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Silicon ameliorates manganese toxicity in cucumber by decreasing hydroxyl radical accumulation in the leaf apoplast

Jelena Dragić Maksimović¹, Miloš Mojović², Vuk Maksimović¹, Volker Römheld³ and Miroslav Nikolić¹,*

¹ Institute for Multidisciplinary Research, University of Belgrade, Kneza Viseslava 1, 11030 Belgrade, Serbia
² Faculty of Physical Chemistry, University of Belgrade, Studentski trg 12–16, 11000 Belgrade, Serbia
³ Institute of Crop Science (340), University of Hohenheim, D-70593 Stuttgart, Germany
* To whom correspondence should be addressed. E-mail: mnikolic@imsi.rs

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Abstract

This work was focused on the role of silicon (Si) in amelioration of manganese (Mn) toxicity caused by elevated production of hydroxyl radicals (·OH) in the leaf apoplast of cucumber (Cucumis sativus L.). The plants were grown in nutrient solutions with adequate (0.5 μM) or excessive (100 μM) Mn concentrations with or without Si being supplied. The symptoms of Mn toxicity were absent in the leaves of Si-treated plants subjected to excess Mn, although the leaf Mn concentration remained extremely high. The apoplastic concentration of free Mn²⁺ and H₂O₂ of high Mn-treated plants was significantly decreased by Si treatment. Si supply suppressed the Mn-induced increased abundance of peroxidase (POD) isoforms in the leaf apoplastic fluid, and led to a rapid suppression of guaiacol-POD activity under excess Mn. The spin-trapping reagent 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide was used to detect ·OH by electron paramagnetic resonance spectroscopy. Although supplying Si markedly decreased the accumulation of ·OH in the leaf apoplast with excess Mn, adding monosilicic acid to the Mn²⁺/H₂O₂ reaction mixture did not directly affect the Fenton reaction in vitro. The results indicate that Si contributes indirectly to a decrease in ·OH in the leaf apoplast by decreasing the free apoplastic Mn²⁺, thus regulating the Fenton reaction. A direct inhibitory effect of Si on guaiacol-POD activity (demonstrated in vitro) may also contribute to decreasing the POD-mediated generation of ·OH.

Key words: Apoplastic fluid, cell walls, cucumber (Cucumis sativus L.), hydroxyl radicals, leaves, manganese toxicity, peroxidase, silicon.

Introduction

Although manganese (Mn) is an essential microelement for plants and all other living organisms, it easily becomes toxic above accepted physiological levels. Mn toxicity in crops is considered to be one of the major factors limiting plant growth, mainly on poorly drained and acidic soils that have high concentrations of readily available Mn⁴⁺ (Marschner, 1995). Visual symptoms of Mn toxicity vary depending on the plant species and the level of tolerance to an excess of this metal. Typical Mn toxicity symptoms seen in many dicots include chlorosis and brown spots on older leaves (starting from the leaf edge), followed by necrosis and finally leaf shedding (Fecht-Christoffers et al., 2007). The brown spots, located primarily in the apoplast of epidermal cells, consist of oxidized Mn (Mn⁴⁺) and oxidized phenols (Wissemeier and Horst, 1992), the formation of both being catalysed by the apoplastic peroxidases (PODs; Fecht-Christoffers et al., 2003, 2006). PODs in the plant apoplast (Class III; EC 1.11.17) act dually, as H₂O₂-producing enzymes using NADH as electron donor (also termed NADH-oxidases), and also as H₂O₂-consuming and phenol-oxidizing enzymes (so-called guaiacol-PODs), thus playing an important role in secondary cell wall formation and lignification (Führs et al., 2009, and references therein). The POD systems appear to be involved in both evolution and avoidance of Mn toxicity in the leaf apoplast (Fecht-Christoffers et al., 2006). The oxidation of Mn²⁺ and phenols by H₂O₂-consuming PODs...
has been proposed as the key reaction leading to Mn toxicity symptoms, because of the formation of highly reactive intermediates, Mn\(^{3+}\) and phenoxy radicals (Horst, 1988; Horst et al., 1999). The stimulating effect of Mn\(^{2+}\) on the generation of \(\text{H}_2\text{O}_2\) (Halliwell, 1978) has recently been confirmed by the enhanced NADH-oxidase activity in the leaf apoplast of cowpea grown with excess Mn (Feucht-Christoffers et al., 2006; Führs et al., 2009).

As a transition metal, Mn can also be involved in the production of reactive oxygen species (ROS), and in excess Mn leads to injury to biological systems (Ali et al., 1995; Stochs and Bachí, 1995; Lindon and Teixeira, 2000). The exact mechanism of catalytic scavenging of the superoxide anion radical (O\(_2^-\)) and \(\text{H}_2\text{O}_2\) in the presence of Mn is, however, not clear, but is thought to involve intermediate steps (Ducic and Polle, 2005). In vitro, Mn\(^{2+}\) ions lead to the production of oxidizing species such as O\(_2^-\) and hydroxyl radicals (OH) (Halliwell, 1977; Stadtman et al., 1990; Sakihama et al., 2002). Production of OH in the mixture \(\text{H}_2\text{O}_2\) and Mn\(^{2+}\) via a Fenton-like reaction has also been proposed, although this is still a matter of controversy (Strlic et al., 2003; Watts et al., 2005). Mn phytotoxicity can induce oxidative stress via several mechanisms, including direct generation of ROS from Mn\(^{2+}\) ions in the presence of light, most probably through the Fenton reaction (González et al., 1998). However, there is no experimental evidence so far showing that excess Mn might induce increased production of highly toxic -OH in the leaf apoplast as one of the causes of Mn toxicity symptoms.

