction coefficient of 155 $mM^{-1}\,cm^{-1}$.

### 4.6. Preparation of enzyme extraction

For enzyme assays, 0.3 g leaves were ground with 3 ml ice-cold 25 mM Hepes buffer (pH 7.8) containing 0.2 mM EDTA, 2 mM AsA and 2% PVP. The homogenate was centrifuged at 4 °C for 20 min at 12,000*g* and the resulting supernatants were used for determination of enzyme activity. All spectrophotometric analyses

were conducted on a SHIMADZU UV-2410PC spectrophotometer.

### 4.7. Assay of SOD activity

The activity of superoxide dismutase was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) (Rao and Sresty, 2000). The 3-ml reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA and 0.1 ml of enzyme extract. Reaction was started by adding 2 μM riboflavin and placing the tubes under 15 W fluorescent lamps for 15 min. A complete reaction mixture without enzyme, which gave the maximal colour, served as control. Reaction was stopped by switching off the light, then the tubes were covered with a black cloth. A non-irradiated complete reaction mixture served as a blank. The absorbance was recorded at 560 nm, and one unit of SOD was defined as being present in the volume of extract that caused inhibition of the photo-reduction of NBT by 50%.

### 4.8. Assay of CAT activity

CAT activity was measured the decline in absorbance at 240 nm due to the decline of extinction of $H_2O_2$. The reaction mixture containing 25 mM sodium phosphate buffter (pH 7.0),10 mM $H_2O_2$ and 0.1 ml enzyme extracts. The reaction was started by adding hydrogen peroxide (Cakmak and Marschner, 1992).

### 4.9. Assay of GPX activity

GPX was based on the method as described by Nickel and Cunningham (1969). Activity was measured by the increase in absorbance at 470 nm due to guaiacol oxidation. The reaction mixture contained 25 mM guaiacol, 10 mM $H_2O_2$ and 0.1 ml supernatant. The reaction was started by adding hydrogen peroxide.

### 4.10. Assay of APX activity

APX was measured according to Nakano and Asada (1981). The assay depends on the decrease in absorbance at 290 nm as ascorbate was oxidized. Two ml reaction mixture contained 25 mM sodium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM hydrogen peroxide, 0.1 mM EDTA and 0.1 ml supernatant. The reaction was started by adding hydrogen peroxide.

### 4.11. Assay of DHAR activity

The assay of DHAR activity was carried out by measuring the increase in absorbance at 265 nm due to AsA formation (Nakano and Asada, 1981). The reaction mixture contained 25 mM sodium phosphate buffter (pH 7.0). 2.5 mM reduced glutathione (GSH), 0.4 mM dehydroascorbate (DHA), and 0.1 ml enzyme extract.

### 4.12. Assay of GR activity

GR activity was measured according to Foyer and Halliwel (1976), which depends on the rate of decrease in the absorbance of NADPH at 340 nm. The reaction mixture consisted of 25 mM Hepes buffer (pH 7.0), 0.5 mM oxidized glutathione (GSSG), 0.12 mM NADPH and 0.2 mM EDTA. The specific activity of GR was expressed as μmol NADPH oxidized $g^{-1}$ FW $min^{-1}$.

### 4.13. Determination of ascorbate concentration

Reduced ascorbate (AsA), dehydroascorbate (DHA) and total ascorbate (AsA + DHA) were determined by a modified method from Hodges et al. (1996). About 0.3 g of frozen leaf sample was ground with inert sand and 2 ml of 5% (v/v) *m*-phosphoric acid with a mortar and pestle. The homogenate was centrifuged at 12,000*g* for 20 min. Total ascorbate was determined in a reaction mixture consisting of 100 μl of supernatant, 500 μl of 150 mM $KH_2PO_4$ buffter (pH 7.4) containing 5 mM EDTA and 100 μl of 10 mM dithiothreitol (DTT) to reduce DHA to AsA. After 10 min at room temperature, 100 l of 0.5% (w/v) N-ethylmaleimide was added to remove excess DTT. AsA was assayed in a similar manner except that 200 μl of deionized $H_2O$ was substituted for DTT and *N*-ethylmaleimide. Colour was developed in both series of reaction mixtures with the addition of 400 μl of 10% (w/v) TCA, 400 μl of 44% (v/v) *o*-phosphoric acid, 400 μl of α,α′-dipyridyl in 70% (v/v) ethanol and 200 μl of 30 g $l^{-1}$ $FeCl_3$. The reaction mixtures were incubated at 40 °C for 1 h and quantified spectrophotometrically at 525 nm. DHA was estimated from the difference of total ascorbate and AsA.

### 4.14. Determination of glutathione

Reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined according to the modified method from Hissin and Hilf (1976). *o*-Phthalaldehyde (OPT) was used as a fluorescent reagent, 0.3 g fresh leaves were ground with 3 ml buffer consisting of TCA:1 mM EDTA:0.1 M HCl (1:1:1, v/v/v), then centrifuged at 12,000*g* for 20 min at 4 °C. For the measurement of GSH, 0.2 ml of the supernatant was mixed with 3.6 ml of 0.1 M phosphate buffer (pH 8.0, including 5 mM EDTA) and 0.2 ml of OPT stock (1 mg $ml^{-1}$ in ethanol) added. For GSSG, 0.2 ml of supernatant was initially mixed with 80 ml of 0.04 M *N*-ethylmaleimide for 30 min and subsequently added to 3.52 ml of 0.1 M NaOH and 0.2 ml of OPT stock, fluorescence was

read at an excitation and emission wavelength of 350 and 420 nm.

### 4.15. Statistical analysis

Data were tested by analysis of variance (ANOVA) using SAS software (SAS Institute, Cary, NC). All data presented are the mean values. The measurement was done with four replicates. Statistical assays were carried out by ANOVA test and means were compared by the least significant difference (LSD) test. Comparisons with $p$ values $<0.05$ were considered significantly different.

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