Silicon (Si) is a beneficial element for most plants, known to alleviate various biotic and abiotic stresses effectively (for reviews, see Epstein, 1999; Ma and Yamaji, 2006; Liang et al., 2007). It has been demonstrated in many studies that Si supply to roots greatly improves tolerance to Mn toxicity in rice (Okuda and Takahashi, 1962; Horiguchi, 1988), barley (Williams and Vlamis, 1957; Horiguchi and Morita, 1987), bean (Horst and Marschner, 1978), cowpea (Horst et al., 1999; Iwasaki et al., 2002a, b; Führs et al., 2009), pumpkin (Iwasaki and Matsumura, 1999), and cucumber (Rogalla and Römheld, 2002; Shi et al., 2005; Dragišić Maksimović et al., 2007). Although the effect of Si detoxification was attributed to a lower uptake of Mn by roots (Islam and Saha, 1969; Bowen, 1972; Galvez et al., 1989), this has not been confirmed in many other studies (e.g. Horst and Marschner, 1978; Iwasaki and Matsumura, 1999; Rogalla and Römheld, 2002; Dragišić Maksimović et al., 2007).

For cucumber, an Si-accumulating dicot (Nikolic et al., 2007), the main mechanism of Si-mediated alleviation of Mn toxicity has been proposed to be an induced increase in cell wall binding capacity for Mn (Rogalla and Römheld, 2002; Wiese et al., 2007). This explanation, however, could only partly account for the alleviating properties of Si in cowpea (Horst et al., 1999; Iwasaki et al., 2002a, b) which has a lower ability to accumulate Si in the shoots. A more direct involvement of Si in detoxification of high Mn in the leaf apoplast has thus been proposed for this species. This includes, for instance, changes in the apoplastic metabolome profile, including PODs and phenols (Führs et al., 2009). Hence, the physiological basis of Si-mediated alleviation of Mn toxicity in plants still remains insufficiently understood.

The study reported here tests the hypothesis that increased accumulation of -OH from free Mn\(^{2+}\) in the leaf apoplast, most probably generated directly through a Fenton-like reaction, may be one of the main causes of Mn toxicity in cucumber leaves. The primary objective of this work was therefore to elucidate Si-mediated detoxification of excess Mn in cucumber in relation to lowering -OH in the leaf apoplast.

### Materials and methods

#### Plant material and growth conditions

Cucumber (Cucumis sativus L., cv. Chinese long) seeds were germinated on filter paper moistened with 2.5 mM CaSO\(_4\) and after 5 d the seedlings were transferred to a full strength nutrient solution (four plants per 2.5 l plastic pot) containing (mM): 0.7 K\(_2\)SO\(_4\), 0.1 KCl, 2.0 Ca(NO\(_3\))\(_2\), 0.5 MgSO\(_4\), 0.1 KH\(_2\)PO\(_4\), and (in \(\mu\)M): 10 H\(_2\)BO\(_3\), 0.5 MnSO\(_4\), 0.5 ZnSO\(_4\), 0.2 CuSO\(_4\), 0.01 (NH\(_4\))\(_2\)MoO\(_4\). Iron was supplied as Fe\(^{3+}\)EDDAH at 20 \(\mu\)M. After 7 d pre-culture at optimal Mn concentration (0.5 \(\mu\)M), plants were subjected to 0.5 \(\mu\)M and 100 \(\mu\)M Mn, respectively, for 2 weeks. Concomitantly, half of the one plants were supplied with 1.5 mM Si as Si(OH)\(_4\) prepared by passing Na\(_2\)SiO\(_4\) through a plastic column filled with cation-exchange resin (Amberlite IR-120, H+-form, Fluka, Deisenhofen, Germany). The nutrient solutions were renewed completely every 3 d and continuously aerated.

Plants were grown under controlled environmental conditions in a growth chamber with a light/dark regime of 16/8 h, temperature regime of 24/20 °C, photon flux density of 250 \(\mu\)mol m\(^{-2}\) s\(^{-1}\), at plant height, and relative humidity of ~70%. After 3 weeks the plants were harvested and the second and third fully developed leaves were used for analyses.

#### Collection of the leaf apoplastic fluid (LAF)

The apoplastic fluid from intact leaves (the second and third fully expanded from the base) was collected 3 h after light onset by the centrifugation method described by Nikolic and Römheld (2003). The first fraction, obtained at a low centrifugal speed of 1500 \(g\) to remove xylem sap and contaminants, was discarded, and the apoplastic fluid was collected by a second centrifugation at 2500 \(g\) for 15 min at 4 °C. The relative activity of malate dehydrogenase (MDH; a mitochondrial marker enzyme) in the apoplastic fluid was <1% of the total activity in the leaf homogenate in all samples (data not shown), which indicated that there was no symptomatic contamination of the apoplastic fluid.

#### Fractionated extraction of Mn

After centrifugation of intact leaves to obtain the LAF (described above), the major midribs were removed, and the leaf segments were infiltrated with hypertonic sucrose solution (0.4 M) under vacuum for 20 min. The plasmolysed leaf tissue was frozen with liquid nitrogen and homogenized in a mortar in 0.4 M sucrose solution. The broken cells were recovered from the homogenate by centrifugation at 1000 \(g\) for 10 min, and the pellet was resuspended in deionized water followed by centrifugation at 2000 \(g\) for 15 min; the supernatants of both centrifugations were mixed, representing the water-extractable Mn fraction. Since the MDH assay test showed a high level of cytosolic contamination, this fraction is considered to be symplastic; however, it also includes a certain proportion of soluble Mn originating from the vacuole as

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**References:**

- Epstein, 1999
- Ma and Yamaji, 2006
- Liang et al., 2007
- Horst and Marschner, 1978
- Iwasaki and Matsumura, 1995
- Stochs and Bachí, 1995
- Lindon and Teixeira, 2000
- Ali et al., 1995
- Williams and Vlamis, 1957
- Horiguchi and Morita, 1987
- Okuda and Takahashi, 1962
- Horiguchi, 1988
- Williams and Vlamis, 1957
- Rogalla and Römheld, 2002
- Shi et al., 2005
- Dragišić Maksimović et al., 2007
- Islam and Saha, 1969
- Bowen, 1972
- Galvez et al., 1989
- Horst et al., 1999
- Iwasaki et al., 2002a, b
- Führs et al., 2009
- Feucht-Christoffers et al., 2006
- Führs et al., 2009
- Horst et al., 1987
- Horst and Marschner, 1978
- Horst et al., 1999
- Watts et al., 2005
- Ducic and Polle, 2005
- Epstein, 1999
- Ma and Yamaji, 2006
- Liang et al., 2007
- Horst et al., 1999
- Iwasaki et al., 2002a, b
- Führs et al., 2009
- Rogalla and Römheld, 2002
- Dragišić Maksimović et al., 2007
- Islam and Saha, 1969
- Bowen, 1972
- Galvez et al., 1989
- Horst and Marschner, 1978
- Iwasaki and Matsumura, 1999
- Rogalla and Römheld, 2002
- Dragišić Maksimović et al., 2007
well as a very small proportion of wall-bound Mn, which can be extracted with water during centrifugation (up to 5% of total Mn; Rogalla and Römhled, 2002). The pellet was subsequently washed three times with 1% (w/v) SDS and centrifuged at 3000 g for 10 min, followed by three washes with deionized water, until these cell wall materials became free of plasma membranes and other cytoplasmic fragments as observed by light microscopy. The collected supernatants were mixed, representing the symplastic Mn fraction bound to the proteins. The final pellet (cell wall material) represented the Mn fraction bound to the cell walls.

All procedures were carried out at 4 °C, except plasmolysis and washing with SDS which were performed at room temperature.

Determination of cation exchange capacity (CEC) in the cell wall material

The cell wall was isolated as previously described by Nikolic and Römhled (2003) and the CEC was determined by incubating fresh cell wall material (~1 g) in 10 ml of 50 mM BaCl₂ (pH 5.0) for 30 min with stirring at 4 °C. After centrifugation at 2000 g, the pellet was resuspended in a fresh BaCl₂ solution and the incubation procedure was repeated. After centrifugation, the pellet was washed three times with deionized water (15 ml) by centrifugation, dried at 65 °C, weighed, microwave digested in 3 ml of HNO₃+2 ml of H₂O₂, and the cation concentrations were determined by inductively coupled plasma optical emission spectrometry (ICP-OES; SpectroGenesis EOP II, Spectro Analytical Instruments GmbH, Kleve, Germany). CEC was calculated as the amount of Ba retained in the cell wall material and expressed as cation charge equivalents displaced by Ba²⁺. No other cations (with the exception of Mn in high Mn treatment) were detected in the cell wall material after BaCl₂ extraction.

Determination of Mn

Collected apoplastic fluid and supernatants (water extracts and SDS extracts) were evaporated and digested to dryness in 5 M HNO₃. The cell wall material was resuspended in deionized water, evaporated to dryness, ashed at 550 °C for 8 h, and digested to dryness in 5 M HNO₃. For determination of the total leaf Mn in order to calculate the relative proportion of Mn fractions, the excised leaves were oven dried at 65 °C for 48 h, weighed, ashed at 550 °C for 8 h, and digested to dryness in 5 M HNO₃. In all samples, Mn was determined by ICP-OES after dissolving the dry residues in 0.5 M HNO₃.

Determination of Si in the LAF and cell wall material

In order to avoid any Si polymerization in the LAF, Si was determined immediately in the fresh samples. After oven drying, the cell wall material was microwave digested with 4 ml of HNO₃+1 ml of HF. Samples were diluted with deionized water in a 25 ml plastic flask and HF was neutralized by adding 2.5 ml of 2% (w/v) H₃BO₃. Si was determined by ICP-OES after a final dilution of the samples of 1:100 (v/v) for cell wall material or 1:10 (v/v) for LAF.

Electron paramagnetic resonance (EPR) determination of Mn²⁺

The samples of powdered dry leaf material (0.3 g) were placed into quartz tubes, while the LAF samples (60 µl) were introduced into Teflon tubes. EPR spectra were recorded at room temperature by an EPR spectrometer (E104-A, Varian Inc., Palo Alto, CA, USA) operating at X-band (9.3 GHz) under the following settings: modulation amplitude, 2 Gauss; modulation frequency, 100 kHz; microwave power, 10 mW; scan range, 1000 Gauss. Spectra were recorded and analysed using the EW software (Scientific Software International, Inc., Lincolnwood, IL, USA).

The EPR signal of so-called free Mn consists of a six hyperfine line spectrum characteristic for freely rotating (aqueous) Mn²⁺ symmetrically coordinated by six water molecules [Mn(H₂O)₆]²⁺ (see Figs 2 and 3). This signal is superimposed on the broad signal of Mn²⁺ bound to the proteins and cell wall macromolecules in the case of dry leaf material (see Fig. 2). Quantitative analysis of the ratio of free (Mn²⁺) and bound Mn was performed by spectral simulations using the program WINPEP SimFonia (Bruker Analytische Messtechnik GmbH, Karlsruhe, Germany).

Determination of guaiacol-POD activity in the LAF

Guaiacol-POD activities in the LAF were determined spectrophotometrically at 470 nm (Shimadzu UV-2501PC, Shimadzu Corp., Kyoto, Japan) using guaiacol as the electron donor substrate, according to the method of Hammerschmidt et al. (1982). LAF samples (5–20 µl; the volume depends on the enzyme activity) were mixed with the assay solution containing 0.25% (v/v) guaiacol in 50 mM phosphate buffer (pH 6.0) and 10 mM H₂O₂. One unit of POD activity was defined as the amount of enzyme which catalyses the conversion of 1 µmol of H₂O₂ min⁻¹.

Determination of guaiacol-POD activity in vitro

Guaiacol-POD activity in vitro was determined by the same method as described above. The final concentration of POD from horseradish (EC 1.11.1.7; Sigma-Aldrich, St Louis, MO, USA) in the assay solution [10 mM guaiacol in 50 mM phosphate buffer (pH 6.0) and 10 mM H₂O₂] was 0.1 µM. The order of adding 0.5 mM Si(OH)₄ as an unspecified component of the reaction mixture, was tested, proving no significant effect on the enzyme activity (data not shown).

Isoelectric focusing (IEF) and staining of the PODs from the LAF

All isozymes were separated by IEF (LKB 2117 Multihor II, LKB Instruments Ltd, South Croydon, Surrey, UK) on a 7.5% polyacrylamide gel containing a solution of ampholites (pH gradient from 3.5 to 10) using purified double-distilled water (18 MΩ, Millipore, Bedford, MA, USA). The prepared LAF samples (20 µl) and markers were applied on the sample application papers (10×5 mm; SERVA Electrophoresis GmbH, Heidelberg, Germany) previously laid on the gel. After completion of electrophoresis, the POD isoenzymes from the LAF were stained with 9.2 mM guaiacol and 5 mM H₂O₂ in sodium acetate buffer (pH 5.5) for 10 min at 25 °C.

Detection of -OH

Detection of -OH was performed by an EPR spin-trapping method using 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO; Alexis Biochemical, Lausen, Switzerland) purified according to the method of Jackson et al. (2002). The samples of either LAF or in vitro incubation media (50 µl) were introduced into 10 cm long gas-permeable Teflon tubes (wall thickness 0.025 mm and i.d. 0.6 mm; Zeus Industries, Raritan, NJ, USA) and folded into 2.5 cm long segments to improve the signal-to-noise ratio (Swartz et al., 1986). The EPR spectra were recorded at room temperature by a Varian E104-A spectrometer operating at X-band (9.3 GHz) with the following settings: modulation amplitude, 2 Gauss; modulation frequency, 100 kHz; microwave power, 10 mW; scan range, 200 Gauss. Spectra were recorded and analysed using the EW software (Scientific Software International, Inc.).

Statistical analysis

Data were subjected to analysis of variance using the statistical software Statistica 6 (StatSoft, Inc., Tulsa, OK, USA), and means were compared by Mann–Whitney non-parametric test at P < 0.05.
Results

The leaf Mn concentration increased significantly as the concentration of Mn in the nutrient solution was raised from 0.5 μM (control) to 100 μM (Table 1). At high Mn supply (100 μM), Si-treated plants (+Si) showed a tendency to accumulate even higher Mn concentrations than non Si-treated plants (–Si). Symptoms of Mn toxicity (e.g., brown spots, small chlorotic regions with necrosis) appeared in the older leaves of –Si plants subjected to excessive Mn, which were accompanied by inhibition of plant growth, estimated as root and shoot dry weight (Table 1). Application of Si, however, stimulated plant growth in both Mn treatments (0.5 μM and 100 μM). Interestingly, the symptoms of Mn toxicity were absent in the leaves of Si-fed plants, despite the extremely high concentration of Mn in their leaves (Table 1).

The study reported here focused on the leaf apoplast with regards to Mn toxicity and Si amelioration of that toxicity. To investigate the effect of Si supply on the Mn binding potential of the leaf apoplast, cucumber leaves were subjected to fractionated extraction of Mn. The fractionated extraction

<table>
<thead>
<tr>
<th>Mn treatment</th>
<th>Si supply</th>
<th>Symptoms of Mn toxicity</th>
<th>Biomass (mg DW per plant)</th>
<th>Leaf Mn concentration (μmol g DW⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Root</td>
<td>Shoot</td>
</tr>
<tr>
<td>0.5 μM</td>
<td>–</td>
<td>0</td>
<td>62±10 b</td>
<td>220±10 b</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0</td>
<td>79±5 c</td>
<td>240±15 c</td>
</tr>
<tr>
<td>100 μM</td>
<td>–</td>
<td>1</td>
<td>40±2 a</td>
<td>73±12 a</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0</td>
<td>64±7 b</td>
<td>200±15 b</td>
</tr>
</tbody>
</table>

Numeric data are means (n=4) ±SD. Different lower case letters within a column denote significant differences at P<0.05.

Table 1. Effect of Si supply to roots on the appearance of leaf Mn toxicity symptoms, plant growth, and leaf Mn concentration of cucumber plants subjected to an adequate (0.5 μM) and excessive (100 μM) external concentrations of Mn. Scale of the visual symptoms of Mn toxicity in older leaves (representative experiment): 0, none; 1, moderate (brown spots, small chlorotic regions with necrosis occasionally).

Table 2. Effect of Si supply to roots on the concentration and relative distribution of Mn fractions in cucumber leaves obtained by fractionated extraction. Water-extractable Mn represents the soluble fraction in the cell walls together with a certain portion of soluble Mn originating from the symplast and vacuole, which cannot be separated during the extraction procedure. The protein-bound Mn fraction originates mostly from the symplast, while the cell wall-bound Mn fraction represents Mn which is fixed to the wall structure. The separately measured total leaf Mn content was used for calculation of the relative proportion of Mn from a fractionated extraction method. Differences of up to 2%, from the sum of the relative proportion of fractionated Mn to 100%, represent the relative proportion of Mn in the LAF.

<table>
<thead>
<tr>
<th>Mn treatment</th>
<th>Si supply</th>
<th>Water extractable (μmol g DW⁻¹)</th>
<th>Protein bound (μmol g DW⁻¹)</th>
<th>Cell wall bound (μmol g DW⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(%)</td>
<td>(%)</td>
<td>(%)</td>
</tr>
<tr>
<td>0.5 μM</td>
<td>–</td>
<td>0.38±0.01 a</td>
<td>56</td>
<td>0.04±0.00 a</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.35±0.02 a</td>
<td>43</td>
<td>0.05±0.00 a</td>
</tr>
<tr>
<td>100 μM</td>
<td>–</td>
<td>4.98±0.08 c</td>
<td>23</td>
<td>2.20±0.04 c</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2.20±0.04 b</td>
<td>9</td>
<td>0.40±0.01 b</td>
</tr>
</tbody>
</table>

Data are means (n=4) ±SD; different lower case letters within a column denote significant differences at P<0.05.

The study reported here focused on the leaf apoplast with regards to Mn toxicity and Si amelioration of that toxicity. To investigate the effect of Si supply on the Mn binding potential of the leaf apoplast, cucumber leaves were subjected to fractionated extraction of Mn. The fractionated extraction method. Differences of up to 2%, from the sum of the relative proportion of fractionated Mn to 100%, represent the relative proportion of Mn in the LAF.
Table 3. Effect of Si supply to roots on the CEC and concentration of non-exchangeable Mn of the cell wall material isolated from cucumber leaves. Exchangeable cations were displaced by incubating the cell wall material with Ba²⁺ (60 mM). Non-exchangeable Mn (strongly wall-bound fraction) represents Mn remaining in the cell wall material after BaCl₂ extraction.

<table>
<thead>
<tr>
<th>Mn supply</th>
<th>Si supply</th>
<th>CEC (μeq g DW⁻¹)</th>
<th>Non-exchangeable Mn (μmol g DW⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 μM</td>
<td>–</td>
<td>467±21 a</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>468±10 a</td>
<td>ND</td>
</tr>
<tr>
<td>100 μM</td>
<td>–</td>
<td>468±11 a</td>
<td>619±12 a</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>469±15 a</td>
<td>968±24 b</td>
</tr>
</tbody>
</table>

Data are means (n=3) ±SD; different lower case letters within a column denote significant differences at P < 0.05. ND, not determined.

Table 4. Effect of Mn treatments on the concentrations of Si in LAF and cell wall material isolated from leaves of cucumber plants grown in the nutrient solutions with or without Si supply.

<table>
<thead>
<tr>
<th>Mn treatment</th>
<th>Si supply</th>
<th>Si concentration LAF (mM)</th>
<th>Cell wall (mmol g DW⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 μM</td>
<td>–</td>
<td>0.03±0.01 a</td>
<td>0.39±0.04 a</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.51±0.13 b</td>
<td>3.97±0.17 b</td>
</tr>
<tr>
<td>100 μM</td>
<td>–</td>
<td>0.04±0.02 a</td>
<td>0.47±0.08 a</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.48±0.26 b</td>
<td>3.54±0.54 b</td>
</tr>
</tbody>
</table>

Different lower case letters within a column denote significant differences at P < 0.05.

The LAF Mn concentrations under both treatments, a significant decrease was found only in the high Mn treatment. The concentration of H₂O₂ in the LAF showed the same pattern but was significantly increased when the supply of external Mn was excessive, while the H₂O₂ concentration of high Mn-treated plants was markedly lower when Si was supplied (Fig. 1B).

Excessive Mn supply significantly increased guaiacol-POD activity in the LAF compared with the control (Fig. 1C). Subsequently, Si supply significantly decreased the activity of guaiacol-POD in LAF at the normal Mn level (2-fold) and especially with the high Mn supply level of 100 μM (10-fold). IEF of the LAF and PODs identified by guaiacol staining revealed various isoforms of this enzyme in the high Mn treatment, as evident from the presence of two distinct bands at pI 3.6 and 4.6, as well as a group of numerous anionic (acidic) bands ranging from pI 5.1 to 6.6 (Fig. 4). Since the inhibitory effect of Si supply on the isoforms and activity of apoplastic Mn toxicity-enhanced PODs is linked to the decrease in free apoplastic Mn (Figs 1–3; Table 2), an additional in vitro experiment was performed in order to demonstrate whether Si affects POD activity directly. As clearly shown in Fig. 5, the presence of monosilicic acid in the reaction mixture significantly decreased the activity of guaiacol-POD in vitro.

Hydroxyl radicals were detected using a spin-trapping reagent DEPMPO, which is capable of distinguishing between oxygen-centred radicals such as O₂⁻ and -OH (Frejaville et al., 1995). EPR spectra of DEPMPO/OH adducts from the LAF of cucumber plants grown under normal and high Mn treatments are shown in Fig. 6. The signal of the DEPMPO/OH adducts progressively increased with high Mn treatment. Root application of Si resulted in a decrease in the formation of DEPMPO/OH adducts in the LAF in both normal and high Mn treatments. The intensity of the EPR signal due to the DEPMPO/OH adducts was lower in the LAF of Si-treated cucumber plants grown with normal Mn treatment (0.5 μM) compared with
the signal recorded for non-Si treated plants (Fig. 6). Even
with the higher Mn supply (100 μM) with treatment with Si,
the formation of DEPMPO/OH adducts was of the same
magnitude as for the control Mn treatment (0.5 μM)
without Si supply (Fig. 6). To elucidate the effect of Si on
the formation of DEPMPO/OH adducts generated through
the Fenton reaction, an in vitro experiment was carried out
using the physiological concentrations of Mn2+ and mono-
meric Si in the LAF (see Fig. 1A; Table 4). As shown in
Fig. 7, increasing the concentration of Mn2+ in the reaction
media from 1 μM to 10 μM resulted in a slight increase in
the signal intensity of DEPMPO/OH adducts. However,
adding monosilicic acid to the reaction media did not affect
the signals of DEPMPO/OH adducts generated at both
Mn2+ concentrations (Fig. 7).

**Discussion**

From the previously published studies on cucumber
(Rogalla and Römheld, 2002; Shi *et al.*, 2005; Dragišić
Maksimović *et al.*, 2007), it is obvious that Si alleviates
the detrimental effect of excess Mn by raising leaf tissue
tolerance rather than by inducing root Mn exclusion via
decreased Mn uptake. In the present study, leaves of Si-
treated cucumber plants subjected to excess Mn were found
to have lower concentrations of free Mn in the LAF
obtained by the centrifugation method and thus a lower
proportion of water-extractable Mn (Fig. 1A; Table 2).
However, the fractionated extraction of leaf Mn might lead
to redistribution of Mn between cell compartments during
the extraction procedures (Table 2; see also the Materials
and methods). EPR spectroscopy has been used as a prom-
ising tool for rapid and relatively non-destructive determi-
nation of free Mn2+ in biological materials including plant

![Fig. 2. EPR spectra of Mn2+ from the leaves of cucumber plants
grown in nutrient solutions with adequate (0.5 μM) and high
(100 μM) Mn concentrations, without (–Si) or with (+Si) supply of
1.5 mM Si. Inset: magnified regions of EPR spectra indicating the
low amplitude Mn2+ signal at an Mn supply of 0.5 μM. The filled
circle indicates the inconsequential central signal at g=2.003 of
delocalized electrons and/or stable free radicals that occur
naturally in plants; the open circle indicates the first of the
characteristic six hyperfine lines of aqueous Mn2+; the dashed line
represents the broad signal from Mn2+ bound to the proteins and
cell wall macromolecules.]

![Fig. 3. EPR spectra of Mn2+ from the LAF of cucumber plants.
The experimental conditions are indicated in the legend of Fig. 2.
The filled circle indicates the inconsequential central signal; the
open circle indicates the first of the characteristic six hyperfine
lines of aqueous Mn2+.]

![Fig. 1. Effect of Si treatments (–Si, black bars; +Si, grey bars) on
the concentrations of Mn (A) and H2O2 (B), and the total activity of
guaiaicol-POD (C) in the leaf apoplastic fluid (LAF) from cucumber
plants subjected to adequate (0.5 μM) and excessive (100 μM)
external concentrations of Mn. If applied, the concentration of
Si(OH)4 in the nutrient solution was 1.5 mM. Data are means (n=4)
±SD. Significant differences at P < 0.05 are indicated by different
letters.]

...
tissues (Bacic et al., 1993; Jücker et al., 1999; Todorović et al., 2008). The intensity of the Mn^{2+} EPR signal increased both in the bulk leaves and in the LAF of cucumber plants subjected to high Mn and showed a tendency to decrease in the Si-treated plants (Figs 2, 3), which is in agreement with the data reported by Jücker et al. (1999) for bean plants. The relative proportion of free and bound Mn, estimated from the EPR signals of Mn^{2+} (Table 5; see also Fig. 2), is consistent with data obtained by fractionated Mn extraction (Table 2), supporting the hypothesis postulated by Rogalla and Römheld (2002) that, in cucumber plants, Si supply increases the Mn binding properties of leaf cell walls.

It is widely accepted that the capacity of the cell wall to bind metal cations in dicots depends mainly on the amount of pectic polysaccharides with abundant carboxylic groups, i.e. homogalacturonans. The binding affinity of pectin for Mn^{2+} is relatively low (e.g. lower than for Cu, Zn, Fe, and Ca; Dronnet et al., 1996; Eliaz et al., 2006, and references therein), so it would be unlikely that such a high amount of Mn (see Table 2) could be bound to the cell wall merely by fixation to the negatively charged carboxylic groups. Although Si can be bound to the pectins and thus contribute to cross-linking of the cell wall structure (Schwarz, 1973), the increased Si content in the leaf cell wall of Si-supplied cucumber plants did not induce an enhancement of negative charges of the cell wall (Table 3). The formation of Zn-silicate precipitated in the leaf epidermal cell wall is proposed to be one of the key mechanisms involved in detoxification of excess Zn in heavy metal-tolerant Minuartia verna (Neumann et al., 1997). Accordingly, if Mn is stabilized as an Mn-silicate complex (like the metal-polysilicate layers proposed in yeast cell walls; Barsser et al., 2006), this cell wall formation could explain the increased levels of cell wall-bound Mn detected in excess Mn conditions (Tables 2, 3). On the other hand, studies in cowpea suggested that the alleviation of Mn toxicity cannot be explained only by a decrease in free leaf apoplastic Mn through its enhanced binding by the cell wall macromolecules in Si-treated plants (see Führs et al., 2009, and references therein). It is not possible, therefore, to generalize that Si-enhanced wall binding of Mn (Rogalla and Römheld, 2002; Wiese et al., 2007; this study) constitutes the universal mechanism of Si alleviation of Mn toxicity in all plant species.

An increased amount of free Mn^{2+} in the leaf apoplast (Figs 1A, 3) was followed by enhanced formation of H_{2}O_{2} (Fig. 1B), most probably as a result of Mn^{II}-stimulated activity of NADH-oxidases (Halliwell, 1978). Also, enhanced superoxide dismutase (SOD) activity of cucumber leaves cannot be excluded as a cause of increased H_{2}O_{2} production at excess Mn (Shi et al., 2005). Stress-induced release of basic PODs (Gaspar et al., 1985) and their proposed role in defensive mechanisms against oxidative stress (Penel and Castillo, 1991) argue in favour of the higher abundance of POD isoforms at excess Mn in our experiments, which decreased under Si treatment (Fig. 4). The soluble PODs of the leaf apoplast, present either in the apoplastic fluid obtained by the centrifugation technique (this study) or in the apoplastic washing fluid (extracted by water using the vacuum infiltration/centrifugation technique; e.g. Fecht-Christoffers et al., 2003), were more affected by excess Mn compared with the cell wall-bound and cytosolic PODs (Fecht-Christoffers et al., 2003, 2006). The increased concentration of H_{2}O_{2} in the leaf apoplast caused by Mn toxicity is accompanied by a guaiacol-POD-catalysed oxidation of monophenols and also probably the co-oxidation of Mn^{III} (demonstrated by Kenten and Mann, 1950), resulting in enhanced evolution of phenoxy radicals and Mn^{III} intermediates, which might also be responsible for the leaf browning (Horst, 1988).

Application of Si decreased the free apoplastic Mn^{2+} due to increasing the proportion of cell wall-bound Mn in cucumber leaves (Table 5; Figs 1A, 3), which in turn led to decreased production of H_{2}O_{2} in the leaf apoplast (Fig. 1B; see also Führs et al., 2009). However, compartmentation and the accurate quantification of the apoplastic H_{2}O_{2} concentration is still a matter of debate, because of the rapid diffusion of H_{2}O_{2} across membranes. It has been demonstrated that Si application prevents the accumulation of free phenols in cucumber apoplast (Dragičić Maksimović et al., 2007), which (particulary p-coumaric acid) may additionally enhance Mn^{2+}-induced NADH-oxidase activity (Führs et al., 2009). Si treatment suppressed the Mn-induced increase in abundance of POD isoenzymes in the LAF (Fig. 4), and led to a rapid suppression of overall guaiacol-POD activity under excess Mn (Fig. 1C). This rapid decrease in POD activity in the LAF of Si-fed plants can be explained by the effect of Si in preventing contact between the enzyme and its phenolic substrate (Swain, 1977;
Iwasaki et al., 2002b) or even by removal of free monophenols as a consequence of the formation of Si-phenol complexes (Dragišić Maksimović et al., 2007), rather than by Si-mediated lowering of apoplastic H$_2$O$_2$ formation. Indeed, this direct inhibitory effect of Si on the activity of guaiacol-POD was further confirmed in the experiment in vitro (Fig. 5).

One of the important physiological roles of -OH that has been proposed is in the loosening of the cell wall and cleavage of polysaccharide polymers (Fry, 1998; Schweikert et al., 2002). The formation and thus the toxicity of extremely reactive -OH strongly depends on the presence of a Fenton catalyst such as metal ions or a peroxidase (Chen and Schopfer, 1999). In this study, high Mn supply led to an increased EPR signal of the DEPMPO/-OH adduct in the LAF (Fig. 6), which was followed by the increased concentrations of apoplastic free Mn$^{2+}$ and H$_2$O$_2$ (Fig. 1A, B). Hence, the Fenton reaction can be a source of -OH not only generated from the in vitro H$_2$O$_2$/Mn$^{2+}$ mixture (Fig. 7), but also for its elevated production in the leaf apoplast at excess Mn (Fig. 6) via the following simplified reaction:

$$\text{Mn}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Mn}^{3+} + \text{OH}^- + \cdot \text{OH}$$

Since POD may be involved in the transformation of H$_2$O$_2$ into the much more toxic -OH (Chen and Schopfer, 1999), enhanced POD activity in the leaf apoplast of cucumber with high Mn treatment (Fig. 1C) might contribute not only to the Mn$^{2+}$-catalysed Fenton reaction, but also to the increased signal of the DEPMPO/-OH adduct (Fig. 6). The present study demonstrated for the first time that apoplastic accumulation of highly toxic -OH can be considered as one of the major factors inducing leaf symptoms of Mn toxicity, which might be synergistically enhanced by toxic Mn$^{2+}$, generated concomitantly via the Fenton reaction and/or by the activity of apoplastic POD.

Si supply markedly decreased the EPR signal of DEPMPO/-OH adducts in the leaf apoplast at high Mn treatment (Fig. 6), which argues in favour of an important role for Si in suppression of -OH generating Mn toxicity. However, the addition of monosilicic acid to the Mn$^{2+}$/H$_2$O$_2$ reaction mixture did not directly affect the Fenton reaction in vitro (Fig. 7). It has been reported that a novel synthetic organosiliceous anionic hydride compound (SiH$_n$D$_2$) shows a strong -OH-scavenging effect in vitro (Stephanson et al., 2003). It is unlikely, however, that such compounds occur in the LAF or cell walls, in which the occurrence of orthosilicic acid (H$_2$SiO$_4$), either free (monomeric) or complexed by organic compounds (e.g. phenolics, lignins, carbohydrates, and peptides), and the hydrated amorphous polymer of orthosilicic acid known in minerals as opal (SiO$_2$)$_n$×nH$_2$O, have been experimentally confirmed (Inanaga et al., 1995; Iwasaki et al., 2002b; Casey et al., 2003; Kauss et al., 2003, and references therein). Thus, neither direct Si interactions with Mn nor -OH scavenging ability of monosilicic acid can be proposed. Rather, Si nutrition contributes indirectly to a lowering of -OH in the leaf apoplast due to a decrease in the free and exchangeable apoplastic Mn$^2+$ (a Fenton catalyst), thus regulating a Fenton reaction. A direct inhibitory effect of Si on the POD activity may also contribute to decreasing the POD-mediated generation of -OH.

In conclusion, the results presented here confirm the previously unsubstantiated hypothesis that the leaf apoplast plays the central role in modulating Mn toxicity and Si-enhanced Mn tolerance in cucumber. The elevated accumulation of -OH in the leaf apoplast appears to be the key...
factor inducing Mn toxicity symptoms, and Si has a protective effect in controlling -OH production under excess Mn.

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